

評価報告書

In vitro 発熱性物質試験

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国立医薬品食品衛生研究所

In vitro 発熱性物質試験

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In vitro 発熱性物質試験の評価会議報告書

2010年9月6日

JaCVAM 評価会議

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In vitro 発熱性物質試験について、第三者評価委員会からの報告を受け、以下の 8 項目について審議した。本 2~8 項目は OECD ガイダンス文書 No. 34 に示された検討項目である。

< 検討項目 >

1. 検討対象の試験法は、日本のどの法規制やガイドラインに関係しているか。
2. 検討対象の試験法とその妥当性を示すデータは、透明で独立な評価を受けているか。
3. 当該試験法で得られるデータは、対象毒性を十分に評価あるいは予測できるものであるか。データは、当該試験法と従来の試験法の、代替法としての繋がりを示しているか。あるいは（同時に）そのデータは、当該試験法と、対象としているあるいはモデルとしている動物種についての影響との繋がりを示しているか。
4. 当該試験法は、ハザードあるいはリスク、あるいはその両方を評価するのに有用であるか。
5. 当該試験法とその妥当性を示すデータは、その試験法で安全性を保証しようとする、行政上のプログラムあるいは関係官庁が対象としている化学物質や製品を、十分広く対象としたものとなっているか。当該試験法が適用できる条件及び適用できない条件が明確であるか。
6. 当該試験法は、プロトコルの微細な変更に対して十分頑健で、適切な訓練経験を持つ担当者と適切な設備のある施設において、技術習得が容易なものであるか。
7. 当該試験法は、時間的経費的に有用性があり、行政上で用いられやすいものであるか。
8. 当該試験法は、従来の試験法と比べて、科学的・倫理的・経済的に、新しい試験法あるいは改訂試験法であることが正当化されているか。

< 審議内容 >

1. 検討対象の試験法は、日本のどの法規制やガイドラインに関係しているか。
第十五改正日本薬局方（15 局，2006 年）及び医療機器の生物学的安全性評価のための試験法（医薬局審査管理課事務連絡，医療機器審査 No. 36）が該当する。
 - ・ 日本薬局方 15 局は、発熱性物質の検出法として「発熱性物質試験法」及び「エンドトキシン試験法」（リムルス試験）を収載している。
 - ・ 日本薬局方 15 局の「製剤総則・注射剤」の項では「エンドトキシン試験法」が優先されているが、「エンドトキシン試験法の適用が困難な場合は、発熱性物質試験法を用いることができる」と規定されている。
 - ・ 「輸液用ゴム栓試験法」（日本薬局方 15 局）及び「医療機器の生物学的安全性評価のための試験法」では「発熱性物質試験法」の実施が要求されている。
2. 検討対象の試験法とその妥当性を示すデータは、透明で独立な評価を受けているか。
 - ・ ICCVAM が、ECVAM の検討結果（EC STATEMENT ON THE VALIDITY OF IN-VITRO TESTS 及び ECVAM Background Review Document (BRD)）を第 3 者評価し、2008 年 5 月に 2 つの報告書（ICCVAM Test Method Evaluation Report 及び ICCVAM Background Review Document (BRD)）を公表した。これらの報告書について JaCVAM 発熱性物質試験評価委員会が評価した。
 - ・ ICCVAM による妥当性評価では、OECD の指針書（GD34）に従って、要確認項目（Modular approach）が精査された。
 - ・ これらの報告書には評価した 5 つの試験のプロトコール及び被験物質が記載されている。

総合評価の資料として取り入れなかった報告については概要を示し、ウサギ「発熱性物質試験法」との相関性については参考資料を挙げて比較したことが示されている。

- ・ 資料には、データ処理の仕方に若干の問題があったが、その結論に与える影響は小さく、ICCVAM の総合評価は受け入れられるものである。
3. 当該試験法で得られるデータは、対象毒性を十分に評価あるいは予測できるものであるか。データは、当該試験法と従来の試験法の、代替法としての繋がりを示しているか。あるいは（同時に）そのデータは、当該試験法と、対象としているあるいはモデルとしている動物種についての影響との繋がりを示しているか。
- ・ *in vitro* 発熱性物質試験法（*in vitro* PBMC 試験法）においては、発熱性物質の存在を評価するために、ヒト末梢血単核細胞あるいは株化ヒト単球様細胞から分泌される炎症性サイトカイン（インターロイキン（IL） -1β あるいは IL-6）を測定する。エンドトキシンを介した発熱の機序には即しており、科学的に妥当である。
 - ・ 試験法として標準的に参照されているウサギの体温上昇を測定する「発熱性物質試験法」、及び「エンドトキシン試験法」との比較を行い、結果が概ね一致していることを確かめている。
 - ・ ヒト由来の細胞を用いることから、異なる動物種を用いる「発熱性物質試験法」や「エンドトキシン試験法」よりもヒトでの発熱性を正確に予測できる可能性はあるが、それは検証されていない。
 - ・ 評価書では、グラム陰性菌由来のエンドトキシンについてのみ検証されており、非エンドトキシンについては十分な検証がなされていない。
4. 当該試験法は、ハザードあるいはリスク、あるいはその両方を評価するのに有用であるか。
当該試験法は、あるエンドトキシン投与量においてサイトカイン量がある値を越えているかで陽性・陰性を評価する試験法であり、ハザードは評価できる。ウサギを用いた発熱性物質試験法と同様、リスク評価を目的とするものではない。
5. 当該試験法とその妥当性を示すデータは、その試験法で安全性を保証しようとする、行政上のプログラムあるいは関係官庁が対象としている化学物質や製品を、十分広く対象としたものとなっているか。当該試験法が適用できる条件及び適用できない条件が明確であるか。
- ・ 当該試験法の正確性及び再現性は合計 13 物質のバリデーション研究によって評価されている。いずれもエンドトキシンを添加した医薬品で、エンドトキシンを添加せずとも発熱性を示す物質、生物学的製品及び医療機器などについては実施されていない。よって、非エンドトキシンの検出力が不明確である。当該試験法は非経口投与される医薬品のエンドトキシンによる発熱性の評価が可能であるが、医療機器や経口曝露される医薬品に対しては不十分であると考えられる。
 - ・ 参考資料として、臨床上で発熱の副作用報告のあったヒト血清アルブミン製剤、第Ⅷ因子製剤、及び血漿分画製剤にエンドトキシンを添加した試料について、ウサギ発熱性物質試験、エンドトキシン試験、*in vitro* PBMC 試験での結果を比較した報告が上げられている。また、当該試験法を用いた各種グルカン、リポテタイコ酸、真菌胞子、各種医療材料、脂質等での結果も報告として示されており、他の試験法では適用困難なものについても検出することが可能であった

としている。しかし、これらは検証的なデータとは言えない。

6. 当該試験法は、プロトコールの微細な変更に対して十分頑健で、適切な訓練経験を持つ担当者として適切な設備のある施設において、技術習得が容易なものであるか。
 - ・ 採血後 4 時間以内のヒト全血、凍結保存血、全血から採取した末梢血単核細胞あるいは株化細胞を用い、IL-1 β または IL-6 を測定する。組み合わせによって 5 つの試験法（全血（Whole Blood：WB）/IL-1、保存血 CryoWB/IL-1、WB/IL-6、PBMC/IL-6、単球細胞株 MM6/IL-6）があり、それらの推奨プロトコールが ICCVAM により提案されている。発熱性の予測性能としては、どの試験法ともほぼ同じ一致率を示している。
 - ・ PBMC/IL-6 及び WB/IL-1 については、変法が評価されている。しかし、その変法がどのような経緯で作られたか明らかではなく、頑健性については評価できない。
 - ・ ELISA キットを用いてサイトカインを測定するので、技術移転の容易性については検討されていないが、経験を有する人が技術習得することは容易であると思われる。WB/IL-1 についての施設間再現性が悪いが、他の試験法の施設間再現性および施設内再現性は良好とされている。
7. 当該試験法は、時間的経費的に有用性があり、行政上で用いられやすいものであるか。
 - ・ 多数の動物を用い、実施まで時間のかかるウサギを用いる「発熱性物質試験法」よりは時間的経費的に優位であると推察される。
 - ・ 「エンドトキシン試験法」と比較したときの時間的経費的な有用性については明らかではない。
 - ・ ヒト血液を用いる場合は、行政的な制約を伴う。
8. 当該試験法は、従来の試験法と比べて、科学的・倫理的・経済的に、新しい試験法あるいは改訂試験法であることが正当化されているか。
 - ・ ウサギを用いる発熱性物質試験と比較した場合、当該試験法は新しい試験法であり、3Rs 原則を満たしており、経済的にも優位性がある。
 - ・ エンドトキシン試験と比較した場合、作用機序の異なる新しい試験法ではあるが、倫理的・経済的には優位性が明らかでない。

以上の審議の結果、JaCVAM 評価会議は、*In vitro* 発熱性物質試験について以下のように結論した。

- ・ 当該試験法は、エンドトキシンによる発熱の機序をもとに考案された方法であり、原理面で科学的な妥当性はある。
- ・ バリデーション研究によって、ウサギ発熱性物質試験法との相関性も概ね高いと思われるが、これを代替法として採用するためには、化合物数を増やし、多施設バリデーションを実施することが必要である。
- ・ 現時点でエンドトキシン試験にかわるものではない。本邦においては、日本薬局方 15 局でウサギを用いる発熱性物質試験からエンドトキシン試験への移行が進められている現状を踏まえると、利用価値は限定的と言える。
- ・ 有望な試験法と考えられるが、現時点では、ただちに代替法として採用することは妥当でない。

参考文献

1. OECD (2005) OECD Series on testing and assessment Number 34, Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment, ENJ/JM/MONO(2005) 14

In vitro 発熱性物質試験の第三者評価報告書

平成 22 年 (2010 年) 8 月 18 日

発熱性物質試験評価委員会

発熱性物質試験評価委員

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用語集

BET : Bacterial Endotoxin Test : Limulus amoebocyte Lysate (: LAL) TESTING (エンドトキシン試験)

BRD: Background Review Document

ECVAM: European Center for the Validation of Alternative methods

ELISA: Enzyme-Linked ImmunoSorbent Assay

GD: Guidance Document

ICCVAM: Interagency Coordinating Committee on the Validation of Alternative Methods

IL: Interleukin (インターロイキン)

IL-1 β : Interleukin 1 β

IL-6: Interleukin 6

IL-8: Interleukin 8

LAL: Limulus amoebocyte Lysate (カプトガニ血球抽出物)

LPS : Lipopolysaccharide (リポ多糖)

LTA:Lipoteichoic acid (リポテイコ酸)

MM: Monocytoid Cell Line Nono Mac (単球細胞株)

OECD : Organization for Economic Co-operation and Development

PBMC : Peripheral Blood Mononuclear Cell (末梢血単核細胞)

RPT : Rabbit Pyrogen Test (ウサギ発熱性物質試験)

TNF- α : Tumor Necrosis Factor- α

WB: Whole Blood(全血)

要旨

当委員会は、製剤の発熱性物質（パイロジェン）の検査法である発熱性物質試験に関して提案されている新規試験法について、その妥当性を検討することを目的とする。この新規試験法は、従来の発熱性物質試験に代わるとされる *in vitro* 発熱性物質試験法であり、採取したヒト末梢血単核細胞（PBMC）から分泌されるサイトカイン（インターロイキン；IL）の量を指標とする。この試験法に対し、各委員が以下のような観点で考察を加え、その妥当性を総合的に評価した。

1) 試験法の科学的、規制の上での妥当性

従来の発熱性物質の検査法であるウサギを用いた発熱性物質試験法とカプトガニの血球抽出成分の凝固反応によるエンドトキシン試験法（リムルス試験）、さらに、今回新たに提案された *in vitro* 発熱性物質試験法（以後、*in vitro* PBMC 試験法と記す）について、検出可能な発熱性物質の種類について考察を加えた。ウサギによる発熱性物質試験ではエンドトキシン、非エンドトキシンを問わず発熱性物質の検出が可能であるが、試験法が煩雑であり、また、検査に多数の動物を必要とする。これに対し、エンドトキシン試験は簡便であり、医薬品の品質管理上問題となるエンドトキシンに対し優れた検出力を有するが、非エンドトキシンの発熱性物質試験には無効である。*in vitro* PBMC 試験法は発熱の生化学的メカニズムに立脚しており、理論上はエンドトキシン、非エンドトキシンの両者に対応が可能であるが、これまでに提出されているデータは主としてエンドトキシンに関するもので、非エンドトキシンの発熱性物質検出に有効であるかどうかは明確には示されていない。エンドトキシンの検出には現在用いられているエンドトキシン試験が有効であるため、動物実験代替法（以下、代替法と記す）としては非エンドトキシンをもカバーするウサギによる発熱性物質試験に代わる役割が期待されるが、個々の事例について検証する必要があると考えられる。また、ヒトの血液等を用いるので、ヒトへの影響を直接的に評価できるという利点も想定されるが、日本人の血液を用いる場合にはその検証が必要となろう。

2) プロトコールの妥当性およびバリデーション研究に用いた被験物質

in vitro PBMC 試験法の5つのプロトコールを詳細に検討し、例数およびそれに基づく解釈についての疑念に加えるとともに、日本における採血実施に伴うウイルス感染への危惧を指摘した。非エンドトキシンの発熱性物質についての考慮不足が懸念された。

3) 試験法の正確性・信頼性についての考察

文献1. ICCVAM Test Method Evaluation Report, および、文献2. ICCVAM Background Review Document の2つのデータを精査し、その問題点を詳細に指摘している。文献1における ICCVAM の総合評価は、“5種の *in vitro* PBMC 試験法は非経口投与によるエンドトキシンの発熱性物質試験として利用可能であるが、他の物質や器具の評価法としては不十分である”，というものであった。これは OECD の指針書 Guidance Document (GD)

34 に従ってなされている。データの質を吟味する上で、恣意的な選択がなされていないか、判定基準が後づけでないか、などの観点からの考慮が必要であり、質の良くないデータを削除した痕跡等の問題が文献1および2に散見された。しかし、文献のデータの吟味を行った13名の委員からなる *in vitro* Pyrogenicity Independent Peer Review Panel の中には統計に詳しいと思われる委員もおり、データの細部に性能を高め評価する偏りが多少見受けられるものの、ICCVAM の総合評価の結果から *in vitro* PBMC 試験法をおおむね受け入れて差し支えないものと考えられた。

4) 試験法の正確性を評価する物質の *in vivo* および参照データ

in vitro PBMC 試験法のバリデーションに用いられた13の物質に関し、比較のためのウサギを用いた発熱性物質試験は行われておらず、その代わりとして過去にドイツで行われたウサギによるデータとの比較がなされていることをまず指摘している。この比較はエンドトキシン試験の結果をも加えて行っている。ここで、実際の臨床使用で発熱が報告された物質に対する応答性を比較したところでは、必ずしもウサギで発熱が認められるとは限らず、例えば、ヒト血清アルブミン製剤については、*in vitro* PBMC 試験法では5例で平均5.52倍の応答が認められたが、ウサギではすべて陰性であった。エンドトキシンをスパイクした試料でのウサギを用いた試験との比較においても、第VIII因子製剤あるいはヒト血清アルブミンの発熱性について、*in vitro* PBMC 試験法ははるかに優れた応答性を示した。また、発熱性を示したゼラチン含有輸液製剤2バッチを用いた比較では、*in vitro* PBMC 試験法はともに陽性を示したが、エンドトキシン試験はともに陰性であり、ウサギは一方が陽性、他方が陰性であった。以上のように、*in vitro* PBMC 試験法はウサギを用いる方法あるいはエンドトキシン試験よりもヒトの発熱性を的確に検出できる可能

性があることが文献的に示された。

しかし、実際の製剤の原料や製造工程において、エンドトキシン以外の発熱性物質の汚染が考えられない場合には、現状のエンドトキシン試験で対応が可能である。また、ウサギによる発熱性物質試験のすべてを今回の新規試験法に切り替えるのが適当とは思われず、例えば、何らかの理由でエンドトキシン試験が適用できない場合に、ウサギによる発熱性物質試験の代替として *in vitro* PBMC 試験法を採用するといった限定的な使用にとどめるべきであるという考察がなされており、本委員会も同様に考える。

5) 試験法の科学的根拠および有用性と限界

in vitro PBMC 試験法以外にも、*in vitro* 発熱性物質試験法は提案されており、発熱あるいはそれに類するバイオマーカーを指標とする多様な系が開発されつつある。しかしながら、*in vitro* PBMC 試験法については、ICCVAM の報告書にもあるように、ウサギ発熱性物質試験との相関についてはエンドトキシン試験を引用しており、また、IL-1、IL-6 といったマーカーが発熱性について普遍性があるとは言えない。よって、*in vitro* PBMC 試験法をウサギ発熱性物質試験の代替法とすることには無理がある。発熱性の作用機序については、*in vitro* のみならず、*in vivo* のウサギにおいても、科学的に十分に解明されているとは言いがたい。最新の知見を踏まえた上で現行の試験法の可否と新たな試験法の必要性を論じる必要がある、という考察がなされており、本委員会も同様に考える。

6) 結論

上記の各考察より、今回提案された *in vitro* PBMC 試験法は、ウサギの使用数を削減可能とし、また、作用機序に立脚した発熱性物質試験の新たな方向性として評価でき、さらに、バリデーションでのデータの扱いに関しても納得のできる基準を満たしていると考えられる。しかし、ウサギを用いた発熱性物質試験との互換性については不明な点が多く、この試験法の代替法とするには、問題がある。国内ではウサギ発熱性物質試験からエンドトキシン試験への移行の問題も残されており、これに優先して独立した代替法とすることには無理がある。しかしながら、エンドトキシン試験が使用できないような特殊な状況やウサギとヒトでの発熱性に関する種差が想定されるような状況においてウサギ発熱性物質試験と併用するなど、限定的な使用については推奨できよう。このような *in vitro* PBMC 試験法が科学的根拠に基づいた研究により、さらに広範囲な状況で使用可能となることが望まれる。

補) エンドトキシン試験と発熱性物質試験に関する実態調査結果報告

2009年1月に日本製薬工業協会の協力を得て、アンケートを行い、36社から回答を得た。エンドトキシン試験法が、日本薬局方で優先すべき試験法として推奨されていることもあり、最も広く用いられていた。エンドトキシン試験法の原理は一連の酵素活性化反応であるため、これらの系に影響する反応干渉因子が存在する。しかし、実際には干渉のメカニズムが既知の物質については、製剤そのものが体温に影響する場合を含め、ほとんどが検証可能であった。今回提案されている新規試験法 (*in vitro* PBMC 試験法) の実施経験の報告は皆無であった。また、ヒトの血液の入手あるいはバイオハザードの問題などがあり、動物愛護の観点等からウサギを用いた発熱性物質試験法から代替法へと切り替えるにしても、エンドトキシン試験法への変更が妥当であると考えられた。上述したように、現状ではエンドトキシン試験法は日本薬局方においてその使用が推奨され最も汎用されている系ではあるが、推奨される以前にウサギによる発熱性物質試験のデータを用いて承認を受けた医薬品に関しては、動物愛護の観点を含めこれをエンドトキシン試験法に変更することが望まれる。しかし、実行するとなると、両者の同等性を示すために多数のウサギを使用しなければならない、というジレンマが生じる。

0. 評価資料

- 1) ICCVAM Test Method Evaluation Report
- 2) ICCVAM Background Review Document (BRD)
- 3) EC STATEMENT ON THE VALIDITY OF IN-VITRO PYROGEN TESTS
- 4) ECVAM BRD

1. 試験法の科学的、規制の上での妥当性

発熱性物質（パイロジェン）は、体温調節中枢である視床下部に直接的あるいは間接的に作用して体温を上昇させる物質である。主に、細菌やウイルス、菌類等の微生物に由来し、生体はこのような物質の暴露に反応して、前炎症性サイトカインである IL-1 や IL-6, TNF- α 等の内因性発熱性物質を産生分泌し、これらがプロスタグランジンの合成を促進して中枢神経系に作用することにより発熱を生じる。発熱性物質のタイプや量、あるいは暴露を受けた個人の感受性によっては、サイトカインが過剰に放出され、ショックや多臓器不全を発症して死に至る場合もあり、注射剤等の医薬品や医療用具の品質および安全性確保の観点から、発熱性物質の存在を適切かつ確実に評価できる試験法を確立することが重要である。

日本薬局方においては、2種類の試験、すなわち、「発熱性物質試験法」および「エンドトキシン試験法」（リムルス試験）が収載されている。試料をウサギの静脈内に投与して体温上昇（発熱）を測定する「発熱性物質試験法」は、前述の微生物由来物質のほか、化学的発熱性物質を含むさまざまなタイプの発熱性物質を検出できるが、実験操作が煩雑であることに加えて、多数の動物を使用することから、その動物実験代替法（以下、代替法と記す）として、カプトガニの血球抽出成分に対する凝固能を指標とした「エンドトキシン試験法」が開発された。

エンドトキシンは極めて活性の高い発熱性物質で、グラム陰性菌の細胞壁成分のリポ多糖であり、環境中に広く存在するとともに、その失活や除去が困難であることから、医薬品等の品質管理を行う上で特に重要である。2006年に告示された第十五改正日本薬局方（15局）の製剤総則・注射剤の項では、「エンドトキシン試験法の適用が困難な場合は、発熱性物質試験法を用いることができる」と規定され、発熱性物質の検出において、「エンドトキシン試験法」が優先され、「発熱性物質試験法」はその補助的な試験法として位置づけられた。しかし、「エンドトキシン試験法」はグラム陰性菌由来のエンドトキシンの検出において感度が高いものの、非エンドトキシン型の発熱性に対しては反応せず、また、ある種の物質（ β -グルカン等）や試料によっては検出系が干渉され、適用が困難な場合もあり、さらに、「輸液用ゴム栓試験法」（15局）や「医療機器の生物学的安全性評価のための試験法」（医薬局審査管理課事務連絡、医療機器審査 No. 36）においては「発熱性物質試験法」の実施が要求されていることから、未だに多くのウサギが試験に用いられているのが現状である。また、ウサギには発熱性を示さないがヒトでは発熱性を示す物質については、「発熱性物質試験法」では検出できない。従って、規制の上からも、発熱性物質の検出において、ウサギでの「発熱性物質試験法」に代わり得る、新規の *in vitro* 発熱性物質試験法の開発は有意義であると考えられる。

今回提案された新規の *in vitro* 発熱性物質試験法（*in vitro* PBMC 試験法）は、ウサギでの「発熱性物質試験法」が *in vivo* での体温上昇（発熱）を測定するのに対して、発熱反応における単球の役割に注目し、ヒト血液（新鮮血／凍結血）由来あるいは株化されたヒト単球様細胞から培養液中に分泌される前炎症性サイトカイン（インターロイキン（IL）-1 β あるいは IL-6）を測定するものである。ウサギでの発熱性物質投与前後のサイトカイン濃度の変動に関するデータは得られておらず、発熱とサイトカインとの関係を直接比較することはできないが、前述のように、発熱性物質による発熱は、主に前炎症性サイトカインの放出を引き金として誘発されることから、これらのサイトカイン濃度と発熱が相関するとの仮定は科学的に妥当と考えられる。さらに、検出系がヒトに由来することから、「エンドトキシン試験法」や「発熱性物質試験法」よりもヒトにおける発熱性を正確に予測できる可能性も示唆される（表1：Biotest社資料より引用）。

しかし、今回 ECVAM（European Center for the Validation of Alternative methods）から提出された試験成績においては、グラム陰性菌由来のエンドトキシンについてのみ検証されており、エンドトキシンに対する個々の試験法の反応性や適切性に関するデータは示されているものの、非エンドトキシン型の発熱性について十分な検証がなされていない。従って、現時点では、*in vitro* PBMC 試験法は、ウサギでの「発熱性物質試験法」の完全な代替法とはなりえず、試料の検出系に対する干渉や試料が非エンドトキシン型の発熱性物質を含む等の理由から「エンドトキシン試験法」の適用が困難であり、「発熱性物質試験法」の実施が求められる個々の事例について、「発熱性物質試験法」と同等以上の検出感度を有することを検証した上で、ケースバイケースで適用することが妥当と考えられる。なお、これらの試験法の国内での実施にあたっては、ヒト試料を用いることによるバイオハザードの問題を解決するとともに、日本人の

血液を用いる場合にはその検証を行う必要がある。

Comparison of Pyrogen Test Methods			
	Rabbit	LAL	PyroDetect
Test principle	Fever reaction in mammal	Defence mechanism	Fever reaction in human
Contamination	Gram-negative (LPS)	+	+
	Gram-positive (LTA)	+	+
	Yeasts & Molds	+	-
	Virus	+/- ¹	-
Application	Pharmaceuticals	+	+
	Biologicals (e.g. gene therapy, recombinant therapeutic proteins)	+	+/- ²
	Medical devices	+ ³	+/- ³
	Cell therapeutics (e.g. monoclonal antibody)	-	+/-

¹ Variable pyrogen results, ² Rabbit test often required, ³ Indirect test with solution in pyrogen-free water

表 1: Comparison of the Rabbit Test, the LAL Test and the PyroDetect System.

2. プロトコールの妥当性

2-1. 試験名

ボランティアから供与を受けた全血を用い、採血後4時間以内に実施すべき試験が2種、保存血（凍結保存）を用いる試験が1種、全血から取り出したPBMC（末梢血単核細胞）を用いる試験が1種、細胞株を用いる試験が1種記載されている。これらを総称して *in vitro* PBMC 試験法とした。

- 1) 全血 (Whole Blood: WB) /IL-1
- 2) 保存血 CryoWB/IL-1
- 3) WB/IL-6
- 4) PBMC/IL-6
- 5) 単球細胞株 MM6/IL-6

2-2. 試験法

試験法として提案されている5種の試験のプロトコールはいずれも明確である。試験法の共通事項を以下にまとめた。試験法の詳細は添付資料2にまとめた。

- 1) 予備試験 エンドトキシンスパイク回収率（50～200%）のために必要な最低希釈濃度を定めるための相互試験
- 2) 使用限界 相互試験の結果による非経口医薬品、生物学製品、医療機器
- 3) 測定キット ELISA(1測定/n=4)
- 4) 陽性対照(PC) 0.5EU/mL WHO-LPS94/580(E.coli0113:h10:K-)
- 陽性製品対照 (PPC) 0.5EU/mLの陽性対照を添加した製品
- 5) 陰性対照(NC) 生理食塩水
- 陰性製品対照 (NPC) 生理食塩水を添加した製品
- 6) 測定値 吸光度の平均値±標準偏差
- 7) 陽性基準 セクション 4.2 で定められた発熱性閾値を基準にした発熱性様反応を示す >0.5EU/mL

これらの相違点のみを表2-1にまとめた。

表 2-1. 試験法の相違点一覧

試験法	WB/IL-1	CryoWB/IL-1	WB/IL-6	PBMC/IL-6	MM6/IL-6
起源	ヒト全血	ヒト全血	ヒト全血	ヒト全血	MM 細胞バンク
培養液	生理食塩水	RPMI1640	生理食塩水	RPMI1640	10 または 2% 非動化した牛胎児血清を含む RPMI1640
測定指標	IL-1 β	IL-1 β	IL-6	IL-6	IL-6
前処理	採血後, 4 時間以内に使用	Konstanz または PEI 法で凍結保存	採血後, 4 時間以内に使用	白血球および PBMC を遠心分離	MM6 細胞 4 \times 10 ⁵ 細胞/mL
被験物質の適用法	20 μ L サンプル + 200 μ L 生理食塩水 + 血液 20 μ L	Konstanz 法 20 μ L サンプル + PRMI 200 μ L + 血液 20 μ L, PEI 法 20 μ L サンプル + RPMI 180 μ L + 血液 40 μ L	50 μ L サンプル + 生理食塩水 100 μ L + 血液 50 μ L	50 μ L サンプル + PRMI-C100 μ L + PBMC 100 μ L	50 μ L サンプル + PRMI-C100 μ L + 細胞 100 μ L 100 μ L
適用時間	10-24 時間	10-24 時間	16-24 時間	16-24 時間	16-24 時間
ELISA 測定物	10000g, 2 分処理後の上清	混合物	混合物	上清	上清
n	1	5 (プール品)	3	3 - 4	1
適合基準 (陽性対照)	陽性対照 OD1.6 倍 > 陰性対照, 陽性製品対照 OD1.6 倍 > 陰性製品対照, 陽性製品対照の OD は陽性対照 OD の 50-200% 内	陽性対照 OD1.6 倍 > 陰性対照, 陽性製品対照 OD1.6 倍 > 陰性製品対照, 陽性製品対照の OD は陽性対照 OD の 50-200% 内	陽性製品対照の OD は陽性対照 OD の 50-200% 内	陽性製品対照の OD は陽性対照 OD の 50-200% 内, 1EU/mL の OD が > 1000 pg/mL IL-6	陽性対照 期待値 (0.5EU/mL) OD \pm 20%, 陽性製品対照の OD は陽性対照 OD の 50-200% 内
適合基準 (陰性対照)	OD \leq 0.1	OD \leq 0.1	OD < 200pg/mL IL-6	OD < 0.15 および OD < 500pg/mL IL-6	OD < 0.2
その他	特許あり (US6696261 B2)	特許あり (USPTO 436518000)	特許あり (US6696261 B2)		Prf. Ziegler-Heitbrock (German Collection of Microorganisms and Cell bank) のライセンス

これに対して, ICCVAM が第三者評価を実施し, 表 2-2 に示すような主に採用基準に関して推奨プロトコールを示している。

表 2-2. ICCVAM が推奨するプロトコール

試験法	WB/IL-1	CryoWB/IL-1	WB/IL-6	PBMC/IL-6	MM6/IL-6
被験物質	最大有効希釈濃度を越えず、干渉のない原液または段階希釈				
ドナー数	最小 3 (個々またはプールされたもの)				適用外
反応干渉の基準	陽性製品対照の平均 OD が 1.0EU/mL EC で 50-200%	陽性製品対照の平均 OD が 0.5EU/mL EC で 50-200%	陽性製品対照の平均 OD が 1.0EU/mL EC で 50-200%	陽性製品対照の平均 OD が 0.25EU/ mL EC で 50-200%	陽性製品対照の平均 OD が 1.0EU/mL EC で 50-200%
培養プレート	陰性対照 (1)				
	エンドトキシンコントロール (5)				
	被験物質 (14)				
	陽性製品対照 (0)				
ELISA プレート	IL-1 β を 7 点およびブランク 2 点		IL-6 を 7 点およびブランク 2 点		
	陰性製品対照 (0)				
アッセイ採用基準	陰性対照の平均 OD \leq 0.15				
	IL-1 β 標準曲線が二次関数で r \geq 0.95		IL-6 標準曲線が二次関数で r \geq 0.95		
	エンドトキシン標準曲線の OE 値がシグモイド濃度反応で上がる				
	適用外	適用外	高反応ドナー (> 200pg/mL IL-6) は除外	高反応ドナー (>200 pg/mL IL-6) または低反応ドナー (1 EU/mL のエンドトキシンコントロール < 1000 pg/mL) は除外	適用外
Dioxin's テストを用いる棄却検定					
発熱性陽性基準	被験物質中のエンドトキシン濃度 > 被験物質中のエンドトキシン測定限界濃度				

以下に評価委員会の意見を記す。

- 1) 日本での実施においては、採血を伴う試験の普及度が気掛かりである。試験者が試験によって未知のウイルスによる感染する恐れも否定できず、可能であれば細胞株の利用を推し進めるべきであろう。
- 2) 指標としては、インターロイキン 1 β (IL-1 β) が 2 種、または IL-6 が 3 種の試験で提案されており、ELISA 法で簡便に測定できる。
- 3) ECVAM では、PBMC の場合、ドナーのバラツキを考慮して n=3~4、保存血の n=5 と妥当な数を推奨している。これらの結果の多数決で判定される。WB/IL-6 が n=3 であるのに対し、WB/IL-1 の n が 1 であることには納得できていない。検体数は ICCVAM が推奨するように、最少でも 3 以上とすべきである。
- 4) 陽性対照は、大腸菌の抽出液 (WHO-リポポリサッカライド 94/580) という標準品 (エンドトキシン) であり、0.25~5.0EU/mL で検量線を求め、その基準と合わせ妥当な選択がなされていると考える。
ECVAM では、0.5 エンドトキシンユニット (EU) /mL を越えた場合を陽性とし、ICCVAM では試験法によって、0.25~1.0 (EU) /mL としている。ICCVAM が試験法毎に基準を変えている理由が明確でなく、判断できない。
- 5) エンドトキシンを添加していない対照品も試験されており、コントロールの設定は適切であると考えられる。
- 6) 本研究は将来、n 数を増やし、エンドトキシンを添加せずとも発熱性物質試験で陽性となるような被験物質を用いた追加バリデーション研究が必要であると考えられる。現状では、発熱性物質試験の完全な代替法とはいえ、補完試験の位置付けである。

3. バリデーション研究に用いた被験物質

表 3-1 および 3-2 に示すように、正確性の確認のために 10 物質、再現性確認のために 3 物質が実験に利用されている。

表 3-1. 正確性を求めるためにバリデーション研究に用いられた 10 物質

Test Substance ²	Source	Active Ingredient	Indication	MVD (-fold)
Beloc [®]	Astra Zeneca	Metoprolol tartrate	Heart dysfunction	140
Binotal [®]	Aventis	Ampicillin	Antibiotic	140
Ethanol 13% (w/w)	B. Braun	Ethanol	Diluent	35
Fenistil [®]	Novartis	Dimetindenmaleat	Antiallergic	175
Glucose 5% (w/v)	Eifel	Glucose	Nutrition	70
MCP [®]	Hexal	Metoclopramid	Antiemetic	350
Orasthin [®]	Aventis	Oxytocin	Initiation of delivery	700
Sostril [®]	GSK	Ranitidine	Antiacidic	140
Drug A - 0.9% NaCl	-	0.9% NaCl	-	35
Drug B - 0.9% NaCl	-	0.9% NaCl	-	70

Abbreviations: MVD = Maximum valid dilution; GSK = GlaxoSmithKline; NaCl = Sodium chloride; w/w = Weight/weight; w/v = Weight/volume

¹Each substance was tested in all five *in vitro* pyrogenicity test methods.

²Each test substance was spiked with 0, 0.25, 0.5, 0.5, or 1.0 EU/mL of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). Each sample contained the appropriate spike concentration when tested at its Maximum Valid Dilution (MVD).

表 3-2. 再現性を確認するためにバリデーション研究に用いられた 3 物質

Test Substance ²	Source	Agent	Indication
Gelafundin [®]	Braun Melsungen	Gelatin	Transfusion
Jonosteril [®]	Fresenius	Electrolytes	Infusion
Haemate [®]	Aventis	Factor VIII	Hemophilia

¹Each substance was tested in all five *in vitro* pyrogenicity test methods.

²Each test substance was spiked with 0, 0.5, or 1.0 EU/mL of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). Each sample contained the appropriate spike concentration when tested at its Maximum Valid Dilution (MVD).

表 3-1 および 3-2 に示すような合計 13 物質を用いたバリデーション研究は少なすぎる。エンドトキシンを製剤にスパイクしたものを被験物質として使用しており、この方式では被験物質を増やしたから適切であるとは言い切れない。選ばれている製品はコード化されたものが、医薬品しか実施されていない。生物製品、医療機器などの結果がない。

いずれもエンドキシンを製品に添加した被験物質を試験に用いているが、エンドトキシンをスパイクせずとも発熱試験で陽性となるような被験物質における結果が必要である。また、エンドトキシンを産生するグラム陰性細菌の評価は可能であるとしても、その他細菌による発熱作用は判別できているとはいえ、その使用は限定的であると考えられる。

4. 試験法の正確性・信頼性を評価するデータについての考察

試験法の正確性や信頼性の評価に用いられているデータの質について述べる。検討したものは、次の二つの冊子の中で説明されているデータとそれを評価に利用したときの方法論である。

文献 1. ICCVAM Test Method Evaluation Report

文献 2. ICCVAM Background Review Document

ICCVAM のこの検討結果は、2008 年 5 月に公開されたものであり、それより前の ECVAM の検討結果を利用している。従って、データを吟味するという面では、最新でかつ最も網羅的なものと考えられる。

実際、文献 2 の § 9 では、ECVAM が 2005 年に「RPT, BET, *in vitro* pyrogen test について、実験データを持っている場合は提供してほしいと呼びかけたが反応は皆無であった」と述べている。そして、文献検索で入手した報告において、試験プロトコールが異なるがゆえに総合評価に用いなかった 10 報告 (§ 9.1) と、被験物質の関係で総合評価に取り入れなかった 9 報告 (§ 9.2) について、概要を述べている。

以上の状況を考えると、5 つの *in vitro* PBMC 試験法の性能評価は、文献 2 に採録されている資料に基づいて行うのが妥当と考えられる。

文献1において ICCVAM は、検討した5試験について、「これらの5試験は経口でない投与におけるグラム陰性エンドトキシンによる発熱性物質を評価・同定するための代替法として、完全ではないが利用可能である（… can be considered for use to detect … in human parenteral drugs on a case-by-case basis）. しかし、他の物質や器具の評価法としては不十分である。」と総合評価している（p. xvi）.

この妥当性評価は、OECDの指針書（GD34）にしたがっている。そこでの要確認項目（Modular approach）は次の通りである。

- (i) 当該試験法の定義（目的、必要性、科学的根拠を含む） Test definition (including purpose, need and scientific basis)
- (ii) 施設内の同等性・再現性 intra-laboratory repeatability and reproducibility
- (iii) 施設間の易移転性 inter-laboratory transferability
- (iv) 施設間の再現性 inter-laboratory reproducibility
- (v) 予測能（正確性） predictive capacity (accuracy)
- (vi) 適用限界 applicability domain
- (vii) 性能標準 performance standards

これらの各確認項目のうち、(i) 試験の標準手順とその正当化、(vi) 適用限界、(vii) 標準性能、については、被験物質や試験条件の技術的吟味が主要で、データの質について議論する事柄はほとんどない。

データの質について着目すべき吟味点は次の通りである。

(ii) 「施設内同等性・再現性」の根拠とされたデータの完全性（integrity）を確認するには、同じときに反復したとされる実験が、「・試料名をコード化してかつ適切に混ぜて行われたものかどうか、・どの程度の時間範囲で行われたか、・恣意的なデータ選択が行われなかったか、・判定基準が後知恵的でなかったか、」などを吟味することが必要である。

(iii) 「施設間の易移転性」を確認する方法論は国際的にも、国内的にも確立していないので、これについてはどのような検討がされているかを吟味することが必要である。

(iv) 「施設間の再現性」については、多くの被験物質を多くの施設で分業的に確認する、という方針を過去の日本でのバリデーション研究で取ってきたが、欧米ではそうではなく、少数の被験物質を多くの施設で共通に確かめることと、多くの被験物質を少数の施設で確かめること、の両方で良いとしてきた。このECVAMとICCVAMの方針に基づいて得られた結果が適切かどうかを、日本の方針に基づいて吟味することが必要がある。

(v) 「予測能（正確性）」については、黄金標準となる真の評価が本当に「真」なものかどうか疑問が生じることが稀でない。実際、人での結果と動物（*in vivo*）での結果の類似性（similarity）は、完全に一致するものではないことが知られている。これについて吟味することが必要である。

(v) で取り上げられている正確度について、文献1は表4-1のデータを示している。ただしこの表では、英語で“accuracy”と書かれているものを一致度（concordance）と置き換えてある。概念としての正確性と、数値としての比率を区別するためである。

表 4-1

Test method	Concordance	Sensitivity	Specificity
Cryo WB/IL-1	92% (110/120)	97% (75/77)	81% (35/43)
MM6/IL-6	93% (138/148)	96% (85/89)	90% (53/59)
PBMC/IL-6	93% (140/150)	92% (83/90)	95% (57/60)
PBMC/IL-6 (Cryo) *	87% (130/150)	93% (84/90)	77% (46/60)
WB/IL-6	92% (136/138)	89% (79/89)	97% (57/59)
WB/IL-1 (Tube)	81% (119/147)	73% (64/88)	93% (55/59)
WB/IL-1 (96-well plate) *	93% (129/139)	99% (83/84)	84% (46/55)

文献の1, 2ではこの種の表に、偽陰性率、偽陽性率等の数値も併記されているが、これらは感度と特異度から引き算で簡単に計算されるものであるから、データの質の吟味には不要である。

評価される試験法が5試験なのに、この表に7試験があるのは、*をつけた試験がそのすぐ上の試験の変法として ECVAM のバリデーション研究で実験が行われ、データが評価に利用されたためである。

表 4-1 に示されている値は、ウサギを用いる発熱性物質試験を黄金標準として示されている。文献 2 ではこれについて、ウサギでの結果とヒトでの結果はおおむね類似している、と述べている。ただし、用量反応関係はウサギよりヒトの方が急峻になる傾向があることを注意している。これがどのように発熱性評価に影響するかは明らかでないが、代替法としての評価には影響しない程度と考えられる。

表 4-1 で示されている予測性能は、PBMC/IL-6 については原法、WB/IL-6 については変法の標準作業手順 (SOP) を用いることで、どの試験法も非常に良いことを示している。

問題としては、この変法がどのような経緯でバリデーション研究に加えられたかである。評価においては、SOP を固定して比較するのが原則だからである。

表 4-1 のデータについては、評価に採用されている実験データの量が、試験毎に異なっていることを注意しなければならない。この理由について、文献 1 は、§ 5.3 “Description of the Statistical Approaches Used to Evaluate the Resulting Data” において次のように述べている。

「再現性解析においては、1 実験 (run) が 4 反復 (quadruplicate) の測定値からなっているので、その変動係数 (coefficient of variation: CV) で反復におけるデータの精度を評価し、試験毎に多少異なる、 $CV < 0.25$ から $CV < 0.45$ という基準でデータの採否を決めた。」

これは、バリデーション研究の実験データから、精度の悪さを反映しているデータを除去して計算したことを意味している。この基準をいつの時点 (事前か事後か) どのような正当化に基づいて導入されたかは、明らかでない。

データの最大量が、10 物質 x 5 施設 x 3 実験、の 150 実験 (runs) であるから、データ量が少ない試験は、それだけ試験法としての再現性が悪かったと評価すべきである。たとえば、Cryo WB/IL-1 や WB/IL-1 (96-well plate) における高い感度は、陽性物質 90 実験の 14%、7% を除外して評価した値であることに注意すべきである。極端な場合として、除外されたものがすべて「適正に評価されなかったもの」とすれば、Cryo WB/IL-1 の感度は、83%になる。

感度と特異度は、取引関係 (trade-off relationship) を持っている。すなわち、判定限界値 (cut-off value) を変えれば、一方が大きくなることで他方が小さくなる、という関係を持っている。このデータに基づいて判定限界値を調整することで、感度や特異度は変更できる。しかしそうすると、バリデーション研究の正統的遂行がおかされる。このデータに基づいて、SOP の変更を行ったとすると、その妥当性を新たに検証しなければならない。

文献 2 の § 5.5 によると、被験物質は基本的にはコードをつけて実験に供されたが、3 物質だけは例外であった。すなわち、「the identity of the three test substances was not blinded」とされている。その理由が明らかでない。

ICCVAM は易移転性についての検討を全くしていない。これは、わずか 3 施設しかバリデーション研究に参加させていなかったためと考えられる。GD34 では、要確認項目としているのに、ECVAM も ICCVAM も、試験法の易移転性は重視していないようである。

実際に易移転性を評価するには、日本のバリデーション研究で行っているように、比較的広い範囲で一般を代表できる施設で実験を行うべきであるが、ECVAM にも ICCVAM にも、そのような視点が存在していないようである。これが問題であることは、日本から提起・発信していくべきではないだろうか。

実験の反復安定性 (repeatability) と施設内再現性 (intra-laboratory reproducibility) は、概念としては違うことが GD34 の用語集 (Glossary) で示されている。しかし ECVAM のバリデーション研究では、この区別がされておらず、文献 2 では、§ 7.2.2 で両方を 3 実験 (runs) での結果の対ごとの一致率で評価している。この評価法自体は悪くないが、その際に都合の悪いデータの除去が行われたようである。これについては元データを吟味する必要がある。

Lab 2 には問題があるようで、データが除外されているだけでなく、“not included” の欄が一つあって、“NI: Not included due to lack of sufficient data. The sensitivity criteria were not met for 1 of 3 substances in run 2, and 1 of 3 substances in run 3” と注記されている。

数値としては悪くないが、Lab 2 での除外が何を意味するか、検討が必要である。

表 4-2

Lab	WB/IL-1			WB/IL-6			PBMC/IL-6			MM6/IL-6		
	Lab1	Lab2	Lab3	Lab1	Lab2	Lab3	Lab1	Lab2	Lab3	Lab1	Lab2	Lab3
1vs2	92%(1/12)	100%(8/8)	100%(12/12)	75%(9/12)	92%(1/12)	100%(12/12)	92%(1/12)	100%(12/12)	100%(12/12)	100%(12/12)	92%(1/12)	100%(12/12)
1vs3	83%(10/12)	88%(7/8)	92%(1/12)	100%(12/12)	92%(1/12)	100%(12/12)	100%(12/12)	100%(12/12)	92%(1/12)	100%(12/12)	92%(1/12)	92%(1/12)
2vs3	92%(1/12)	NI	92%(1/12)	75%(9/12)	92%(1/12)	100%(12/12)	92%(1/12)	100%(12/12)	92%(1/12)	100%(12/12)	100%(12/12)	92%(1/12)
All	83%		92%	75%	92%	100%	92%	100%	94%	100%	92%	92%

施設間再現性 (inter-laboratory reproducibility) も施設内再現性と同一ように、一致率で検討がされている。結果は表 4-3 にまとめられている。

表 4-3

Lab.	Test Method				
	WB/IL-1	Cryo WB/IL-1	WB/IL-6	PBMC/IL-6	MM6/IL-6
1vs2	92%(77/84)	92%(11/12)	72%(78/108)	81%(87/108)	97%(105/108)
1vs3	77%(83/108)	92%(11/12)	75%(81/108)	86%(93/108)	89%(96/108)
2vs3	68%(57/84)	92%(11/12)	97%(105/108)	89%(96/108)	86%(93/108)
All3	58%(167/288)	92%(11/12)	72%(234/324)	78%(252/324)	86%(279/324)

特定の施設が他とかけ離れている、という現象は起こっていない。WB/IL-1 β の施設間再現性が悪いようである。

データの吟味は、13人の委員からなる、*in vitro* Pyrogenicity Independent Peer review Panel (Panel) で行われた。委員の中には統計に詳しいと思われる委員が一人、日本人が一人含まれている。

文献1の Executive Summary (p. xv) の中段に「The Panel ... to review the ICCVAM draft BRD for errors and omissions and to discuss the current validation status to the five *in vitro* test methods.」という記載がある。この中の、「errors and omissions」がデータの何を意味するのであれば、データの取舍選択が問題になったことになる。

文献2の § 5.3 では、測定値の除外の方法についての約1ページにわたる記載があるが、最終的に定められた基準が、データを見てからの後付原則であるか、事前討議の結果であるか明らかでない。文脈をみると、前者のように感じられる。

以上、データの細部には、性能を高め評価する偏りの存在が見受けられるが、その影響はそれほど大きいものではない。ICCVAM の総合評価の結果は、おおむね受け入れて差し支えないと考えられる。

試験法の正確性を評価する物質の *in vivo* および参照データ

・ *In vivo* データについて

WB/IL-1, CRYO WB/IL-1, WB/IL-6, PBMC/IL-6, MM6/IL-6の5種類の *in vitro* PBMC試験法に関する ECVAM BRD March, 2006 Section 3.3 (ICCVAM BRD Appendix A1 ~ A5 May 2008 に収載) および ICCVAM BRD May, 2008 (Section 3.3) には、評価に使用した13種類の物質の一覧が記載されているが、ECVAM BRD March, 2006 Section 4.1 および ICCVAM BRD May 2008 (Section 4.2) によれば、これらの物質でのバリデーションには、倫理的な理由からウサギを使用した発熱性物質試験は実施されていない。

これは、エンドトキシンをスパイクした13種類の物質についてのウサギ発熱性物質試験での発熱に関する干渉作用の有無は明らかにされなかったことを意味する。これと同様に、13種類の物質でのエンドトキシン以外の発熱物質についての反応に対する干渉作用の有無が明らかではない。

その代わりに他の研究成果によるデータが参考資料として使用されている。これは、ドイツの

Paul-Ehrlich Instituteによって得られたデータで、エンドトキシンを用いた171羽のウサギ(Chinchilla Bastards種)による発熱閾値の調査で、被験ウサギの50%が0.55°Cの体温上昇を示す体重1kgあたりのエンドトキシン量は5EUであったとしている。

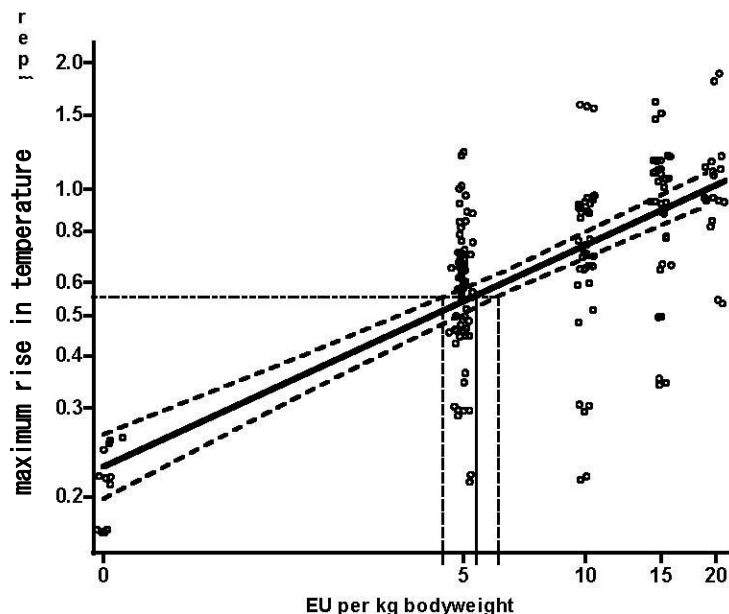


図4-1

ECVAM BRD March, 2006 Section 4.1 Figure 4.1.1 "Dose-Temperature of standard endotoxin applied to Chinchilla Bustards (n=171)"より

これは、ヒト男性ボランティアにエンドトキシンを投与した研究と同等の結果であり、エンドトキシンに対するウサギとヒトの発熱反応が類似であるとしている。(ECVAM BRD March, 2006 Section 4.5)

また、5種類のECVAM BRD March, 2006 Section 9およびECVAM Response to ICCVAM Questions (ICCVAM BRD Appendix Bに収載)にて、*in vitro* PBMC試験法、ウサギ発熱性物質試験、エンドトキシン試験についての過去の文献による比較が報告された。

ここでは、実際の臨床使用において発熱の副作用報告のあったヒト血清アルブミン製剤、第Ⅷ因子製剤、および血漿分画製剤にエンドトキシンをスパイクした試料を用いて、ウサギ発熱性物質試験、エンドトキシン試験と、*in vitro* PBMC試験法の報告があり、非エンドトキシン性発熱物質やウサギで発熱を起こさないがヒトで発熱を起こす物質について、*in vitro* PBMC試験法により検出可能であったとしている。さらに、エンドトキシンと共に各種グルカンやリポテイコ酸(LTA)等での報告や、真菌孢子、各種医療材料、脂質等での報告があり、ここでも*in vitro* PBMC試験法で検出が可能であったとしている。内容を以下にまとめた。

- ・発熱の副作用報告のあったヒト血清アルブミン製剤について、ウサギ発熱性物質試験およびエンドトキシン試験では全て陰性であったが、*in vitro* PBMC試験法のうち、WBを用いる試験法では、ドナー数5の平均値が対照ロットの平均値と5.52倍の差が見られた事が示された。

表4-4

Donor	Incriminated batch IL-1 (pg/ml)	Control batch IL-1 (pg/ml)	Quotient incriminated/control
1	79.0	4.0	19.75
2	14.1	3.9	3.61
3	44.3	15.0	2.95
4	20.9	14.9	1.4
5	71.9	3.9	18.44
Mean	46.04	8.34	5.52

ECVAM Response to ICCVAM Questions 1. a "Fever reactions caused by a batch of Human Serum Albumin Table: Incriminated Human Serum Albumin" (ICCVAM BRD Appendix B)より

- ・第Ⅷ因子製剤について、6製造業者の製剤各3ロットに、エンドトキシンをスパイクした時のウサギ発熱性物質試験およびWBを用いる試験法による比較では、ウサギの発熱閾値に相当する5 IU/kg体重のエンドトキシンを投与されたウサギでは明らかな陽性の結果が得られなかったが、WBを用いる試験法(WB/IL-1)では全て陽性の結果が示された。

表4-5

Endotoxin (WHO Standard)		Rabbit Pyrogen Test 3 animals per test			WB n = 4 donors each	
Rabbit	IVPT	positive	repetition possible	negative	positive	negative
IU LPS/kg (1mL/kg)	IU LPS/mL	> 2.65 °C	> 1, 15 < 2.65 °C	< 1.15 °C		
0	0	0	0	18	0(1)*	72(71)*
5	5	0	17	1	72	0
15	15	11	7	0	72	0

ECVAM Response to ICCVAM Questions 1. b "Coagulation Factor VIII Concentrates Table: Comparison study Factor VIII (18 batches, 162 rabbits)" (ICCVAM BRD Appendix B) より

* testing the same sample, the blood of 3 donors remained negative, the blood of 1 donor reacted slightly positive in the first experiment, the repetition was negative

(* 初回の試験で1ドナーの血液のみ弱い陽性反応を示したため、同じ試料で再試験を実施した結果は陰性、他の3ドナーの血液も陰性。)

- ・発熱を起こした血漿分画製剤について、ウサギ発熱性物質試験で陽性、エンドトキシン試験で陰性であったと報告された。(図表なし: ECVAM Response to ICCVAM Questions 1. c "Pyrogenic batch of a plasma derivative" (ICCVAM BRD Appendix B) より)
- ・ヒト血清アルブミンについて、5製造業者の各種濃度製剤各3バッチ(合計29バッチ)に、エンドトキシンをスパイクした時のウサギ発熱性物質試験およびWBを用いる試験法による比較では、ウサギの発熱閾値に相当する5 IU/kg体重のエンドトキシンを投与されたウサギでは、29バッチの製剤中5バッチのみ陽性であったが、その他のバッチでは明らかな陽性の結果が得られなかった。一方、WBを用いる試験法では全て陽性の結果が示された。

表4-6

Endotoxin (WHO Standard)		Rabbit Pyrogen Test 3 animals per test			WB	
Rabbit	IVPT	positive	repetition possible	negative	positive	negative
IU LPS/kg (1mL/kg)	IU LPS/mL	> 2.65 °C	> 1, 15 < 2.65 °C	< 1.15 °C		
0	0	0	0	29	0	29
5	5	5	23	1	29	0
15	15	21	8	0	29	0

ECVAM Response to ICCVAM Questions 1. e "Table: Comparison study Human Serum Albumin (29 batches, 261 rabbits)" (ICCVAM BRD Appendix B) より

- ・発熱の副作用報告のあったゼラチン含有輸液製剤について、副作用事例の生じていない対照バッチと共に、エンドトキシン試験、ウサギ発熱性物質試験およびWBを用いる試験法で比較を行った結果、WBではヒトで発熱を起こした2つのバッチと陰性対照を識別できたのに対して、エンドトキシン試験では全て陰性、ウサギ発熱性試験ではヒトで発熱を起こした2バッチの内1バッチが陰性で、ヒトに発熱性を有するがウサギには発熱を起こさない事例が示された。

表 4-7

batch	LAL test	rabbit test	fever in patients	WB		
				IL-1 (pg/mL)	IL-6 (pg/mL)	TNF (pg/mL)
A	negative	negative	no	8.5	28.0	28.2
B	negative	positive	yes	142.6	654.4	67.6
C	negative	negative	yes	421.5	9444.0	116.7
cut off:				32.6	127.6	43.6

ECVAM Response to ICCVAM Questions 1. f (ICCVAM BRD Appendix B) "Table 5: Incriminated infusion solution containing gelatine"より

- 11の物質について、それぞれの試験法で反応を示した最小濃度 (ng/mL) が報告された。3種類のグルカンは、エンドトキシン試験で偽陽性の結果となり、エンドトキシン試験で偽陽性のグルカンとカードランに反応せず、エンドトキシン試験で偽陰性のLTAに反応したと報告された。

表4-8

	WBT/IL-1	WB/IL-6	PBMC/IL-6	MM6/IL-6	THP-1/Neo
Curdlan	1000	1000	100	1000	1000
Glucan-Barley	negative	negative	negative	negative	negative
Glucan-Yeast	not done	negative	negative	negative	negative
Zymosan	negative	10000	10000	negative	10000
PHA-L	100	10000	100	100	1000
PHA-E	negative	negative	negative	negative	negative
Lipid A	10000	1000	10000	1000	negative
Glucan STD	negative	negative	negative	negative	undiluted
Endotoxin-C	4	40	0.4	4	4
Endotoxin-G	0.4	40	4	4	4
LTA	1000	1000	100	1000	1000

ECVAM Response to ICCVAM Questions 2. (ICCVAM BRD Appendix B) "Table: Smallest concentration [ng/mL] or dilution of substances active in the respective method"より

- 12人のドナー血液によるエンドトキシン (0.5EU/mL) と B. subtilis 由来LTAの反応性について、いずれも明らかな陽性反応を示したと報告された。

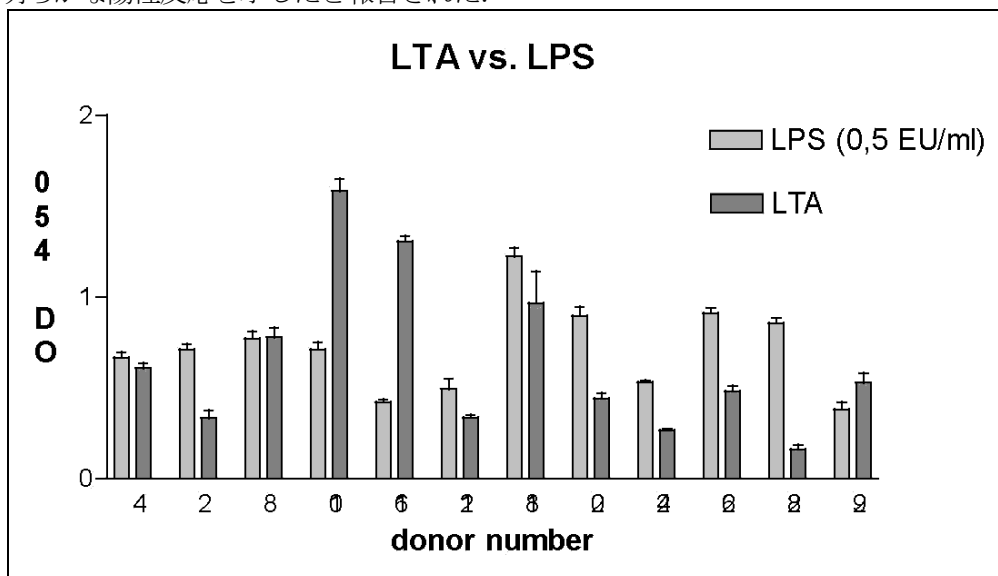


図4-2

ECVAM Response to ICCVAM Questions 2. "Fig. Reactivity of 12 donors towards LPS E. coli 0113: H10 (0.5 EU/mL) and LTA from B. subtilis"(ICCVAM BRD Appendix B)より

- 真菌胞子によるヒト全血のIL-1 β 放出は、リポ多糖 (LPS) 阻害物質であるポリミキシンBによって抑制されないことからエンドトキシンによる反応では無いことが示され(図4-3および4-4), 一方, *E. coli* 由来のLPSはポリミキシンBによってIL-1 β 放出が抑制される. したがって, 真菌由来の非エンドトキシン・発熱性に対しても活性を有することが示された.

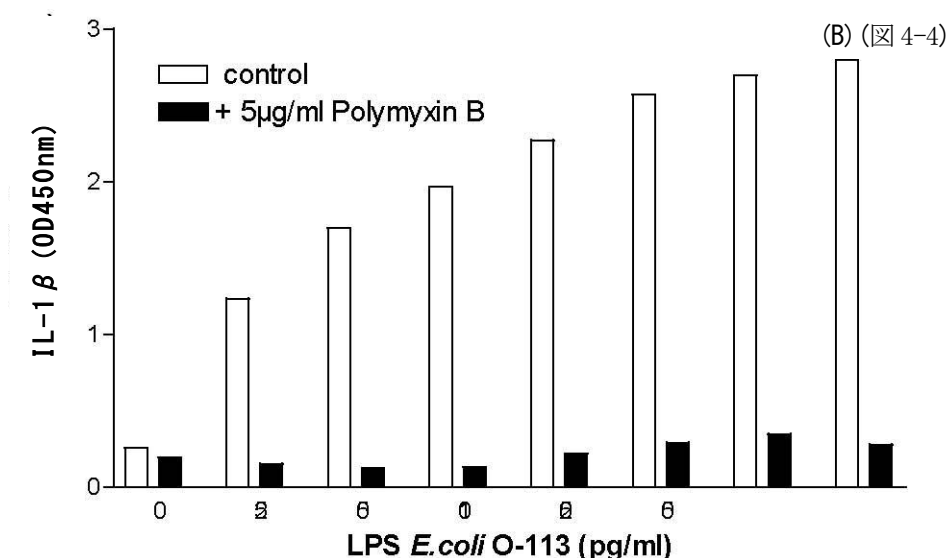
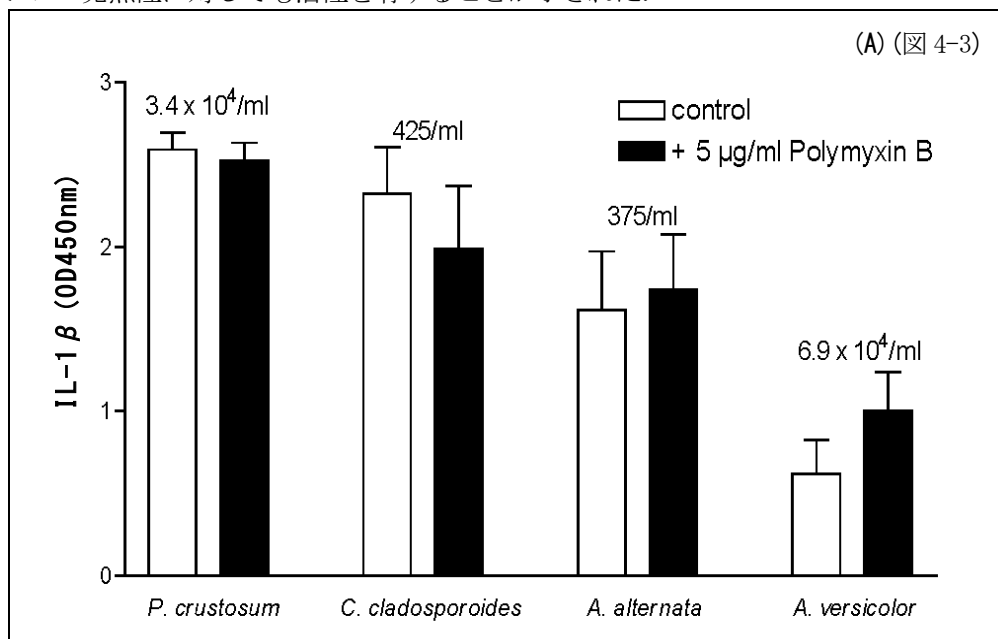


図4-3および4-4

ECVAM Response to ICCVAM Questions 2. "Figure 8: IL-1 release in human whole blood in response to fungal spores (A) is not inhibited by 5 μ g/ml polymyxin B, mean of 4 donors (\pm SEM), numbers above the bars indicate the spore counts employed. In contrast (B), the response to LPS is inhibited over a wide concentration range, mean of double values." (ICCVAM BRD Appendix B) より

- 各種医療材料について, それら疑わしい物質の抽出液をウサギに移植することは, 侵襲性により発熱性汚染と関連しない反応を引き起こす可能性があり, 倫理面や科学的見地から問題があると述べている. *in vitro* PBMC試験法は抽出液が必須ではなく, 血液に直接接触させることで, エンドトキシンだけではなく全ての関連した発熱性を検出可能との報告が示された.

ECVAM Response to ICCVAM Questions 3. "Adaptation of the WB/IL-1 to biocompatible materials" (ICCVAM BRD Appendix B) より

- ・透析装置および透析液について、米国ではダイアライザーを再処理して使用している透析施設は72%あり、また、透析施設で透析液の調製に使用する純水や透析液、フィルターや透析装置内配管のデッドスペース、等による細菌の増殖に起因する腎不全患者の透析終了後の発熱が問題となり、Association for the

Advancement of Medical Instrumentation (AAMI) によって1993年に処理水と透析液の従属栄養細菌に関する基準が設けられたが、1994年ドイツ、ギリシャ1998年、米国1990年、カナダ1995年の調査結果では、このAAMIの緩い基準でさえ完全に達成できていないことが示された。多数のグラム陽性菌汚染の報告もあり、透析チューブ内のバイオフィルム片を調査したところ、*in vitro* PBMC試験法では、エンドトキシン試験の20倍高い反応を示す調査結果も示された。エンドトキシンだけではなく菌体外毒素やペプチドグリカン等のサイトカイン誘導物質が透析膜を通過する可能性も指摘され、米国での1991年の調査では1年間に全透析施設の20%で複数の臨床的な発熱が報告されている。こうした慢性的に刺激を受けた患者の血液のサイトカインレベルは健常者のそれよりも高いとの報告が示された。(図表なし)

ECVAM Response to ICCVAM Questions 3. "Dialysis"(ICCVAM BRD Appendix B)より

- ・脂質製剤について、ビタミン剤やステロイド剤などの一部の製剤は非親水性の性状や筋注や皮下注等の投与経路の違いから、ウサギの静脈内に投与することが出来ず、また、エンドトキシン試験も水性試料を対象としているため試験が困難であるが、試験に対して非干渉性の油脂で希釈することにより、*in vitro* PBMC試験法の適用が可能となることが示された。(図表なし)

ECVAM Response to ICCVAM Questions 3. "Lipidic formulations"(ICCVAM BRD Appendix B)より

- ・発熱性物質の標準品について

in vitro PBMC試験法に関するECVAM BRD March, 2006 Section 2.1(ICCVAM BRD Appendix A1~A5 May 2008に収載)およびICCVAM BRD May 2008 Section 2.1によれば、発熱性物質の標準品として、WHO-LPS エンドトキシン標準品[(94/580, 0113:H10:K-)]が使用されており、エンドトキシンの由来となる大腸菌の株はU. S. Pharmacopoeia (USP) エンドトキシン標準品と同じものである。

発熱性物質の標準品としてエンドトキシンを使用した理由は、エンドトキシンが安定的に入手可能で高い検出精度を有する標準化された発熱物質であるからとされている。これは、既に広く普及しているカプトガニ血球成分から成るライセート試薬を用いたエンドトキシン試験において、このエンドトキシン標準品が使用されているからであるが、この選択理由は妥当と考える。ここで、エンドトキシン標準品の表示単位がエンドトキシン試験で得られた値であることに留意する必要があるが、過去に得られたウサギの結果でエンドトキシン投与量と体温上昇の直線性が確認され、ここで得られた発熱閾値を利用して、*in vitro* PBMC試験法は「ヒトに発熱性」または「ヒトに非発熱性」を判定している(ECVAM BRD March, 2006 Section 2.2.13)ことから、エンドトキシン試験という異なる尺度での表示単位で表された標準品を使用しても問題ないと考えられる。

- ・考察

in vitro PBMC試験法で、過去の文献により非エンドトキシン性発熱性の検出が確認されている。よって、この試験法は他の発熱機序によるものは別として、ヒトに特異的な発熱物質の検出法の一つとして有用であると思われる。ただし、原料や製造工程でエンドトキシン以外の発熱物質の汚染が考えられない場合には、エンドトキシン試験で代替可能と思われることから、現在、ウサギを用いて発熱性物質試験を行っている試験対象について、発熱性物質の検出の全てを*in vitro* PBMC試験法に代替を集約するのではなく、何らかの理由からエンドトキシン試験が適用できず、ウサギによる試験でしか発熱性物質の検出が可能な試料にのみ、この*in vitro* PBMC試験法を適用すべきと考える。

5. 他の科学的な報告

In vitro 発熱性物質試験に関してヒト末梢血由来細胞などを用いた IL-1 β , IL-6 測定系以外にもウサギ発熱性物質試験の代替として可能性のある試験法が提案されている。例を挙げると、末梢血前血を用いた IL-8 産生能に基づいた試験法は IL-1 β , IL-6 より感受性が高いとされ、多くの発熱性の受容体である Toll 様受容体 (Toll-like receptor : TLR) の強制発現細胞株などはその特異性は非常に高いことが知られている。(例 : Journal of Immunological Methods 336 (2008)45-55)

また、発熱、もしくはそれに類する毒性のバイオマーカーとして多様な評価系が提案、もしくは開発研究を開始している。そのなかでも、Embryonic Stem (ES) 細胞や人工多能性幹細胞 (Induced pluripotent stem cells : iPS) 細胞を利用した毒性試験やトキシコパノミクス (トランスクリプト

ーム、プロテオーム、メタボロームを含めた網羅的な毒性解析)を利用した毒性試験などは *in vitro* と *in vivo* 試験それぞれの利点、欠点を補うものとしてその迅速な開発が期待されている。

6. 3Rs への関与

ヒト血液を用いるあるいは細胞を用いる試験法は、動物を用いた発熱性物質試験に置き換わる方法として動物福祉面で貢献するものである。

7. 本試験法の有用性と限界

in vitro PBMC 試験法のような人の白血球や細胞株によるサイトカインの産生量計測に基づく試験はウサギや人の発熱の誘導に相関はしているといえよう。しかしながら、ICCVAM の報告書にあるように、(ICCVAM (1.2.3)) 「ウサギ発熱性物質試験は(多様な原因、メカニズムに基づいて誘導される全身の発熱を検出するものであるが、本代替法は培養細胞の1-2種類のサイトカインを計量しているに過ぎない。)現時点で、IL-1 β およびIL-6が全身の発熱反応に必須であるという科学的根拠が欠落している。」とあり、「少なくともヒトの発熱反応とヒト由来細胞の培養におけるIL-1 β 、IL-6の産生との相関に関する情報をさらに追加する必要がある。特に異なる内毒素(エンドトキシン)に対する反応の仕方の違い等に留意する必要がある」と述べており、本代替法には科学的根拠が少ない、もしくは十分でないとの認識が強い。

ICCVAM の報告書でも述べられているが、ウサギ発熱性物質試験と *in vitro* PBMC 試験法の相関性に加え、他の類似点、相違点に関し多くの文献にて精査されていることも事実である。なかでも報告書が目し言及している点として、それらの文献の多くが、ウサギ発熱性物質試験の結果はヒトの発熱反応とよく相関している事実を再確認したことになっている点や、*in vitro* PBMC 試験法のウサギ発熱性物質試験との相関を示唆するデータはすべてグラム陰性細菌由来のエンドトキシンのみを引用していることがあげられる。言い換えれば代替法である *in vitro* PBMC 試験法があまりにかけ離れていてウサギ発熱性物質試験の「代替法」と定義、解釈することに無理があると思われる。

in vitro PBMC 試験法が開発された時点、ECCVAM の報告書、さらには ICCVAM の報告がなされた後も、自然免疫研究成果が新たに集積している。しかしながら、そのような最新の知識をもってしても、IL-1 β 、IL-6の本代替法はおろか、ウサギ発熱性物質試験においても、どのような「発熱性」があることが知られていて、それらがどのような細胞の受容体、シグナル伝達経路、遺伝子発現制御、転写後制御、細胞間相互作用などを介して、全身の「発熱」という計測可能なパラメーターに影響を及ぼしているか、科学的に見て明らかになったとは到底言い難い。ただし、上記の一部、たとえばエンドトキシンの自然免疫受容体、シグナル経路などは明らかになってきており、本代替法を上回る感受性、特異性をもった検出方法も知られるようになった(上記参照)。

ECCVAM、ICCVAM の報告は発表された時点で、その効力が及ぶ地域の文化的な背景に基づけば、ある程度妥当な意見が述べられている。しかし科学的な観点、および倫理的な観点から鑑みた場合、また、時間的な経過による影響も加味した上で、(必ずしも *in vitro* 発熱性物質試験の必要性があると断定はせずに)新たな発熱性テストの開発の可能性と、現行のテストの限界を独自に議論する必要があると考えられる。

8. 結論

上記の各考察より、今回提案された *in vitro* PBMC 試験法は、ウサギの使用数を削減可能とし、また、作用機序に立脚した発熱性物質試験の新たな方向性として評価でき、さらに、バリデーションでのデータの扱いに関しても納得のできる基準を満たしていると考えられる。しかし、ウサギを用いた発熱性物質試験との互換性については不明な点が多く、この試験法の代替法とするには、問題がある。国内ではウサギ発熱性物質試験からエンドトキシン試験への移行の問題も残されており、これに優先して独立した代替法とすることには無理がある。しかしながら、エンドトキシン試験が使用できないような特殊な状況やウサギとヒトでの発熱性に関する種差が想定されるような状況においてウサギ発熱性物質試験と併用するなど、限定的な使用については推奨できよう。このような *in vitro* PBMC 試験法が科学的根拠に基づいた研究により、さらに広範囲な状況で使用可能となることが望まれる。

エンドトキシン試験と発熱性物質試験に関する実態調査結果報告

日本薬局方に収載されているカプトガニ血球抽出物を用いたエンドトキシン試験法とウサギを用いた発熱性物質試験法、および *in vitro* 発熱性物質試験について、2009年1月に日本製薬工業協会の協力を得て「エンドトキシン試験と発熱性物質試験に関する現状調査のためのアンケート」と題した実態調査を行い36社(1社につき複数事業所含む)の回答を得たので、この調査結果を基に報告する。

一般にエンドトキシン試験での反応干渉因子には、ライセート試薬とエンドトキシンの一連の酵素活性化反応を阻害するものとして、酵素活性阻害剤、タンパク変性剤、キレート剤などがある。また、エンドトキシンに作用して反応を阻害するものとして、界面活性剤や、鉄、アルミニウムなどの金属イオンがある。一方、ライセート試薬とエンドトキシンの一連の酵素活性化反応を促進させるものとして、セリンプロテアーゼなどが挙げられる。その他に試料溶液の濁りや着色は光学的測定法に影響を及ぼすと言われている。また、リポソーム製剤なども反応を阻害する要因となる。実態調査の結果では、これらのエンドトキシン試験での反応干渉因子であっても、未確認の事例を除いて発熱性物質試験は実施可能であった。

日局発熱性物質試験の反応干渉因子として考えられるものは、製剤そのものが発熱または体温下降を起こす場合が考えられる。実態調査においてこのような反応干渉の報告があった3例は、未確認の事例を除いてエンドトキシン試験は実施可能であった。

In vitro 発熱性物質試験について、36社中に試験の実施経験を有する回答は無く、実例による反応干渉因子の報告は得られなかった。

実態調査で今回の評価に際して自由記入の形式で意見を募ったところ、多くの意見が寄せられた。まず、発熱性物質試験については、感度その他の問題や動物愛護の観点から、エンドトキシン試験を含めた何らかの代替法に切り替えたいという意見が多かった。

ただし、今回のヒト血液を用いる *in vitro* 発熱性物質試験 (*in vitro* PBMC 試験法) では、ヒト血液の入手方法やバイオハザードの問題、反応阻害や促進等の反応干渉因子試験の必要性を指摘する意見があった。

日本薬局方では発熱性物質試験法よりもエンドトキシン試験法を優先する規定があり、発熱性物質試験についてはエンドトキシン試験の適用が困難な品目に限り設定することとなっている。実際に新規製造品目の試験規格設定時ではこの規定が適用されており、実態調査においても最終製品での実施例について発熱性物質試験よりもエンドトキシン試験の実施頻度が高い結果となっている。しかし、日本薬局方でエンドトキシン試験が優先する規定が出来る以前は、容量10mLを超える注射剤には発熱性物質試験を適用する規定があったことから、この時期に製造(販売)承認を得た注射剤では発熱性物質試験が適用されているものが現在でも多く存在すると考えられる。これらの発熱性物質試験が適用された既承認医薬品に対して、原料や直接容器等にエンドトキシン以外の発熱性が存在する可能性がある品目やエンドトキシン試験が困難な品目を除き、現行の日本薬局方に則ってエンドトキシン試験への変更を促進することは理にかなうものと考えられる。しかし、これら発熱性物質試験が既に設定された既承認医薬品では、発熱性物質試験をエンドトキシン試験に変更するためには、変更申請時にウサギに大量のエンドトキシンを投与して得られる発熱データの提出を求められ、ウサギの個体差による感度のバラつきや動物愛護の観点等の問題で、エンドトキシン試験へ変更する際の障害となる場合もある。

エンドトキシン以外の発熱性が存在する可能性がある品目やエンドトキシン試験が困難な品目について、現状ではウサギを用いた発熱性物質試験しか方法が無い代替法の確立を望む意見が多く見受けられた。

現在行われている発熱性物質試験のすべてを今回のヒト血液を用いた代替法に切り替えるのではなく、エンドトキシン試験で代替可能なものはエンドトキシン試験に変更することが妥当と考えられ、実態調査における最終製品でのウサギ使用数内訳から見ても、エンドトキシン試験への変更だけでも相当数の発熱性物質試験に使用するウサギを減少させることが可能と考える。

エンドトキシン試験と発熱性物質試験に関する実態調査結果

2009年1月調査実施

*回答企業36社の形態：

国内企業(本調査に該当する海外向け製品あり) : 10社
 国内企業(本調査に該当する海外向け製品なし) : 17社
 海外企業(米国系) : 4社
 海外企業(欧州系) : 5社

*日局エンドトキシン試験および日局発熱性物質試験の実施経験について

36社中(複数事業所を有する企業を含む)

エンドトキシン試験の実施経験あり : 32社
 エンドトキシン試験の実施経験なし : 4社
 発熱性物質試験の実施経験あり : 24社
 発熱性物質試験の実施経験なし : 12社

内訳：両試験共に経験あり : 24社
 エンドトキシン試験のみ経験あり : 8社
 発熱性物質試験のみ経験あり : 0社
 両試験共に経験なし : 4社

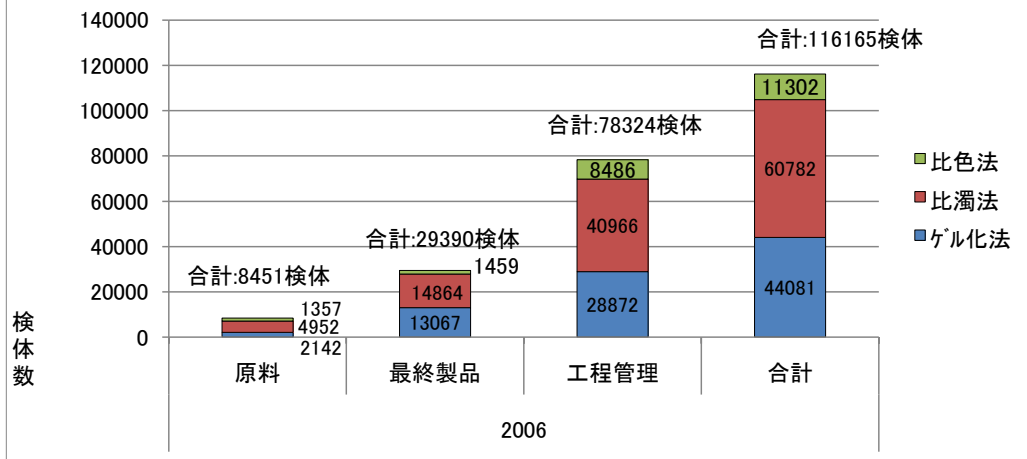
*日局エンドトキシン試験検体数内訳

36社集計		ゲル化法	比濁法	比色法	合計
2006年	原料	2142	4952	1357	8451
	最終製品	13067	14864	1459	29390
	製造工程管理	28872	40966	8486	78324
	合計	44081	60782	11302	116165
2007年	原料	3024	5912	1244	10180
	最終製品	14465	14536	1635	30636
	製造工程管理	34105	54875	8428	97408
	合計	51594	75323	11307	138224
2008年	原料	3285	5821	1189	10295
	最終製品	14461	13745	1936	30142
	製造工程管理	29408	44310	9351	83069
	合計	47154	63876	12476	123506

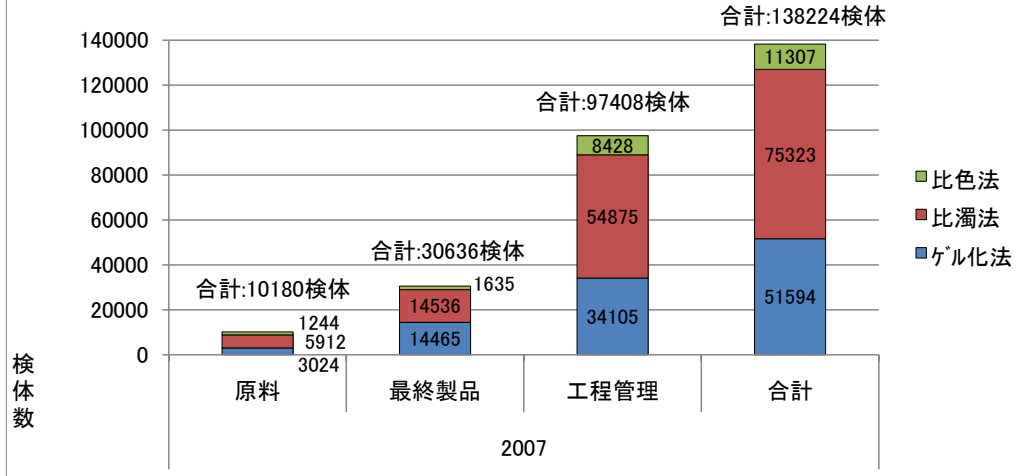
*日局発熱性物質試験検体数内訳

36社集計		検体数	ウサギ使用数(羽)
2006年	ゴム栓	561	1435
	原料	615	1570
	最終製品	2519	5715
	合計	3695	8720
2007年	ゴム栓	537	1356
	原料	495	1406
	最終製品	2618	6107
	合計	3650	8869
2008年	ゴム栓	608	1527
	原料	580	1442
	最終製品	2483	7293
	合計	3671	10262

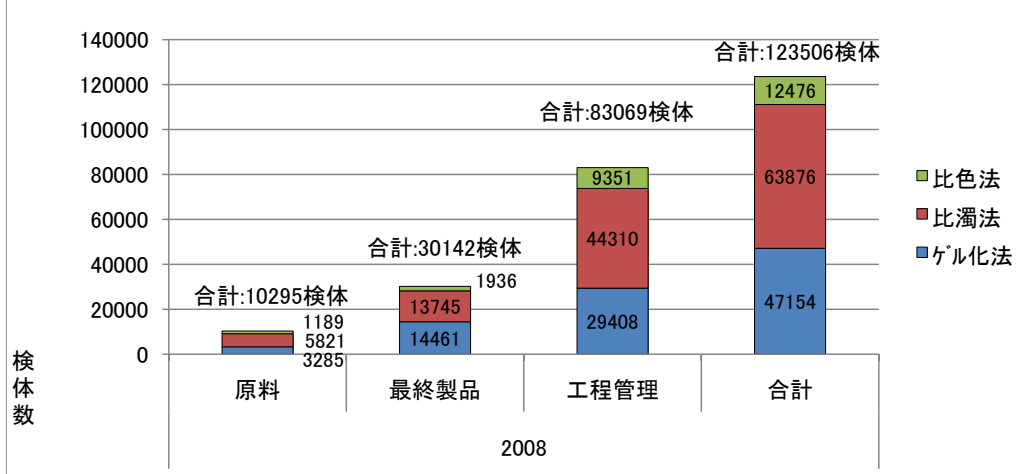
2006年 エンドトキシン試験検体数 32社集計



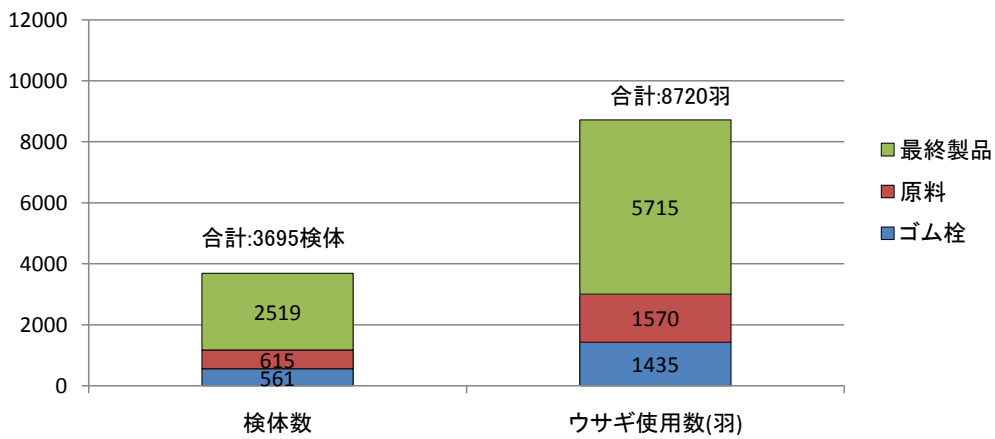
2007年 エンドトキシン試験検体数 32社集計



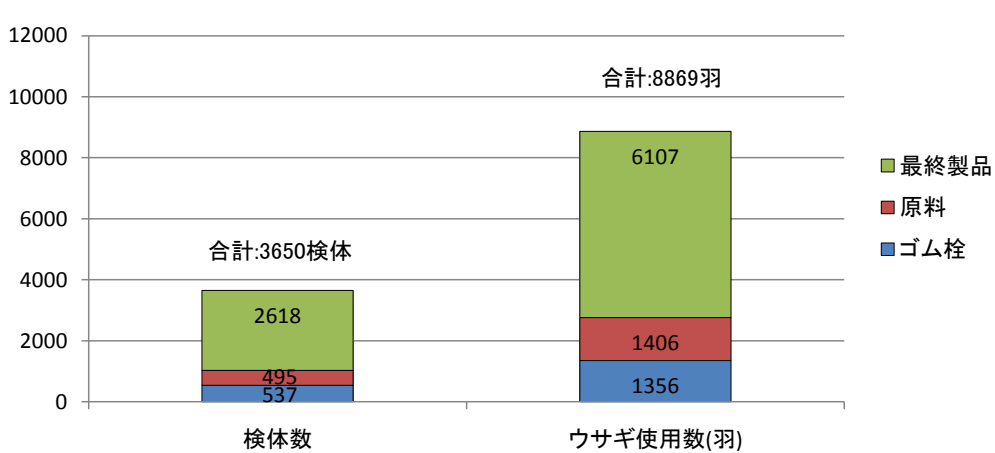
2008年 エンドトキシン試験検体数 32社集計



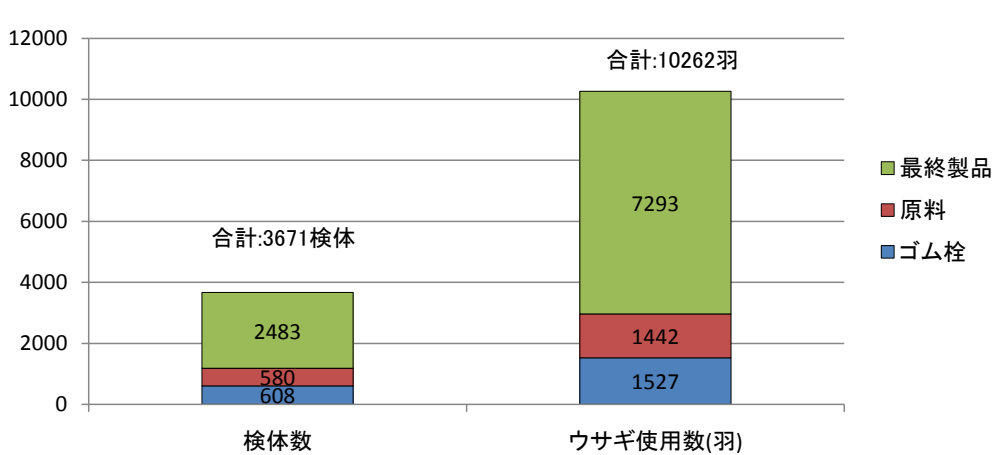
2006年 発熱性物質試験検体数 24社集計

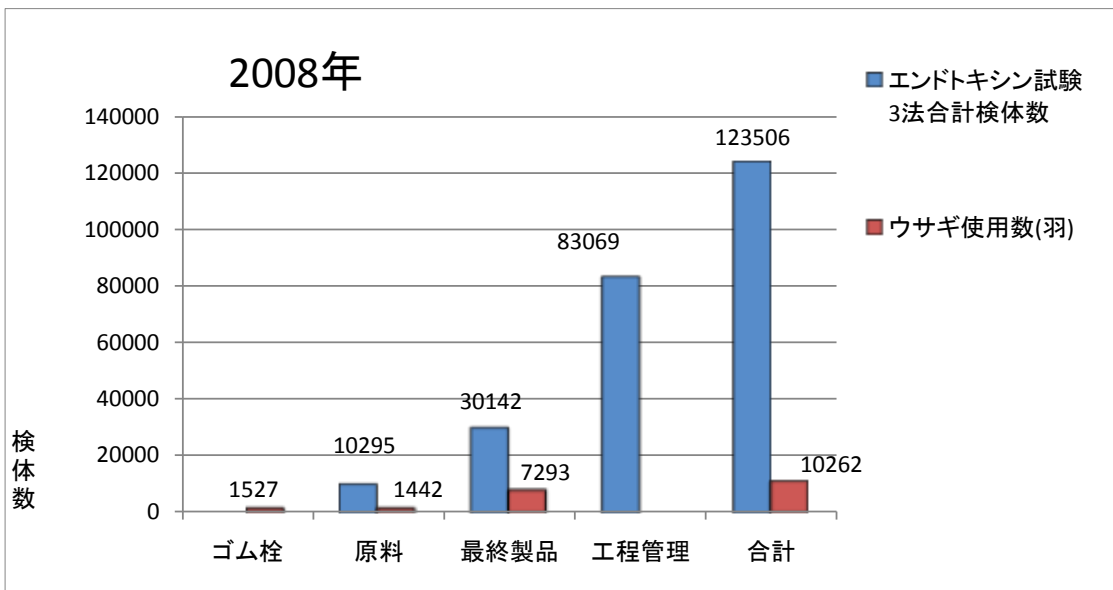
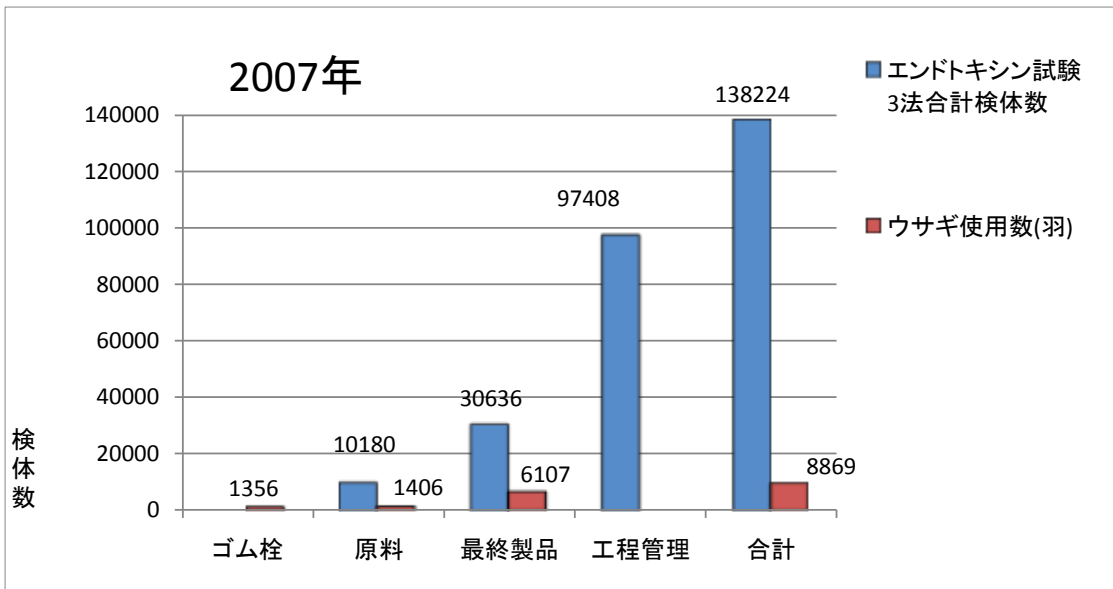
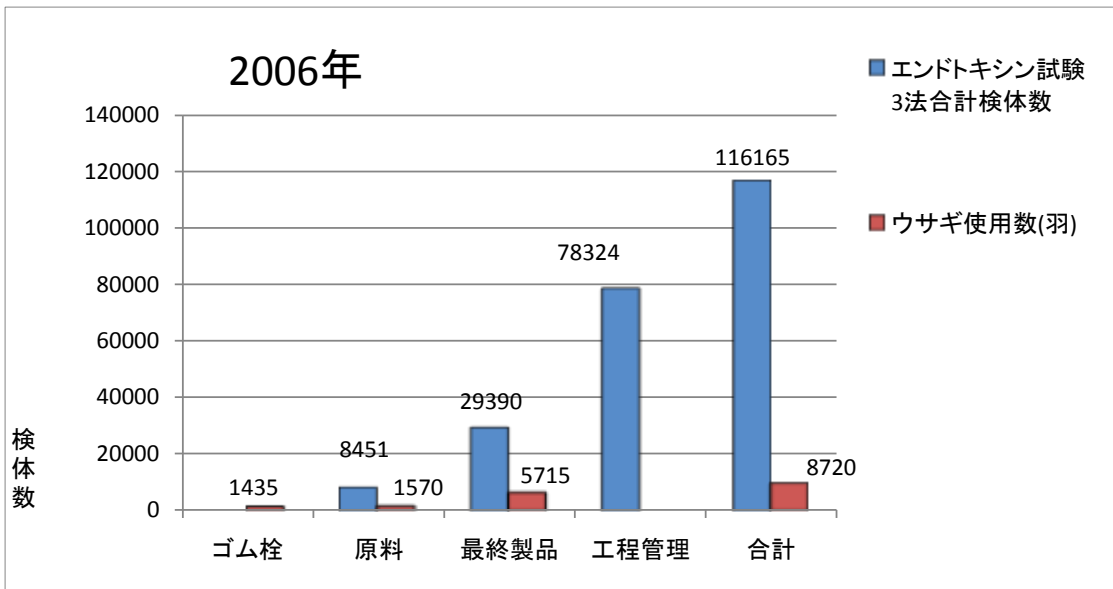


2007年 発熱性物質試験検体数 24社集計



2008年 発熱性物質試験検体数 24社集計





*日局エンドトキシン試験の実施が不可能であった事例について
 経験あり：8社
 経験なし：24社

*日局エンドトキシン試験の実施が不可能であった事例の内訳

事例	要因	薬効分類等	事例を確認した試験法	日局発熱性物質試験法の適用可否
反応阻害	アルミニウムゲルのため	(非公開)	比色法	不明
反応阻害	リポソームのため	(非公開)	ゲル化法	試験可能
反応阻害	原薬の酵素阻害作用のため	蛋白分解酵素阻害剤	ゲル化法 比濁法	試験可能
反応阻害	エマルジョン製剤であり、水層と油層の分離が困難	中枢神経系用薬	ゲル化法	試験可能
反応阻害	原薬の酵素阻害作用のため	蛋白分解酵素阻害剤	ゲル化法 比濁法 比色法	試験可能
反応阻害	リポソームのため	(非公開)	ゲル化法 比濁法 比色法	試験可能
反応促進	タンパクのため	血漿分画製剤	ゲル化法	試験可能
反応阻害	リポソームのため	抗生物質製剤	不明	試験可能

*日局発熱性物質試験の実施が不可能であった事例について
 経験あり：3社
 経験なし：21社

*日局発熱性物質試験の実施が不可能であった事例の内訳

事例	薬効分類等	日局エンドトキシン試験法の適用可否
発熱作用あり	制癌剤	試験可能
ウイルスの性状に起因した発熱作用	生物学的製剤	試験可能
抗発熱作用(阻害)	アミノ酸	不明

**in vitro* 発熱性物質試験(エンドトキシン試験以外)の実施経験について
 経験あり：0社
 経験なし：36社

*エンドトキシン試験，発熱性物質試験，*in vitro* 発熱性物質試験についての意見
 意見あり：17社
 意見なし：19社

*エンドトキシン試験，発熱性物質試験，*in vitro* 発熱性物質試験についての意見

- ・ウサギを使った眼刺激性試験と同様、可能であれば *in vitro* 試験に変更すべき。
- ・局方で発熱性物質試験を規定されていない限りは、(新たな開発品に対して)発熱性物質試験規格は設定せず、エンドトキシン試験規格を設定する方針。
- ・実施経験がないが、今後必要となった場合には *in vitro* 試験での評価系が確立されていれば有用と考える。
- ・*in vitro* 発熱性物質試験について検討を考えたい。具体的な手順等を教えて頂ければありがたい。
- ・輸液用ゴム栓以外の発熱性評価は、エンドトキシン試験で十分評価できると考える。
また、動物愛護の観点から動物の使用を削減し代替法の導入が推奨されている。従って、輸液用ゴム栓以外の発熱性物質試験は必要ないと考え。
- ・発熱性物質試験はゴム栓で外部へ試験委託を行ってきたが、ここ3年間には行ってない。
- ・日局一般試験法、輸液用ゴム栓試験法および医薬品各条の一部に規定されている発熱性物質試験について、エンドトキシン試験への置き換えを要望する。
- ・今回の趣旨とは異なるが、日・EU相互承認協定(MRA)締結内容に早期の無菌医薬品および生物学的製剤の追加を望む。
- ・動物愛護と試験の効率化の観点から、発熱性物質試験からエンドトキシン試験への変更や、新規品目については開発当初からエンドトキシン試験を設定する事例が多いと考える。エンドトキシンは最も強力な発熱性物質に相違ないが、エンドトキシン試験では検出できない発熱性物質の存在を否定するために、エンドトキシン試験導入前に、発熱性物質試験においても問題ない結果が得られることを確認しておくことは重要と考える。
- ・発熱性物質試験の早期国際調和(EP, USP, JP)を希望する。
- ・エンドトキシン試験は、エンドトキシン以外の発熱性物質を検出できない課題がある一方、発熱性物質試験は感度が低く、動物愛護の面での問題も多い。よって、*in vitro* 発熱性物質試験が、将来にこれらの問題を解決する有力な手段であるならば、業界として積極的に導入することを期待する。
- ・発熱性物質試験について、当施設では、日局準拠ではないが、ほぼ同等の手順による「医療機器の生物学的安全性試験の基本的考え方に関する参考資料について(事務連絡 医療機器審査 No. 36)」に基づき、医療機器の中間・最終製品もしくは原料・部材の安全性を評価する目的でウサギを用いた発熱性物質試験を実施している。医療機器の安全性評価の一環として実施する発熱性物質試験は、医療機器または原材料から抽出した抽出液中に、エンドトキシンおよび非エンドトキシン性発熱性物質が存在しないことを確認することを目的としており、特に、ウサギを用いた試験は化学物質等による「非エンドトキシン性発熱性物質」を検出する方法として、医療機器の承認・認証申請において審査当局から試験実施が規定されている。現在、公知の代替方法がないために、当施設では医療機器の安全性評価においてウサギを使用しており、動物愛護の面からも *in vitro* 発熱性物質試験のような動物を使用しない代替方法が受け入れられ、選択肢の1つとして拡充することに期待する。
- ・エンドトキシン試験は、比濁時間分析法で定量的にエンドトキシン濃度が判明するので、工程におけるエンドトキシン汚染の迅速管理に有効であることと、動物愛護的観点と動物飼育経費の削減に寄与してきた。また、発熱性物質試験は発熱増強活性^{*1}がない製剤に関しては、エンドトキシン試験に移行している。

(^{*1}:発熱性物質試験を実施したとき、製剤に0.5 EU/mLになるようにエンドトキシンを加えた液が、生理食塩液に0.5 EU/mLになるようにエンドトキシンを加えた対照液に比べて有意に発熱性を認めた場合、発熱増強活性があるものと判断。)

in vitro 発熱性物質試験の話題に変えると、ヒト単球を用いていることから、糖濃度が高いと、末梢血単球からのインターロイキン8(好中球、Tリンパ球に選択時に働く走化性因子である)の分泌が低下し、白血球の遊走能が低下することが知られている。このことから、製剤の成分が単球のサイトカインの分泌制御に影響し、*in vitro* 発熱性物質試験にも反応阻害などに対する反応干渉因子試験は必要なものと考え。

また、*in vitro* 発熱性物質試験は、生きた単球の安定供給、バイオハザード的観点にも問題があり、工程管理のためのエンドトキシン試験も含めてすべての試験を *in vitro* 発熱性物質試験に切り替えるのは疑問である。

医療用具および医用材料の基礎的な生物学的試験のガイドラインに記載されている発熱性物質試験は、医療用具又は医用材料から抽出した抽出液中に、材料に由来するエンドトキシン以外の発熱性物質が存在しないことを確認する試験と記載されている。(エンドトキシンを確認するには、エンドトキシン試験が別途必要)。この観点からすると、医療機器の発熱性物質試験をエンドトキシン試験に置き換えるには、生産している製品材料に文献的に既知の発熱性化学物質(例えば、ゴムの老化防

止剤 n-phenyl- β -naphthylamine, aldol- α -naphthylamine (現在は使用禁止)などが含まれないことを確認して、試験法をエンドトキシン試験法に変更していく必要があると考える。よって、*in vitro* 発熱性物質試験が化学物質の発熱性を確認する手段となれば、医療用具の(材料に由来するエンドトキシン以外の発熱性物質が存在しないことの確認を目的とした)発熱性物質試験の有効な代替法になるものと考えます。

- 有限かつ高価な天然物試薬であるライセート試薬の低減化として、日局規定の各種試験の試験回数規定を試験の信頼性を欠くことなく最小限での試験を認めて欲しい。

(例えば)

(ゲル化法予備試験のライセート試薬の表示感度試験)

エンドトキシン標準原液を現行4濃度から3濃度以上とし、繰返し4回を3回以上とする。

(ゲル化法予備試験の反応干渉因子試験)

添加エンドトキシン濃度を現行4濃度から3濃度以上とし、繰返し回数を現行2および4回をすべて2回以上とする。

(ゲル化法限度試験法) エンドトキシン無添加の陰性試験を現行2回から1回以上とする。

(ゲル化法定量試験法)

現行エンドトキシン希釈倍数を4条件から3条件以上とする。エンドトキシン無添加の陰性試験を現行2回から1回以上とする。

(比濁法の予備試験の反応干渉因子試験)

エンドトキシン添加濃度を現行3濃度から2濃度以上とする。

- 日局では発熱性物質試験よりもエンドトキシン試験が優先される規定になっているが、このような規定が出来る以前に製造販売承認を取得した注射剤には、未だに発熱性物質試験が設定された製品が多い。一般の注射剤等で考えられる微生物汚染は、製造工程での水棲菌(主にグラム陰性菌)によるものが殆どと考えられるため、原料等がエンドトキシン以外の発熱性物質で汚染される可能性があるもの以外は、速やかにエンドトキシン試験へ変更できるよう(変更申請時に必要とされるデータの簡略化を)考慮すべきと考える。発熱性物質試験も動物を使用しない方法が良いと思うが、日常試験として実施するには、今回のようなヒト血液を用いる方法では、その入手方法やバイオハザード対策等で不安がある。動物を使用せずに代替するのであれば、輸液用ゴム栓などでは(輸液用プラスチック容器試験法からプラスチック製医薬品容器試験法への改正時のように)発熱だけに絞ったものでなく、既に日局にも収載されている細胞毒性試験などで代替することは出来ないか。
- 発熱性物質試験からエンドトキシン試験へ試験法を代替する場合に過剰な実験動物データを必要とされるため代替が困難である。現在検討されている *in vitro* 発熱性物質試験には、どのようなものがあるかご教示ください。
- ライセート試薬を使用しているが、高価な試薬なので安価になれば良いと思う。比濁法で実施しているが、試験管も使い捨てのため Reuse できる洗浄方法などがあれば良いと思う。
- 輸液用ゴム栓試験での発熱性物質試験はエンドトキシン試験への代替が可能なのか？

以上



STATEMENT ON THE VALIDITY OF IN-VITRO PYROGEN TESTS

At its 24th meeting, held on 20-21 March 2006 at the European Centre for the validation of alternative methods (ECVAM), Ispra, Italy, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC)¹ unanimously endorsed the following statement:

Following a review of scientific reports and peer reviewed publications on the following range of in-vitro pyrogen tests:

1. Human Whole Blood IL-1,
2. Human Whole Blood IL-6,
3. PBMC IL-6,
4. MM6 IL-6, and
5. Human Cryopreserved Whole Blood IL-1,

it is concluded that these tests have been scientifically validated for the detection of pyrogenicity mediated by Gram-negative endotoxins, and quantification of this pyrogen, in materials currently evaluated and characterized by rabbit pyrogen tests.

These methods have the potential to satisfy regulatory requirements for the detection and quantification of these pyrogens in these materials subject to product-specific validation.

The test methods have the capacity of detecting pyrogenicity produced by a wider range of pyrogens, but the evidence compiled for, and considered within this peer review and validation process, is not sufficient to state that full scientific validation of this wider domain of applicability has been demonstrated and confirmed.

Thus, the above test methods can currently be considered as full replacements for the evaluation of materials or products where the objective is to identify and evaluate pyrogenicity produced by Gram-negative endotoxins, but not for other pyrogens.

This endorsement takes account of the dossiers prepared for peer review; the views of independent experts who evaluated the dossiers against defined validation criteria; supplementary submissions made by the Management Team; and the considered view of the Peer Review Panel appointed to oversee the process.

Thomas Hartung
Head of Unit
ECVAM
Institute for Health & Consumer Protection
Joint Research Centre
European Commission
Ispra

21 March 2006

1. The ESAC was established by the European Commission, and is composed of nominees from the EU Members States, industry, academia and animal welfare, together with representatives of the relevant Commission services.

This statement was endorsed by the following Members of the ESAC:

Prof Helmut Tritthart (Austria)
Dr Dagmar Jírová (Czech Republic)
Prof Elisabeth Knudsen (Denmark)
Dr Timo Ylikomi (Finland)
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The following Commission Services and Observer Organisations were involved in the consultation process, but not in the endorsement process itself.

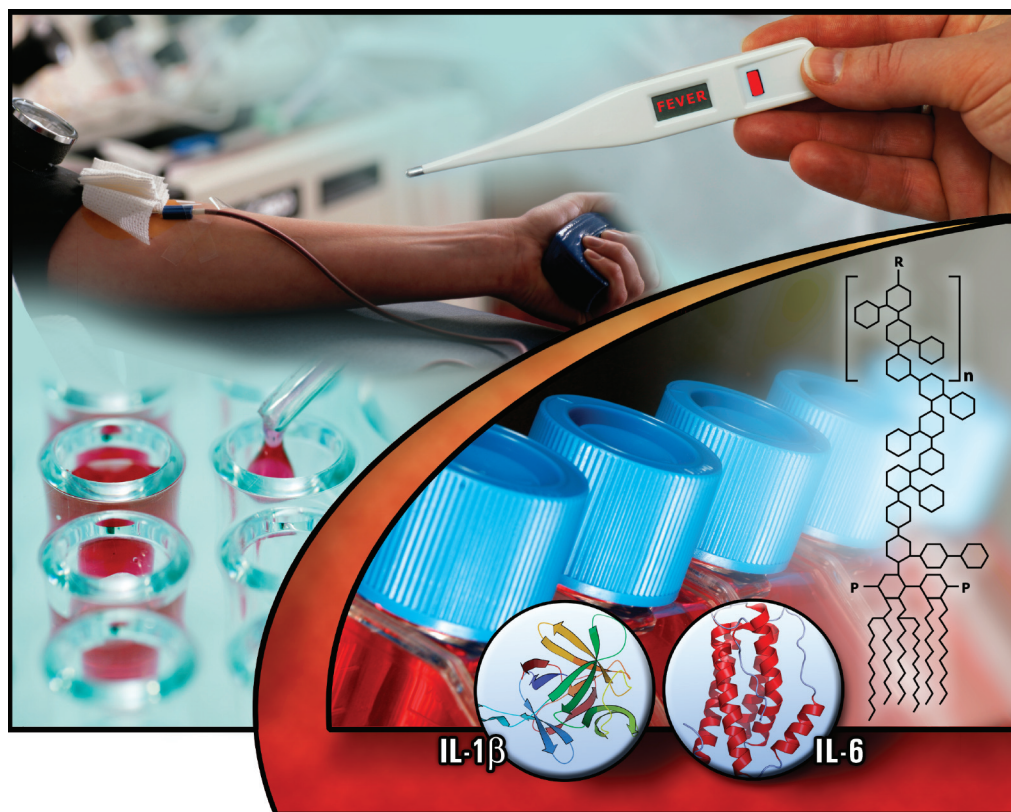
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Annex

The novel pyrogen tests are based on the human fever reaction. Monocytoid cells, either primary from human blood or as propagated cell lines, detect pyrogens of different chemical nature and respond by the release of inflammatory mediators such as cytokines. Since lipopolysaccharides from Gram-negative bacteria are the only type of proven pyrogen, for which an International reference material is available, the tests were standardised to detect the presence of significantly less than 0.5 Endotoxin Units of this preparation, which is considered to be the threshold level for fever induction in the most sensitive rabbit species according to pharmacopoeia test procedures.

The five tests which were sufficiently reproducible and exceeded the rabbit test with regard to sensitivity and specificity for the detection of lipopolysaccharide spiked samples, differ with regard to cell source and preparation, cryopreservation and cytokine measured. The tests have been described elsewhere (1-4). The concept of the validation study (5) and the international validation studies are available (6-7).

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7. Schindler S, Spreitzer I, Loschner, Hoffmann S, Hennes K, Halder M, Brügger P, Frey E, Hartung T and Montag T. (2006) International validation of pyrogen tests based on cryopreserved human primary blood cells. *J. Immunol. Meth.* 316, 42-51.



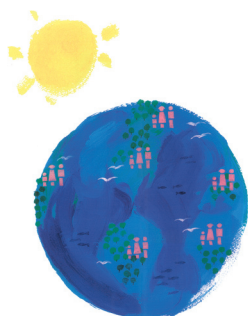
ICCVAM TEST METHOD EVALUATION REPORT

Validation Status of Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
National Institutes of Health
U. S. Public Health Service
Department of Health and Human Services



**About the Interagency Coordinating Committee on the Validation of Alternative Methods
(ICCVAM)
and
The National Toxicology Program Interagency Center for the Evaluation of Alternative
Toxicological Methods (NICEATM)**

In 1997, the National Institute of Environmental Health Sciences (NIEHS), one of the National Institutes of Health (NIH), established ICCVAM to:

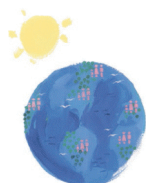
- Coordinate interagency technical reviews of new and revised toxicological test methods, including alternative test methods that reduce, refine, or replace the use of animals
- Coordinate cross-agency issues relating to validation, acceptance, and national and international harmonization of new, modified, and alternative toxicological test methods

On December 19, 2000, the ICCVAM Authorization Act (Public Law 106-545, 42 U.S.C. 2851-3) established ICCVAM as a permanent interagency committee of NIEHS under NICEATM.

ICCVAM is comprised of representatives from 15 U.S. Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability. ICCVAM promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety or hazards of chemicals and products and that refine (decrease or eliminate pain and distress), reduce, and/or replace animal use. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. More information about ICCVAM and NICEATM can be found on the NICEATM-ICCVAM web site (<http://iccvam.niehs.nih.gov>) or obtained by contacting NICEATM (telephone: [919] 541-2384, e-mail: niceatm@niehs.nih.gov).

The following Federal regulatory and research agencies are ICCVAM members:

- Consumer Product Safety Commission
- Department of Agriculture
- Department of Defense
- Department of Energy
- Department of Health and Human Services
 - Centers for Disease Control and Prevention
 - Agency for Toxic Substances and Disease Registry
 - National Institute of Occupational Safety and Health
 - Food and Drug Administration
 - National Institutes of Health
 - Office of the Director
 - National Cancer Institute
 - National Institute of Environmental Health Sciences
 - National Library of Medicine
- Department of the Interior
- Department of Labor
 - Occupational Safety and Health Administration
- Department of Transportation
- Environmental Protection Agency



The NICEATM-ICCVAM graphic symbolizes the important role of new and alternative toxicological methods in protecting and advancing the health of people, animals, and our environment.

**ICCVAM Test Method Evaluation Report:
Validation Status of Five *In Vitro* Test Methods Proposed for
Assessing Potential Pyrogenicity of Pharmaceuticals and Other
Products**

**Interagency Coordinating Committee on the Validation of Alternative
Methods (ICCVAM)**

**National Toxicology Program (NTP) Interagency Center for the
Evaluation of Alternative Toxicological Methods (NICEATM)**

**National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services**

**May 2008
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List of Abbreviations and Acronyms

APHIS	Animal and Plant Health Inspection Service (USDA)
BET	Bacterial Endotoxin Test
BRD	Background Review Document
CBER	Center for Biologics Evaluation and Research (FDA)
CFR	Code of Federal Regulations
Cryo	Cryopreserved
CV	Coefficient of variation
EC	Endotoxin control
ECVAM	European Centre for the Validation of Alternative Methods
ELC	Endotoxin limit concentration
ELISA	Enzyme-linked immunosorbent assay
EP	European Pharmacopeia
ESAC	ECVAM Scientific Advisory Committee
EU	European Union
EU/mL	Endotoxin Units per milliliter
FDA	U.S. Food and Drug Administration
FR	<i>Federal Register</i>
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IL	Interleukin
ILS	Integrated Laboratory Systems
ISO	International Organization for Standardization
LAL	Limulus Amebocyte Lysate test method
LLNA	Murine local lymph node assay
LPS	Lipopolysaccharide
MM6	Mono Mac 6
MVD	Maximum valid dilution
MRC	Medical Research Council (U.K.)
NA	Not applicable
NC	Not calculated
ND	Not done
NI	Not included
NICEATM	National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NPC	Negative product control
NSC	Negative saline control
NTP	National Toxicology Program
OECD	Organization for Economic Co-operation and Development
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PEI	Paul Ehrlich Institut
PPC	Positive product control

PWG	Pyrogenicity Working Group
RPT	Rabbit pyrogen test
RSE	Reference standard endotoxin
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SC	Standard curve
SOP	Standard operating procedure
TS	Test substance
TLR	Toll-like receptors
U.S.	United States
U.K.	United Kingdom
USP	U.S. Pharmacopeia
WB	Whole blood
WHO	World Health Organization

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Preface

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is charged by the ICCVAM Authorization Act of 2000 with evaluating the scientific validity of new, revised, and alternative toxicological test methods applicable to U.S. Federal agency safety testing requirements (ICCVAM 2000). ICCVAM is required to provide recommendations to U.S. Federal agencies regarding the usefulness and limitations of test methods based on this scientific evaluation. This Test Method Evaluation Report provides ICCVAM recommendations for five *in vitro* test methods proposed for assessing the potential pyrogenicity of pharmaceuticals and other products. These recommendations are based on a comprehensive evaluation of the current validation status of these test methods.

In March 2005, the European Centre for the Validation of Alternative Methods (ECVAM), a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre, submitted background review documents (BRDs) to ICCVAM for five *in vitro* test methods, which were proposed as replacements for the rabbit pyrogen test. The information in the BRDs was based on validation studies financed by the European Commission within the 5th Framework Programme of Directorate General Research, the results of which were recently published (Hoffmann et al. 2005a; Schindler et al. 2006). The five test methods are:

- The Human Whole Blood (WB)/Interleukin (IL)-1 β *In Vitro* Pyrogen Test
- The Human WB/IL-1 β *In Vitro* Pyrogen Test: Application of Cryopreserved Human WB
- The Human WB/IL-6 *In Vitro* Pyrogen Test
- The Human Peripheral Blood Mononuclear Cell/IL-6 *In Vitro* Pyrogen Test
- The Monocytoid Cell Line Mono Mac 6/IL-6 *In Vitro* Pyrogen Test

In June 2005, ICCVAM initiated evaluation of the validation status of these five test methods. An ICCVAM Pyrogenicity Working Group (PWG) was established to work with the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to carry out this review. Dr. Marlies Halder was designated by ECVAM as their liaison to the PWG. Following a NICEATM pre-screen evaluation of the ECVAM BRDs, NICEATM, ICCVAM and the ICCVAM PWG requested additional information and clarification from ECVAM on a number of issues. In March 2006, ECVAM provided revised BRDs and responses addressing these issues.

NICEATM, in conjunction with the PWG, prepared a comprehensive BRD to combine the available data and information for each of the five *in vitro* test methods into one document. The ICCVAM BRD describes the current validation status of these test methods, including what is known about their reliability and accuracy, the scope of the substances tested, and the availability of standardized protocols for each test method. The ICCVAM BRD was based on the ECVAM BRDs, but also includes other relevant data and analyses, including data and information submitted to NICEATM in response to a *Federal Register (FR)* Notice (Vol. 70, No. 241, pp. 74833-74834, December 16, 2005). The ICCVAM draft BRD was made available to the public on December 12, 2006 (announced in *FR* Vol. 71, No. 238, pp. 74533-

74534, December 12, 2006) for comment and a public peer review panel meeting on February 6, 2007 was announced.

The independent scientific peer review panel (Panel) met in public session on February 6, 2007 at the National Institutes of Health in Bethesda, Maryland. The Panel first reviewed the ICCVAM draft BRD for errors and omissions and then discussed the current validation status of the five *in vitro* test methods. The Panel also reviewed the extent that the information in the ICCVAM BRD supported the ICCVAM draft test method recommendations for proposed test method uses, standardized protocols, test method performance standards, and future studies. Throughout the review process, interested stakeholders from the public were provided opportunities to provide comments including oral comments at the Panel meeting. The Panel considered these comments as well as public comments submitted in advance of the meeting before concluding their deliberations. The final independent Panel report was made available to the public (<http://iccvam.niehs.nih.gov/docs/pyrogen/PrRevPanFinRpt.pdf>) for review and comment on May 9, 2007 (announced in *FR* Vol. 72, No. 89, pp. 26395-26396).

The ICCVAM draft BRD and draft recommendations, the Panel report, and all public comments were made available to ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and comments were provided at their meeting on June 12, 2007.

ICCVAM and the PWG considered the Panel report, all public comments, and the comments of SACATM in preparing the final ICCVAM test method recommendations provided in this report. This report will be made available to the public and provided to U.S. Federal agencies for consideration, in accordance with the ICCVAM Authorization Action of 2000 (ICCVAM 2000). Agencies must respond to ICCVAM within 180 days after receiving an ICCVAM test method recommendation. These responses will be made available to the public on the NICEATM/ICCVAM website (<http://iccvam.niehs.nih.gov>) as they are received.

The efforts of the many individuals who contributed to the preparation, review and revision of this report are gratefully acknowledged. We greatly appreciate the careful preparation of the BRDs by ECVAM and their prompt response to requests for additional information. We especially recognize all of the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Karen Brown for serving as the Panel Chair and to Drs. Jack Levin, Melvyn Lynn, Anthony Mire-Sluis, and Jon Richmond for their service as Evaluation Group Chairs. The efforts of the PWG were invaluable for assuring a meaningful and comprehensive review. We especially thank the Chair of the PWG, Dr. Richard McFarland (FDA, Center for Biologics Evaluation and Research) for his effective leadership. The efforts of the NICEATM staff and support contractor in preparing the BRD, organizing the Panel meeting, and preparing this final report are greatly appreciated. We acknowledge Drs. David Allen and Elizabeth Lipscomb, Catherine Sprankle, James Truax, and Doug Winters of Integrated Laboratory Systems, Inc., the NICEATM support contractor, for their assistance. We also thank Dr. Raymond Tice, Deputy Director of NICEATM, for his efforts on this project.

This comprehensive ICCVAM evaluation of the validation status of these five test methods and the accompanying recommendations should aid agencies in providing guidance on their future use for regulatory safety testing. The ICCVAM recommendations for future studies are expected to advance broader applicability of these methods, which may further reduce animal use while ensuring continued or better protection of human health.

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Executive Summary

This Test Method Evaluation Report, prepared by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), summarizes ICCVAM's evaluation of the validation status of five *in vitro* test methods proposed for assessing the potential pyrogenicity of pharmaceuticals and other products, as potential replacements for the *in vivo* rabbit pyrogen test (RPT). The five test methods are:

- The Human Whole Blood (WB)/Interleukin (IL)-1 β *In Vitro* Pyrogen Test
- The Human WB/IL-1 β *In Vitro* Pyrogen Test: Application of Cryopreserved (Cryo) Human WB
- The Human WB/IL-6 *In Vitro* Pyrogen Test
- The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test
- The Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6 *In Vitro* Pyrogen Test

This report also provides ICCVAM's recommendations for current uses and limitations for each test method, as well as recommendations for standardized protocols, future studies, and performance standards. In support of this evaluation, ICCVAM prepared a draft Background Review Document (BRD) and ICCVAM draft test method recommendations, which were provided to an independent scientific peer review panel (Panel) and the public for consideration and comment. The ICCVAM draft BRD was prepared using data from validation studies that had been conducted by the European Centre for the Validation of Alternative Methods (ECVAM), a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre. The ECVAM submission, prepared according to the ICCVAM submission guidelines (ICCVAM 2003), included five individual BRDs (i.e., one BRD for each test method), which summarized the validation studies for each of the five *in vitro* test methods.

The Panel met on February 6, 2007 to review the ICCVAM draft BRD for errors and omissions and to discuss the current validation status of the five *in vitro* test methods. The Panel also reviewed the extent that the information contained in the ICCVAM draft BRD supported the ICCVAM draft test method recommendations. In finalizing the test method recommendations presented here, ICCVAM considered the conclusions and recommendations of the Panel as well as comments from the public and its Scientific Advisory Committee on Alternative Toxicological Methods.

ICCVAM Recommendations: Test Method Uses and Limitations

Based on this evaluation, ICCVAM recommends that, although none of these test methods can be considered a complete replacement for the RPT for all testing situations for the detection of Gram-negative endotoxin, they can be considered for use to detect Gram-negative endotoxin in human parenteral drugs on a case-by-case basis, subject to validation for each specific product to demonstrate equivalence to the RPT, in accordance

with applicable U.S. Federal regulations (e.g., U.S. Food and Drug Administration [FDA] ^{*}†). When used in this manner, these methods should be able to reduce the number of animals needed for pyrogenicity testing. Pyrogenicity testing may involve more than slight or momentary pain or distress when a pyrogenic response occurs. Accordingly, alternative test methods must be considered prior to the use of animals for such testing, as required by U.S. Federal animal welfare regulations and policies. Therefore, these and other *in vitro* alternative test methods should be considered prior to the use of animals in pyrogenicity testing and should be used where determined appropriate for a specific testing situation. Use of these methods, once appropriately validated, will support improved animal welfare while ensuring the continued protection of human health.

ICCVAM developed a recommended standardized protocol for each test method based primarily on ECVAM standard operating procedures (SOPs). ICCVAM also provided recommendations for further research and development, optimization, and validation efforts. These recommendations should be helpful to various stakeholders (e.g., applicable U.S. Federal regulatory agencies, the international regulatory community, the pharmaceutical industry) for determining when these test methods might be useful.

The Panel concluded that the validation criteria were adequately addressed in the ICCVAM BRD to determine the usefulness and limitations of these test methods to serve as a substitute for the RPT to identify Gram-negative endotoxin on a case-by-case basis, subject to validation for that specific product. However, the Panel stated the performance of these test methods in terms of their reliability and relevance did not support this proposed use.

In March 2006, the ECVAM Scientific Advisory Committee (ESAC) endorsed a statement of validity for these five *in vitro* pyrogen test methods (see **Appendix E**). Like ICCVAM, ESAC concluded that these five methods can detect Gram-negative endotoxin in materials currently tested with the RPT, and, therefore, may be useful for regulatory decisions, subject to validation for that specific product. Both ICCVAM and ESAC also concluded that the currently available database does not support the use of these test methods to detect a wider range of pyrogens, as suggested in the original ECVAM submission. However, ESAC concluded that these tests "can currently be considered as full replacements for the evaluation of materials or products where the objective is to identify and evaluate pyrogenicity produced by Gram-negative endotoxins, but not for other pyrogens." ICCVAM has concluded that the current validation database for these test methods is inadequate to support such a definitive statement based on the ECVAM validation study design, which did not include biologics or medical devices and evaluated only a limited range and number of pharmaceutical products. Additionally, no RPT data were generated with the same test samples used in the *in vitro* test methods (i.e., parallel testing).

*Mechanisms exist for test method developers to qualify their method on a case-by-case basis. The use of any recommended method will be subject to product-specific validation to demonstrate equivalence as recommended by the FDA (e.g., U.S. Code of Federal Regulations (CFR) 21 CFR 610.9 and 21 CFR 314.50(d)(1)(ii)(a)).

†Substances other than endotoxin may induce the cellular release of IL-1 β and/or IL-6. For this reason, users of these test methods should be aware that the presence of other materials might erroneously suggest the presence of endotoxin and lead to a false positive result.

Accuracy and Reliability

The accuracy of *in vitro* pyrogen test methods for detecting Gram-negative endotoxin was based on the results for 10 parenteral pharmaceuticals, each spiked with four concentrations of endotoxin (0, 0.25, 0.5, or 1.0 Endotoxin Units [EU]/mL, with 0.5 EU/mL tested in duplicate). As shown in **Table 1**, accuracy among the test methods ranged from 81% to 93%, sensitivity ranged from 73% to 99%, specificity ranged from 77% to 97%, false negative rates ranged from 1% to 27%, and false positive rates ranged from 3% to 23%.

Table 1 Accuracy of *In Vitro* Pyrogen Test Methods¹

Test Method	Accuracy ²	Sensitivity ³	Specificity ⁴	False Negative Rate ⁵	False Positive Rate ⁶
Cryo WB/IL-1 β	92% (110/120)	97% (75/77)	81% (35/43)	3% (2/77)	19% (8/43)
MM6/IL-6	93% (138/148)	96% (85/89)	90% (53/59)	5% (4/89)	10% (6/59)
PBMC/IL-6	93% (140/150)	92% (83/90)	95% (57/60)	8% (7/90)	5% (3/60)
PBMC/IL-6 (Cryo) ⁷	87% (130/150)	93% (84/90)	77% (46/60)	7% (6/90)	23% (14/60)
WB/IL-6	92% (136/148)	89% (79/89)	97% (57/59)	11% (10/89)	3% (2/59)
WB/IL-1 β (Tube)	81% (119/147)	73% (64/88)	93% (55/59)	27% (24/88)	7% (4/59)
WB/IL-1 β (96-well plate) ⁸	93% (129/139)	99% (83/84)	84% (46/55)	1% (1/84)	16% (9/55)

Abbreviations: Cryo = Cryopreserved; EU/mL = Endotoxin units per milliliter; IL = Interleukin; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

¹Data shown as a percentage (number of correct runs/total number of runs), based on results of 10 parenteral drugs tested in each of three different laboratories. Samples of each drug were tested with or without being spiked with a Gram-negative endotoxin standard (0, 0.25, 0.5, or 1.0 EU/mL, with 0.5 EU/mL tested in duplicate).

²Accuracy = the proportion of correct outcomes (positive and negative) of a test method.

³Sensitivity = the proportion of all positive substances that are classified as positive.

⁴Specificity = the proportion of all negative substances that are classified as negative.

⁵False negative rate = the proportion of all positive substances that are falsely identified as negative.

⁶False positive rate = the proportion of all negative substances that are falsely identified as positive.

⁷A modification of the PBMC/IL-6 test method that uses Cryo PBMCs.

⁸A modification of the WB/IL-1 β test method that uses 96-well plates instead of tubes for the test substance incubation.

Repeatability within individual laboratories was determined for each *in vitro* test method, using saline and various endotoxin spikes to evaluate the closeness of agreement among optical density (OD) readings for cytokine measurements at each concentration. The results indicated that the variability in OD measurements increased with increasing endotoxin concentration. However, the variability was low enough that the threshold for pyrogenicity could still be detected (i.e., the 0.5 EU/mL spike concentration could still be distinguished from the lower concentrations).

Reproducibility within individual laboratories was evaluated using three marketed pharmaceuticals spiked with various concentrations of endotoxin. Three identical, independent runs were conducted in each of the three testing laboratories, with the exception

of the Cryo WB/IL-1 β test method[‡]. The correlations (expressed as percentage of agreement) between pairs of the independent runs (i.e., run 1 vs. run 2; run 1 vs. run 3; run 2 vs. run 3) were determined, and the mean of these three values was calculated. Agreement across three runs within a single laboratory ranged from 75% to 100%.

Reproducibility across all laboratories was evaluated in two different studies in which each run from one laboratory was compared to all other runs of another laboratory. The proportion of equally qualified samples provided a measure of reproducibility. In the first reproducibility study, three marketed pharmaceutical products were spiked with either saline control or various concentrations of endotoxin, and each sample was tested in triplicate in each of three different laboratories, except for the Cryo WB/IL-1 β . In the second study, reproducibility was determined using the results from the 10 substances used in the accuracy analysis. Each drug was spiked with four concentrations of endotoxin and tested once in each of three laboratories. The extent and order of agreement among laboratories were similar in both studies: the WB/IL-1 β test method showed the least agreement (57% to 58%), and the Cryo WB/IL-1 β test method showed the most (88% to 92%).

ICCVAM Recommendations: Test Method Protocols

ICCVAM recommends standardized protocols for each test method that should be used for validation of specific products on a case-by-case basis for U.S. regulatory consideration. These recommended protocols, provided in **Appendix C**, are primarily based on ECVAM SOPs for each test method. ICCVAM has updated these protocols to address inadequacies identified by the Panel, including modifications to standardize essential test method components across the five *in vitro* test methods. These modifications are not expected to reduce or otherwise impact test method accuracy and reliability.

The Panel concluded that the information provided in the ICCVAM draft BRD supported the ICCVAM draft recommended protocols for these five *in vitro* test methods, as long as inadequacies identified by the Panel with respect to reliability and relevance are fully addressed.

ICCVAM Recommendations: Future Studies

ICCVAM recognizes that these test methods could be applicable for detection of a wider range of pyrogens (i.e., endotoxin and pyrogens other than endotoxin) and test materials, provided that they are adequately validated for such uses. Test materials that have been identified clinically as pyrogenic might be invaluable for use in future validation studies and might allow such studies to be conducted without the use of animals. Wherever possible, historical data generated with the same test samples in both *in vitro* and *in vivo* studies (i.e., parallel testing data) should be retrospectively evaluated, or *in vitro* testing should be performed in parallel with RPT and/or bacterial endotoxin tests (BET) conducted for

[‡]The ECVAM Cryo WB/IL-1 β test method BRD stated that there was no direct assessment of intralaboratory reproducibility because such an evaluation was performed in the WB/IL-1 test method, and the authors assumed that variability would not be affected by the use of cryopreserved blood.

regulatory purposes[§]. Future validation studies should include the following considerations:

1. Both endotoxin-spiked and non-endotoxin spiked samples should be included. Non-endotoxin pyrogen standards should be characterized prior to their use in any study, if possible.
2. All aspects of the studies should comply with Good Laboratory Practices.
3. Future studies should include products that have intrinsic pro-inflammatory properties in order to determine if these tests can be used for such substances.
4. Optimally, a study that includes three-way parallel testing, with the *in vitro* assays being compared to the RPT and the BET, should be conducted to comprehensively evaluate the relevance and comparative performance of these test methods. These studies may be conducted with historical RPT data provided that the same substances (i.e., same lot) are tested in each method. Based on ethical and scientific rationale, any *in vivo* testing should be limited to those studies that will fill existing data gaps.
5. Test substances that better represent all categories of sample types (e.g., pharmaceuticals, biologicals, and medical devices) intended for testing by the methods should be included.
6. The hazards associated with human blood products should be carefully considered, and all technical staff should be adequately trained to observe all necessary safety precautions.
7. Formal sample size calculations should be made to determine the required number of replicates needed to reject the null hypothesis at a given level of significance and power. For reliability assessments, formal hypothesis testing is essential with the alternative hypothesis being no difference between groups.

The Panel agreed with ICCVAM that any future studies should be performed using the ICCVAM recommended test method protocols. The Panel also provided other suggestions and recommendations for future studies (see **Appendix A**). Like ICCVAM, the Panel also recognized that these test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.

ICCVAM Recommendations: Performance Standards

As indicated above, these test methods have not yet been adequately evaluated for their ability to detect Gram-negative endotoxin in parenteral pharmaceuticals, biological products, and medical devices compared to the RPT or the BET. For this reason, ICCVAM does not consider it appropriate at this time to develop performance standards that can be used to evaluate the performance of other test methods that are structurally and functionally similar.

[§]In order to demonstrate the utility of these test methods for the detection of non-endotoxin pyrogens, either an international reference standard is needed (as is available for endotoxin [i.e., WHO-LPS 94/580 *E. coli* O113:H10:K-]) or, when a positive non-endotoxin-mediated RPT result is encountered, this same sample should be subsequently tested *in vitro*.

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1.0 Introduction

In June 2005, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) initiated a review of the validation status of five *in vitro* pyrogen test methods proposed as replacements for the rabbit pyrogen test (RPT). The test methods were submitted by the European Centre for the Validation of Alternative Methods (ECVAM), a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre. This submission was based on a validation study financed by the European Commission within the 5th Framework Programme of Directorate General Research and was recently published (Hoffmann et al. 2005a; Schindler et al. 2006). The proposed test methods are:

- The Human Whole Blood (WB)/Interleukin (IL)-1 β *In Vitro* Pyrogen Test
- The Human WB/IL-1 β *In Vitro* Pyrogen Test: Application of Cryopreserved (Cryo) Human WB
- The Human WB/IL-6 *In Vitro* Pyrogen Test
- The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test
- The Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6 *In Vitro* Pyrogen Test

For simplicity, the submitted studies are referred to collectively as the ECVAM validation study in this document.

ICCVAM, which is charged with coordinating the technical evaluations of new, revised, and alternative test methods with regulatory applicability (ICCVAM 2000), unanimously agreed that the five submitted *in vitro* test methods should have a high priority for evaluation. An ICCVAM Pyrogenicity Working Group (PWG) was established to work with the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to carry out these evaluations; Dr. Marlies Halder was the ECVAM liaison to the PWG. Following a NICEATM pre-screen evaluation of the comprehensive background review documents (BRDs) submitted by ECVAM, NICEATM, ICCVAM and the ICCVAM PWG requested additional information and clarification from ECVAM on a number of issues. In March 2006, in response to this request, ECVAM submitted revised BRDs and a list of responses to address these issues.

NICEATM, which administers ICCVAM and provides scientific support for ICCVAM activities, subsequently prepared a comprehensive draft BRD that provided information and data from the validation studies and scientific literature to enable a peer review of the validation status of each of the five *in vitro* test methods. A request for any other data and information on these test methods and for nominations to serve on an independent, scientific pyrogenicity review panel (Panel) was made through a 2005 *Federal Register* (FR) notice (Vol. 70, No. 241, pp. 74833-74834, December 16, 2005, available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E5_7410.pdf), through the ICCVAM electronic mailing list, and through direct requests to over 100 stakeholders. Panel nominations were received, but no additional data or information was submitted in response to this request.

Announcement of a public Panel meeting to review the validation status of the five *in vitro* pyrogen test methods and availability of the ICCVAM BRD was made through a 2006 *FR* notice (Vol. 71, No. 238, pp. 74533-74534, December 12, 2006, available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E6_21038.pdf). The draft BRD was made publicly available on the NICEATM/ICCVAM website (<http://iccvam.niehs.nih.gov>). Additional information provided by ECVAM in response to a request from Panel was appended to this BRD. All of the information provided to the Panel was also made publicly available. Comments from the public and scientific community are available on the NICEATM/ICCVAM website.

The adequacy of the data and information contained in the ICCVAM BRD to support the ICCVAM draft test method recommendations were discussed by the Panel in a public meeting on February 6, 2007 at the National Institutes of Health campus in Bethesda, MD. A report of the Panel's recommendations (see **Appendix A**; Panel Report, available at <http://iccvam.niehs.nih.gov/docs/pyrogen/PrRevPanFinRpt.pdf>) was made available for public comment on the NICEATM/ICCVAM website (see *FR* notice [Vol. 72, No. 89, pp. 26395-26396, May 9, 2007], available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_8896.pdf).

The ICCVAM draft BRD, the Panel report, and all public comments were made available to ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), for review and comment at their meeting on June 12, 2007.

ICCVAM and the PWG then considered the Panel report, all public comments, and the comments of SACATM in preparing the final BRD and the final test method recommendations that are provided in this ICCVAM Test Method Evaluation Report. This report will be made available to the public and provided to U.S. Federal agencies for consideration (ICCVAM 2000). The ICCVAM final BRD, revised in response to the Panel and PWG comments, will also be provided as background information and technical support for this report. Agencies with applicable testing regulations and guidelines (see **Appendix B**) are required by law to respond to ICCVAM within 180 days of receiving an ICCVAM test method recommendation. These responses will be made available to the public on the NICEATM/ICCVAM website (<http://iccvam.niehs.nih.gov>) as they are received.

2.0 ICCVAM Recommendations for *In Vitro* Pyrogen Test Methods

ICCVAM evaluated the validation status of the five *in vitro* pyrogen test methods as potential replacements for the RPT. ICCVAM was unable to evaluate these tests as possible replacements for the Bacterial Endotoxin Test (BET) because the validation studies were not designed for this purpose.

2.1 ICCVAM Recommendations: Test Method Uses and Limitations

The ability of the WB/IL-1 β , Cryo WB/IL-1 β , WB/IL-6, PBMC/IL-6, and MM6/IL-6 test methods to detect Gram-negative endotoxin in a limited number of human parenteral drugs have been tested in recent validation studies. The performance assessment for these five test methods, and the drugs included in the associated validation studies are detailed in **Section 3.0**. Based on a review of the available data, these test methods have not been adequately evaluated for their ability to detect Gram-negative endotoxin in parenteral pharmaceuticals, biological products, and medical devices compared to the RPT or the BET. This is based on the fact that the validation study only evaluated a limited range and number of pharmaceutical products and did not evaluate the potential to detect endotoxin in biologics or medical devices. Therefore, none of the test methods should be considered as a complete replacement for the RPT or the BET for the detection of Gram-negative endotoxin. However, these test methods can be considered for use to detect Gram-negative endotoxin in human parenteral drugs on a case-by-case basis, subject to product-specific validation to demonstrate equivalence to accepted pyrogen tests in accordance with applicable U.S. Federal regulations (e.g., U.S. Food and Drug Administration [FDA] ^{*}†). Potential users should consider the false negative/false positive rates as well as ease of use in selecting any test method for possible use. In addition, while the scientific basis of these test methods suggests that they have the capability to detect pyrogenicity mediated by non-endotoxin sources, there is insufficient data to support this broader application. Users should be aware that the performance characteristics for these *in vitro* pyrogen test methods might be revised based on additional data. Therefore, ICCVAM recommends that test method users routinely consult the NICEATM/ICCVAM website (<http://iccvam.niehs.nih.gov/>) and other appropriate sources to ensure that the most current information is considered.

2.1.1 Independent Peer Review Panel Conclusions and Recommendations

The Panel agreed that the applicable validation criteria have been adequately addressed in the ICCVAM draft BRD in order to determine the usefulness and limitations of these test methods to serve as a substitute for the RPT, for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product-specific validation. However, the Panel generally agreed that the performance of these test methods in terms of their reliability and relevance did not support this proposed use (see **Appendix A**).

^{*}Mechanisms exist for test method developers to qualify their method on a case-by-case basis. The use of any recommended method will be subject to product-specific validation to demonstrate equivalence as recommended by the FDA (e.g., 21 CFR 610.9 and 21 CFR 314.50(d)(1)(ii)(a)).

[†]Substances other than endotoxin may induce the cellular release of IL-1 β and/or IL-6. For this reason, users of these test methods should be aware that the presence of other materials might erroneously suggest the presence of endotoxin and lead to a false positive result.

While ICCVAM agreed with the Panel that these test methods cannot be considered complete replacements for the RPT, they did recommend their use to detect Gram-negative endotoxin in human parenteral drugs on a case-by-case basis, subject to product-specific validation to demonstrate equivalence to the RPT.

2.1.2 ECVAM Scientific Advisory Committee (ESAC) Statement of Validity

In March 2006, the ESAC unanimously endorsed a statement of validity for these five *in vitro* pyrogen test methods, which describes their recommendations on test method uses (see **Appendix E**). Like ICCVAM, ESAC concluded that these five methods can detect pyrogenicity mediated by Gram-negative endotoxin in materials currently tested in the RPT, and that they may be useful for regulatory decisions, subject to product-specific validation. Both ICCVAM and ESAC also concluded that the currently available database does not support their use to detect a wider range of pyrogens, as was suggested in the original ECVAM submission.

However, ESAC concluded that these tests have been scientifically validated for the detection of pyrogenicity mediated by Gram-negative endotoxins, and quantification of this pyrogen, in materials currently evaluated and characterized by rabbit pyrogen tests. In contrast, as described in **Section 2.1**, ICCVAM has concluded that the current validation database for these test methods is inadequate to support such a definitive statement based on the ECVAM validation study design, which did not include biologics or medical devices and evaluated only a limited range and number of pharmaceutical products and additionally did not include parallel testing with the RPT.

2.2 ICCVAM Recommendations: Test Method Protocols

ICCVAM recommends that when testing is conducted, the *in vitro* pyrogen test method protocols should be based on the standardized test method protocols provided in **Appendix C**. These ICCVAM recommended protocols, summarized in **Table 2-1**, are based primarily on ECVAM Standard Operating Procedures (SOPs) for each test method, with modifications made by NICEATM and ICCVAM in an effort to standardize essential test method components across protocols where possible. These modifications are not expected to reduce test method performance. A table summarizing the differences between the ICCVAM recommended protocol and the relevant ECVAM protocol/SOP is provided as an introduction to each protocol included in **Appendix C**.

By comparison, the Panel concluded that the information provided in the ICCVAM draft BRD supported the ICCVAM draft recommended protocols for these five *in vitro* test methods, providing that the list of inadequacies identified by the Panel with respect to reliability and relevance are fully addressed. The revised ICCVAM recommended protocols (see **Appendix C**) have been updated to address many of the Panel's concerns.

Using these recommended standardized protocols will facilitate collection of consistent data and expand the current validation database. Exceptions and/or changes to the recommended standardized test method protocols should be accompanied by a scientific rationale. Users should be aware that the test method protocols could be revised based on future optimization and/or validation studies. Therefore, test method users should consult the NICEATM/ICCVAM website (<http://iccvam.niehs.nih.gov>) or other appropriate sources to ensure use of the most current recommended test method protocol.

Table 2-1 Summary of ICCVAM Recommended *In Vitro* Pyrogen Test Method Protocols

Protocol Component	ICCVAM Recommended <i>In Vitro</i> Pyrogen Protocols				
	WB/IL-1 β	Cryo WB/IL-1 β	WB/IL-6	PBMC/IL-6	MM6/IL-6
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD				
Number of Blood Donors	Minimum of 3 (independent or pooled)				NA
Decision Criteria for Interference	Mean OD ¹ of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 0.5 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 0.25 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC
Incubation Plate (The number of samples or controls measured in quadruplicate)	NSC (1)				
	EC (5)				
	TS (14)				
	PPC ² (0)	PPC (0)	PPC (0)	PPC (0)	PPC ³ (0)
	NPC ² (0)	NPC (0)	NPC (0)	NPC (0)	NPC (0)
ELISA Plate	Includes seven point IL-1 β SC and blank in duplicate		Includes seven point IL-6 SC and blank in duplicate		
Assay Acceptability Criteria	Mean OD of NSC ≤ 0.15				
	Quadratic function of IL-1 β SC $r \geq 0.95^3$		Quadratic function of IL-6 SC $r \geq 0.95$		
	EC SC produces OD values that ascend in a sigmoidal concentration response				
	NA	NA	High responder blood donors (i.e., >200 pg/mL IL-6) may be excluded	High responder blood donors (i.e., > 200 pg/mL IL-6) or low responder blood donors (i.e., Mean OD of 1EU/mL EC is significantly less than that of 1000 pg/mL IL-6) may be excluded	NA
	Outliers rejected using Dixon's test ⁴				
	Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC ⁵ TS			

Abbreviations: Cryo = Cryopreserved; EC = Endotoxin control; ELC = Endotoxin Limit Concentration; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL= Interleukin; MM6 = Mono Mac 6; MVD = Maximum valid dilution; NA = Not applicable; NPC = Negative product control; NSC = Negative saline control; OD = Optical density; PBMC = Peripheral blood mononuclear cell; PPC = Positive product control; SC = Standard curve; TS = Test substance; WB = Whole blood

¹In WB/IL-1 β and MM6/IL-6 test methods, the mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

²In the ICCVAM protocols (see **Appendix C**), PPC and NPC are assessed in the interference test described in Section 4.2, which is performed prior to the ELISA.

³Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

⁴Dixon 1950.

⁵Where unknown, the ELC is calculated (see **Appendix C**).

2.3 ICCVAM Recommendations: Future Studies

ICCVAM recognizes that these test methods could be applicable for the detection of a wider range of pyrogens (i.e., endotoxin and non-endotoxin) and test materials, provided that they are adequately validated for such uses. Test materials identified clinically as pyrogenic might be invaluable for use in future validation studies and might allow such studies to be conducted without the use of animals. Wherever possible, historical data from parallel *in vivo/in vitro* studies should be retrospectively evaluated, or parallel *in vitro* testing should be conducted with RPT and/or BET tests that are performed for regulatory purposes[‡]. Future validation studies should include the following considerations:

1. Both endotoxin-spiked and non-endotoxin spiked samples should be included. Non-endotoxin standards should be characterized prior to their use in any study, if possible.
2. All aspects of the studies should be compliant with Good Laboratory Practice.
3. Future studies should include products that have intrinsic pro-inflammatory properties in order to determine if such substances are amenable to these tests.
4. Optimally, a study that includes 3-way parallel testing, with the *in vitro* assays being compared to the RPT and the BET, should be conducted to allow for a comprehensive evaluation of the relevance and comparative performance of these test methods. These studies may be conducted with historical RPT data provided that the same substances (i.e., same lot) are tested in each method. Based on ethical and scientific rationale, any *in vivo* testing should be limited to those studies that will fill existing data gaps.
5. Test substances that better represent all categories of sample types (e.g., pharmaceuticals, biologicals, and medical devices) intended for testing by the methods should be included.
6. The hazards associated with human blood products should be carefully considered, and all technical staff should be adequately trained to observe all necessary safety precautions.
7. Formal sample size calculations should be made to determine the required number of replicates needed to reject the null hypothesis at a given level of significance and power. For reliability assessments, formal hypothesis testing is essential with the alternative hypothesis being no difference between groups.

The Panel agreed that any future studies should be performed using the ICCVAM proposed protocols. Like ICCVAM, the Panel also recognized that these test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.

[‡]In order to demonstrate the utility of these test methods for the detection of non-endotoxin pyrogens, either an international reference standard is needed (as is available for endotoxin [i.e., WHO-LPS 94/580 *E. coli* O113:H10:K-]) or, when a positive non-endotoxin-mediated RPT result is encountered, this same sample should be subsequently tested *in vitro*.

The Panel also recommended other studies for consideration:

1. A proposed strategy for the Cryo WB/IL-1 β test method is to retest if a test fails because of too much variability. The statistical properties of this multistage procedure should be characterized.
 - ICCVAM note: This comment, which pertains to the ECVAM Catch-Up Validation SOP for the Cryo WB/IL-1 β pyrogen test, is not relevant to the ICCVAM recommended protocol.
2. The effects of direct administration of IL-1 β and IL-6 to rabbits and the comparison of the resulting pyrogenic response with endotoxin-mediated pyrogenicity should be evaluated. In addition, the correlation of IL-1 β and IL-6 levels in the *in vitro* tests with levels produced in rabbits using similar doses of endotoxin should be evaluated.
 - ICCVAM note: This information would certainly be interesting and possibly useful in the comparison of the responses of the *in vitro* human cells to that of the *in vivo* rabbit. However, ICCVAM did not consider that the information gained could justify the additional resources and animals that would be required to perform such studies, and therefore, ICCVAM has not included this specific recommendation.
3. The endotoxin-spike concentrations used for the performance assessment studies should not be so close to the positive test concentration limit, especially considering the relatively large enhancement and inhibition range permitted in the sample specific qualification investigations.
 - ICCVAM note: ECVAM has previously commented that, "The study design, using borderline spikes, aimed to profile differences in pyrogen tests (i.e., RPT, BET, and *in vitro* tests), but does not reflect routine test situations. Furthermore, the threshold chosen represents the endotoxin limit, where 50% of the rabbits using the most sensitive rabbit strain react with fever." Therefore, the validation study was designed to maximally challenge the sensitivity of the *in vitro* pyrogen tests. For this reason, and because the *in vitro* test methods are being recommended for consideration on a case-by-case basis, subject to product-specific validation, ICCVAM has not included this specific recommendation.
4. A 'limit' test design protocol and a 'benchmark reference lot comparison' test design protocol for each assay should be included.
 - ICCVAM note: Because these *in vitro* test methods are being recommended for consideration on a case-by-case basis, subject to product-specific validation, ICCVAM did not consider the additional resources required to perform both study designs practical.

2.4 ICCVAM Recommendations: Performance Standards

As indicated above, these five *in vitro* test methods have not been adequately evaluated for their ability to detect Gram-negative endotoxin compared to the RPT or the BET in a

sufficient number and range of parenteral pharmaceuticals, and in no biological products and medical devices. For this reason, it is not feasible at this time to develop performance standards that can be used to evaluate the performance of other test methods that are structurally and functionally similar.

3.0 Validation Status of *In Vitro* Pyrogen Test Methods

The following is a synopsis of the information in the ICCVAM BRD, which reviews the available data and information for each of the five test methods. The ICCVAM BRD describes the current validation status of the five *in vitro* pyrogen test methods, including what is known about their reliability and accuracy, the scope of the substances tested, and standardized protocols used for the validation study. The ICCVAM BRD may be obtained electronically from the NICEATM/ICCVAM website (<http://iccvam.niehs.nih.gov/>) or by contacting NICEATM via email at niceatm@niehs.nih.gov. A hard copy of the ICCVAM BRD may be requested by email or by mail to NICEATM, NIEHS, P.O. Box 12233, Mail Drop EC-17, Research Triangle Park, NC 27709.

3.1 Test Method Description

According to the ECVAM submission, these *in vitro* pyrogen test methods are intended for the detection of Gram-negative endotoxin contained in substances intended for parenteral use (e.g., pharmaceuticals, biologics, medical devices). These methods are based on the detection of the release of proinflammatory cytokines (i.e., IL-1 β or IL-6) from human monocytes or monocytoid cells induced by exposure to a product contaminated with Gram-negative endotoxin.

3.1.1 General Test Method Procedures

The *in vitro* pyrogen test methods measure cytokine release from monocytes or monocytoid cells (i.e., WB, PBMCs, or the MM6 cell line) by using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for either IL-1 β or IL-6. The amount of endotoxin present is determined by comparing the values of endotoxin equivalents produced by WB cells exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)⁴ or an equivalent standard expressed in Endotoxin Units (EU)/mL. A product is considered to be pyrogenic if the endotoxin concentration exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

3.1.2 Protocol Similarities and Differences

Although there are differences among the five *in vitro* pyrogen test methods, the basic procedural steps are consistent across all test methods:

- The test substance is mixed with a suspension of human-derived cells.
- The mix of cells and test product is incubated for a specific time.
- The concentration of pro-inflammatory cytokines (e.g., IL-1 β , IL-6) is measured with an ELISA by comparison to a standard curve.

⁴RSEs are internationally harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; U.S. Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

- The endotoxin content is calculated by comparing the measured concentration of pro-inflammatory cytokines to an endotoxin standard curve.
- A test substance is considered pyrogenic if the estimated endotoxin concentration of the test substance exceeds the ELC for the test substance.

3.2 Validation Database

The test substances selected for use in the validation studies were marketed parenteral pharmaceuticals. No biological or medical device products were included in the validation study. A total of 13 test substances were included in the performance analysis of each of the five *in vitro* test methods. Ten substances (**Table 3-1**), each spiked with four concentrations of endotoxin (0, 0.25, 0.5, and 1.0 EU/mL, with 0.5 EU/mL tested in duplicate), were used to evaluate accuracy. Three substances (**Table 3-2**), each spiked with three concentrations of endotoxin (0, 0.5, and 1.0 EU/mL, with 0 EU/mL tested in duplicate), were used to assess intralaboratory reproducibility. Interlaboratory reproducibility was evaluated in two different studies. The first study tested the substances listed in **Table 3-2** in triplicate in each of three laboratories. In the second study, interlaboratory reproducibility was tested using the substances in **Table 3-1**, which were tested once in each of three laboratories.

Table 3-1 Parenteral Drugs Used in the Validation Studies for Determining Test Method Accuracy¹

Test Substance ²	Active Ingredient	Source	Lot Number(s)	Indication	MVD (-fold)
Beloc®	Metoprolol tartrate	Astra Zeneca	DA419A1	Heart dysfunction	140
Binotal®	Ampicillin	Grünenthal	117EL2	Antibiotic	140
Ethanol 95%	Ethanol	B. Braun	2465Z01	Diluent	35
Fenistil®	Dimetindenmaleat	Novartis	21402 26803 ³	Antiallergic	175
Glucose 5%	Glucose	Eifelfango	1162 3132 ³	Nutrition	70
MCP®	Metoclopramid	Hexal	21JX22	Antiemetic	350
Orasthin®	Oxytocin	Hoechst	W015	Initiation of delivery	700
Sostril®	Ranitidine	Glaxo Wellcome	1L585B 3H01N ³	Antiacidic	140
Syntocinon®	Oxytocin	Novartis	S00400	Initiation of delivery	-
Drug A - 0.9% NaCl	0.9% NaCl	-	-	-	35
Drug B - 0.9% NaCl	0.9% NaCl	-	-	-	70

Abbreviations: MVD = Maximum valid dilution

¹Each substance was tested in all five *in vitro* pyrogen test methods.

²Each test substance was spiked with 0, 0.25, 0.5, or 1.0 Endotoxin Units (EU)/mL of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]), with 0.5 EU/mL tested in duplicate. Each sample contained the appropriate spike concentration when tested at its MVD.

³Indicates the lot numbers used in the catch-up validation study for the Cryopreserved whole blood/Interleukin-1β test method.

Table 3-2 Parenteral Drugs Used in the Validation Studies for Determining Test Method Reproducibility¹

Test Substance ²	Source	Agent	Indication
Gelafundin®	Braun Melsungen	Gelatin	Transfusion
Haemate®	Aventis	Factor VIII	Hemophilia
Jonosteril®	Fresenius	Electrolytes	Infusion

¹Each substance was tested in all five *in vitro* pyrogen test methods.

²Each test substance was spiked with 0, 0.5, or 1.0 Endotoxin Units (EU)/mL of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]), with 0 EU/mL tested in duplicate. Each sample contained the appropriate spike concentration when tested at its maximum valid dilution.

3.3 Reference Test Method Data

The historical RPT studies were conducted at the Paul Ehrlich Institut (PEI), which supports regional German regulatory authorities, provides marketing approval of certain marketed biological products (e.g., sera, vaccines, test allergens), and functions as a World Health Organization (WHO) collaborating center for quality assurance of blood products and *in vitro* diagnostics. The unit for pyrogen and endotoxin testing of the PEI is accredited following the International Organization for Standardization (ISO) and the International Electrotechnical Commission 17025 (ISO 2005). In a request for additional information from ECVAM, it was stated that the RPT data was generated according to the European Pharmacopeia (EP) monograph, but the detailed protocol used by this laboratory was not provided.

These data were generated for internal quality control studies from 171 rabbits (Chinchilla Bastards). Chinchilla Bastards are reported to be a more sensitive strain than the New Zealand White rabbit strain for pyrogenicity testing (Hoffmann et al. 2005b). However, the USP (USP 2007) and the EP (EP 2005) do not prescribe a specific rabbit strain for the RPT.

3.4 Test Method Accuracy

The ability of the *in vitro* pyrogen test methods to correctly identify the presence of Gram-negative endotoxin was evaluated using parenteral pharmaceuticals spiked with endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). As described in **Section 3.2**, 10 substances (see **Table 3-1**) spiked with four concentrations of endotoxin (i.e., 0, 0.25, 0.5, or 1.0 EU/mL, with 0.5 EU/mL tested in duplicate) were used for the evaluation. The individual spike concentrations in each substance were tested once, using each test method, in three different laboratories, providing a total of 150 runs (i.e., 10 substances x 5 spike solutions x 3 laboratories = 150). Outliers were identified using Dixon's test (i.e., significance level of $\alpha = 0.01$) and subsequently excluded from the evaluation, which resulted in fewer than a total of 150 runs per evaluation (Dixon 1950; Barnett et al. 1984). A comparison of the results for the *in vitro* test methods indicates that the number of runs excluded was greatest for the Cryo WB/IL-1 β and WB/IL-1 β (plate method) test methods, which had 30 and 11 runs excluded, respectively. No other test method had more than three runs excluded.

As described in **Section 3.3**, no RPTs were conducted in parallel with the *in vitro* pyrogen test methods during the ECVAM validation studies. Instead, historical RPT data from rabbits tested with endotoxin were used to establish a threshold pyrogen dose (i.e., the endotoxin

dose at which fever was induced in 50% of the rabbits). This historical data were subsequently used to establish the limit of detection (i.e., 0.5 EU/mL) that the *in vitro* test methods being validated must meet. Accordingly, the *in vitro* call was compared to the "true status" (based on the known endotoxin spike concentration) of the sample. The resulting calls were used to construct 2x2 contingency tables, which were used to calculate the resulting test method performance values.

The accuracy of each *in vitro* pyrogen test method for correctly identifying samples spiked with 0.5 or 1.0 EU/mL endotoxin as positive and samples spiked with 0 or 0.25 EU/mL endotoxin as negative was evaluated. As provided in **Table 3-3**, accuracy ranged from 81% to 93%, sensitivity ranged from 73% to 99%, specificity ranged from 77% to 97%, false negative rates ranged from 1% to 27%, and false positive rates ranged from 3% to 23%.

Table 3-3 Accuracy of *In Vitro* Pyrogen Test Methods¹

Test Method	Accuracy ²	Sensitivity ³	Specificity ⁴	False Negative Rate ⁵	False Positive Rate ⁶
Cryo WB/IL-1β	92% (110/120)	97% (75/77)	81% (35/43)	3% (2/77)	19% (8/43)
MM6/IL-6	93% (138/148)	96% (85/89)	90% (53/59)	5% (4/89)	10% (6/59)
PBMC/IL-6	93% (140/150)	92% (83/90)	95% (57/60)	8% (7/90)	5% (3/60)
PBMC/IL-6 (Cryo) ⁷	87% (130/150)	93% (84/90)	77% (46/60)	7% (6/90)	23% (14/60)
WB/IL-6	92% (136/148)	89% (79/89)	97% (57/59)	11% (10/89)	3% (2/59)
WB/IL-1β (Tube)	81% (119/147)	73% (64/88)	93% (55/59)	27% (24/88)	7% (4/59)
WB/IL-1β (96-well plate) ⁸	93% (129/139)	99% (83/84)	84% (46/55)	1% (1/84)	16% (9/55)

Abbreviations: Cryo = Cryopreserved; EU/mL = Endotoxin units per milliliter; IL = Interleukin; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

¹Data shown as a percentage (number of correct runs/total number of runs), based on results of 10 parenteral drugs tested in each of three different laboratories. Samples of each drug were tested with or without being spiked with a Gram-negative endotoxin standard (0, 0.25, 0.5, or 1.0 EU/mL, with 0.5 EU/mL tested in duplicate).

²Accuracy = the proportion of correct outcomes (positive and negative) of a test method.

³Sensitivity = the proportion of all positive substances that are classified as positive.

⁴Specificity = the proportion of all negative substances that are classified as negative.

⁵False negative rate = the proportion of all positive substances that are falsely identified as negative.

⁶False positive rate = the proportion of all negative substances that are falsely identified as positive.

⁷A modification of the PBMC/IL-6 test method that uses Cryo PBMCs.

⁸A modification of the WB/IL-1β test method that uses 96-well plates instead of tubes for the test substance incubation.

3.5 Test Method Reliability

Intralaboratory repeatability was evaluated by testing saline spiked with various concentrations of endotoxin (0, 0.06, 0.125, 0.25, 0.5, and 1.0 EU/mL) and then evaluating the closeness of agreement among OD readings for cytokine measurements at each concentration. For each test method, each experiment was conducted up to three times. From 5 to 32 replicates per concentration were tested and results indicated that variability in OD measurements increased with increasing endotoxin concentration. However, the variability

did not interfere with distinguishing the 0.5 EU/mL spike concentration (i.e., the threshold for pyrogenicity) from the lower concentrations.

Intralaboratory reproducibility was evaluated using three marketed pharmaceuticals spiked with three concentrations of endotoxin (i.e., 0, 0.5, and 1.0 EU/mL, with 0 EU/mL tested in duplicate). Three identical, independent runs were conducted in each of the three testing laboratories, with the exception of the Cryo WB/IL-1 β test method⁵. The correlations (expressed as a percentage of agreement) between pairs of the independent runs (i.e., run 1 vs. run 2; run 1 vs. run 3; run 2 vs. run 3) were determined and the mean of these three values was calculated. In all reproducibility analyses, a single run consisted of each of the products assayed in quadruplicate. Acceptability criteria for each run included a Coefficient of Variation (CV) analysis to remove highly variable responses from the analyses. The criterion used to identify outliers ranged from CV <0.25 to CV <0.45, depending on the method being considered, and was arbitrarily set based on results using saline spiked with endotoxin. As an example, for the MM6/IL-6 test method, the CV for any single spike concentration was ≤ 0.12 , and therefore, the outlier criterion was set at 0.25. Agreement between different runs was determined for each substance in three laboratories. As shown in **Table 3-4**, the agreement across three runs in an individual lab ranged from 75% to 100%.

Interlaboratory reproducibility was evaluated in two different studies. In both studies, each run from one laboratory was compared with all runs of another laboratory. The proportions of similarly classified samples provide a measure of reproducibility. In the first study, the interlaboratory reproducibility was evaluated using results from three marketed pharmaceuticals spiked with endotoxin and tested in triplicate in each of the three laboratories. As shown in **Table 3-5**, the agreement across three laboratories for each test method, where three runs per laboratory were conducted, ranged from 58% to 86%, depending on the test method considered (excludes the Cryo WB/IL-1 β test method, which used only one run per laboratory). However, if the WB/IL-1 β tube method is excluded, the range of agreement across laboratories is 72% to 86%. In comparison, the agreement across three laboratories for the Cryo WB/IL-1 β test method, for which only one run per laboratory was conducted, was 92%.

⁵The ECVAM Cryo WB/IL-1 β test method BRD stated that there was no direct assessment of intralaboratory reproducibility because such an evaluation was performed in the WB/IL-1 β test method, and the authors assumed that variability would not be affected by the use of cryopreserved blood.

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Table 3-4 Intralaboratory Reproducibility of *In Vitro* Pyrogen Test Methods

Run Comparison ¹	WB/IL-1β			Cryo WB/IL-1β			WB/IL-6			PBMC/IL-6			MM6/IL-6		
	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3
1 vs 2	92% (11/12)	100% (8/8)	100% (12/12)	ND ³	ND	ND	75% (9/12)	92% (11/12)	100% (12/12)	92% (11/12)	100% (12/12)	100% (12/12)	100% (12/12)	92% (11/12)	100% (12/12)
1 vs 3	83% (10/12)	88% (7/8)	92% (11/12)	ND	ND	ND	100% (12/12)	92% (11/12)	100% (12/12)	100% (12/12)	100% (12/12)	92% (11/12)	100% (12/12)	92% (11/12)	92% (11/12)
2 vs 3	92% (11/12)	NI ⁴	92% (11/12)	ND	ND	ND	75% (9/12)	92% (11/12)	100% (12/12)	92% (11/12)	100% (12/12)	92% (11/12)	100% (12/12)	100% (12/12)	92% (11/12)
Mean	89%	NC	95%	ND	ND	ND	83%	92%	100%	95%	100%	95%	100%	95%	95%
Agreement ² across 3 runs	83%	NC	92%	ND	ND	ND	75%	92%	100%	92%	100%	92%	100%	92%	92%

Abbreviations: Cryo = Cryopreserved; IL = Interleukin; MM6 = Mono Mac 6; NC = Not calculated; ND = Not done; NI = Not included; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

¹Comparison among 3 individual runs within each laboratory.

²All possible combinations of runs among the 3 laboratories were compared.

³Not done. The ECVAM Cryo WB/IL-1β BRD states that an assessment of intralaboratory reproducibility was performed using the WB IL-1β (fresh blood) test method, and it was assumed that intralaboratory variability would not be affected by the change to cryopreserved blood assayed in 96-well plates.

⁴Not included due to lack of sufficient data. The sensitivity criteria were not met for 1 of 3 substances in run 2, and 1 of 3 substances in run 3.

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Table 3-5 Interlaboratory Reproducibility of *In Vitro* Pyrogen Test Methods: Study One

Lab Comparison ¹	Agreement Between Laboratories ¹				
	WB/IL-1 β (Tube)	Cryo WB/IL-1 β	WB/IL-6	PBMC/IL-6	MM6/IL-6
1 vs 2	92% (77/84) ²	92% (11/12) ³	72% (78/108)	81% (87/108)	97% (105/108)
1 vs 3	77% (83/108)	92% (11/12) ³	75% (81/108)	86% (93/108)	89% (96/108)
2 vs 3	68% (57/84) ²	92% (11/12) ³	97% (105/108)	89% (96/108)	86% (93/108)
Mean	79%	92%	81%	85%	90%
Agreement across 3 labs ⁴	58% (167/288) ²	92% (11/12) ³	72% (234/324)	78% (252/324)	86% (279/324)

Abbreviations: Cryo = Cryopreserved; IL = Interleukin; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

¹Data from three substances (see **Table 3-2**) spiked with endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]) at 0, 0.5 and 1.0 EU/mL, with 0 EU/mL spiked in duplicate, were tested three times in three different laboratories, with the exception of Cryo WB/IL-1 β (only the preliminary run from each laboratory used for analysis).

²Some of the runs did not meet the assay acceptance criteria and therefore were excluded from the analysis.

³For the Cryo WB/IL-1 β test method, each substance tested only once in each laboratory.

⁴All possible combinations of runs among the 3 laboratories were compared (with the exception of Cryo WB/IL-1 β , which was only tested once in each laboratory, resulting in only one possible combination per substance).

In the second study, interlaboratory reproducibility was evaluated with the same 10 substances used for evaluating accuracy. In this study, each of the substances was spiked with four concentrations of endotoxin (0, 0.25, 0.5, and 1.0 EU/mL, with 0.5 EU/mL spiked in duplicate) and tested once in each of three laboratories. As shown in **Table 3-6**, the agreement across three laboratories for each test method ranged from 57% to 88%, depending on the test method considered. The extent and order of agreement among laboratories was the same for both studies; the WB/IL-1 β test method showed the least agreement (57-58%) and the Cryo WB/IL-1 β test method showed the most (88-92%).

Table 3-6 Interlaboratory Reproducibility of *In Vitro* Pyrogen Test Methods: Study Two

Lab Comparison ¹	Agreement Between Laboratories ¹						
	WB/IL-1 β (Tube)	WB/IL-1 β (Plate)	Cryo WB/IL-1 β	WB/IL-6	PBMC/IL-6	PBMC/IL-6 (Cryo)	MM6/IL-6
1 vs 2	73% (35/48)	88% (37/42)	84% (38/45)	85% (41/48)	84% (42/50)	96% (48/50)	90% (45/50)
1 vs 3	82% (40/49)	90% (35/39)	88% (21/24)	85% (41/48)	86% (43/50)	76% (38/50)	90% (43/48)
2 vs 3	70% (33/47)	92% (43/47)	100% (25/25)	88% (44/50)	90% (45/50)	80% (40/50)	83% (40/48)
Mean	75%	90%	91%	86%	87%	84%	88%
Agreement across 3 labs	57% (27/47)	85% (33/39)	88% (21/24)	79% (38/48)	80% (40/50)	76% (38/50)	81% (39/48)

Abbreviations: Cryo = Cryopreserved; IL = Interleukin; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

¹Data from 10 substances spiked with endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]) at 0, 0.25, 0.5, and 1.0 EU/mL, with 0.5 EU/mL spiked in duplicate, were tested once in three different laboratories.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The currently accepted pyrogen test methods require the use of rabbits or horseshoe crab hemolymph. The proposed *in vitro* pyrogen test methods use monocytoid cells of human origin, obtained either from WB donations or from an immortalized cell line. The capability of these five *in vitro* assays to detect Gram-negative endotoxin suggests that they may reduce or eventually replace the use of rabbits and/or horseshoe crab hemolymph for pyrogen testing. However, at the present time, the RPT detects classes of pyrogens that have neither been examined nor validated with the *in vitro* pyrogen test methods and thus, the RPT will still be required for most test substances.

Human blood donations are required for four of the five *in vitro* test methods (WB/IL-1 β , WB/IL-6, Cryo WB/IL-1 β , and PBMC/IL-6) proposed as replacements for the RPT, and as such, no animals will be used when these assays are appropriate for use. While the collection of human blood is a common medical procedure, the many aspects of human blood collection must be considered to ensure that human donors are treated appropriately, and that such collection and use is in accordance with all applicable regulations, policies, and guidelines.

4.0 ICCVAM Consideration of Public and SACATM Comments

In response to three *FR* notices that were released between December 2005 and May 2007, eight public comments were received (see **Appendix D**). Comments received in response to or related to the *FR* notices are also available on the NICEATM/ICCVAM website (<http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm>). The following sections, delineated by *FR* notice, provide a brief discussion of the public comments received.

4.1 Public Comments in Response to *FR* Notice (70FR74833, December 16, 2005): Peer Panel Evaluation of *In Vitro* Pyrogenicity Testing Methods: Request for Comments, Nominations of Experts, and Submission of *In Vivo* and *In Vitro* Data

NICEATM, in an *FR* notice (Vol. 70, No. 241, pp. 74833-74834, December 16, 2005), requested (1) public comments on the appropriateness and relative priority of convening an independent peer review panel (Panel) to evaluate the validation status of five *in vitro* pyrogen test methods, (2) the nomination of scientists with relevant knowledge and experience to potentially serve on the Panel should it be convened, and (3) submission of data from the RPT, the BET, and *in vitro* pyrogenicity testing using any of the five *in vitro* pyrogen test methods under consideration by NICEATM.

In response to this *FR* notice, NICEATM received two comments. No additional data or information was submitted in response to this request. One nomination requested consideration of three potential panelists.

One commenter provided a reference for an *in vitro* pyrogen test method that measured TNF- α (Martinez et al. 2004). The comment and article were provided to the Panel. However, the reference was not included in the ICCVAM BRD because the *in vitro* pyrogen methods being evaluated by NICEATM measured only IL-1 β and IL-6.

A second commenter requested an expeditious review of the *in vitro* pyrogen test methods and described limitations of the currently used *in vivo* pyrogen test methods (i.e., the RPT and the BET). This commenter also stated that the peer review of the *in vitro* test methods is appropriate, necessary, and should be given extremely high priority.

4.2 Public Comments in Response to *FR* Notice (71FR74533, December 12, 2006): Announcement of an Independent Scientific Peer Review Meeting on the Use of *In Vitro* Pyrogenicity Testing Methods; Request for Comments

NICEATM, in an *FR* notice (Vol. 71, No. 238, pp. 74533-4, December 12, 2006), announced (1) an independent scientific peer review meeting to evaluate the validation status of five *in vitro* pyrogen test methods proposed as replacements for the RPT, and (2) the availability of an ICCVAM draft BRD on five *in vitro* pyrogen test methods, which describes the current validation status of these methods and contains all of the data and analyses supporting their current validation status, and ICCVAM draft recommendations on the proposed use of these test methods, draft test method protocols, and draft performance standards. NICEATM invited the submission of written comments on the ICCVAM draft BRD and on the ICCVAM draft test method recommendations. In response to this *FR* notice, NICEATM received four comments.

One commenter expressed that it was not clear why ICCVAM was neither considering the *in vitro* pyrogen test methods for detection of non-endotoxin pyrogens nor for replacement of both the RPT and the BET. The commenter suggested that exclusion of these broader uses would minimize the impact of these test methods on reduction in animal use and urged ICCVAM "to significantly revise its recommendations and BRD to more accurately reflect the potential use of these methods as full replacements for both the {BET} and RPT." Furthermore, they "strongly encouraged ICCVAM to delete the recommendation regarding the conduct of *de novo* RPTs to further demonstrate *in vivo/in vitro* concordance." ICCVAM appreciates the concern for the proposed limited use of these test methods. However, neither data comparing the *in vitro* test methods to the BET nor data directly comparing non-endotoxin pyrogens to the BET or the RPT were included in the validation studies submitted by ECVAM. Therefore, ICCVAM was unable to consider the *in vitro* test methods as replacements for the BET or to propose the use of these test methods for non-endotoxin pyrogens. However, ICCVAM did identify and recommend future studies that could fill these data gaps and in turn, potentially broaden the applicability of these test methods to that suggested by the commenter.

Several commenters argued that the scope of the test substances was limited and the data provided were inadequate to support the intended use of the *in vitro* test methods (i.e., as a complete replacement for the RPT). These commenters emphasized that additional testing is needed before these test methods can be recommended for this broader application. ICCVAM agreed with these comments, which are reflected in the ICCVAM recommended future studies.

One commenter provided data on an alternative *in vitro* pyrogen test method that is based on the measurement of reactive oxygen species from the human HL-60 promyelocytic leukemia cell line (Blatteis 2006; Timm et al. 2006). The comment and articles were provided to the Panel. However, these data were not included in the ICCVAM BRD because the *in vitro* pyrogen methods being evaluated by NICEATM measured only IL-1 β and IL-6.

4.3 Public Comments in Response to *FR* Notice (72FR26395, May 9, 2007): Peer Review Panel Report on Five *In Vitro* Pyrogen Test Methods: Availability and Request for Public Comments

NICEATM, in an *FR* notice (Vol. 72, No. 89, pp. 26395-26396, May 9, 2007), announced the availability of the Panel report and invited the submission of written comments on the report. In response to this *FR* notice, NICEATM received two comments.

One commenter indicated that several of the Panel's observations and recommendations were "nonsensical, irrelevant, or inappropriate." This commenter also expressed concern about the "random" selection of Panel members and recommended both simplification of the questions posed to the Panel and an orientation meeting to provide the panelists with background information and focus. It was recommended that "ICCVAM coordinate with the pharmaceutical and medical devices industry to conduct product-specific validation on a set of pre-selected products and devices to serve as further validation work." ICCVAM appreciates comments related to the evaluation process of new alternative test methods. ICCVAM notes that Panel members were selected from nominations received in response to an *FR* notice (Vol. 70, No. 241, pp. 74833-74834, December 16, 2005), in conjunction with recommendations from the ICCVAM PWG, which includes a liaison from ECVAM.

Additionally, orientation sessions are routinely convened for the Panel to provide background information on the ICCVAM test method evaluation process.

A second commenter outlined responses to specific comments and/or recommendations made in the Panel report. These comments provided rationale for the design of the ECVAM validation study and summarized existing data to address many of the Panel's concerns. ICCVAM appreciates these written responses and clarifications to specific Panel comments. ICCVAM considered all comments prior to finalization of the ICCVAM BRD and in preparation of the ICCVAM test method evaluation report.

4.4 Public and SACATM Comments: SACATM Meeting on June 12, 2007

The June 12, 2007 SACATM Meeting included a discussion of the ICCVAM review of the *in vitro* pyrogen test methods. At this meeting, three public comments and four SACATM comments were presented.

One public commenter reiterated the written comments submitted in response to the *FR* notice announcing the availability of the Panel report (see **Section 4.3**, first commenter).

A second public commenter (who was also the Chair of the ICCVAM peer review panel) stated that, "given more time to discuss these methods, the Panel might have been able to provide a stronger recommendation for one or more of the assays." ICCVAM appreciates comments related to the evaluation process and now intends to extend the time allocated for Panel meetings to ensure that sufficient time is allotted.

A third public commenter noted that the long list of future studies recommended by the Panel were impractical and not feasible to complete, particularly considering the expense that had already been invested in the validation effort. This commenter also provided additional comments relevant to the criticisms of these *in vitro* test methods made by the Panel (e.g., the limitations of the *in vitro* methods were not fairly compared to the limitations of the RPT and BET; only endotoxin was included in the validation study because no non-endotoxin reference standard is available; and false positives were recorded because the assays are too sensitive). ICCVAM considered many of these comments in the revisions of the ICCVAM BRD and in the preparation of the ICCVAM test method evaluation report.

One SACATM member expressed concern with the high false negative rates reported for some of the assays, the proprietary issues associated with using the Novartis IL-6 ELISA, the lack of concordance assessment between the RPT and the *in vitro* data, and the range of substances included in the validation studies. A second SACATM member provided comments on the statistical analyses used to assess the *in vitro* data. ICCVAM agrees with many of these concerns, which are reflected in the ICCVAM test method recommendations.

A third SACATM member recommended that multiple test methods not be reviewed simultaneously. As stated above, ICCVAM plans to allocate additional time for deliberation at Panel meetings.

A fourth SACATM member suggested the concept of "core panelists" who are knowledgeable about the ICCVAM evaluation process for ICCVAM reviews with the addition of *ad hoc* experts for specific methods. ICCVAM also appreciates this suggestion and makes every effort to include in each panel individuals with direct experience with the ICCVAM evaluation process as well as experts in the subject matter being evaluated.

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Appendix A

Independent Scientific Peer Review Panel Assessment

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Appendix A1

Peer Review Panel Report: The Use of Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products

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**Independent Peer Review Panel Report:
Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity
of Pharmaceuticals and Other Products**

April 2007

**Interagency Coordinating Committee on the Validation of Alternative Methods
(ICCVAM)**

**National Toxicology Program (NTP) Interagency Center for the Evaluation of
Alternative Toxicological Methods (NICEATM)**

**National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Services
Department of Health and Human Services**

**This document is available electronically at:
<http://iccvam.niehs.nih.gov/docs/pyrogen/PrRevPanFinRpt.pdf>**

The findings and conclusions of this report are those of the Independent Scientific Peer Review Panel and should not be construed to represent the official views of ICCVAM or its member agencies.

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PREFACE

This document is an independent report of the *In Vitro* Pyrogenicity Peer Review Panel ('Panel') evaluation of the validation status of five *in vitro* test methods for pyrogenicity testing. The Panel was convened as a National Institutes of Health (NIH) Special Emphasis Panel by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to provide advice to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). This report summarizes the discussions, conclusions, and recommendations of the Panel's public meeting convened at the NIH in Bethesda, MD on February 6, 2007. ICCVAM and the ICCVAM Pyrogenicity Working Group (PWG) will consider the Panel report, along with comments from the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and prepare final test method recommendations for U.S. Federal agencies. ICCVAM test method recommendations will be forwarded to U.S. Federal agencies for consideration and action, in accordance with the ICCVAM Authorization Act of 2000 (42 U.S.C. 285f-3, available at http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf).

The Panel considered five *in vitro* test methods submitted to ICCVAM by the European Centre for the Validation of Alternative Methods (ECVAM), a unit of the Institute for Health and Consumer Protection (IHCP) at the European Commission's Joint Research Centre. ECVAM submitted background review documents (BRDs) for these test methods to ICCVAM for consideration as replacements for the rabbit pyrogen test (RPT) in June 2005. The proposed test methods are:

- The Human Whole Blood (WB)/IL-1 *In Vitro* Pyrogen Test
- The Human WB/IL-1 *In Vitro* Pyrogen Test: Application of Cryopreserved Human WB
- The Human WB/IL-6 *In Vitro* Pyrogen Test
- The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test
- An Alternative *In Vitro* Pyrogen Test Using the Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6

ICCVAM established an ICCVAM PWG to work with NICEATM to carry out the evaluation of these test methods. The ICCVAM PWG developed draft test method recommendations and questions for consideration by the Panel. The ICCVAM PWG also collaborated closely with ECVAM throughout the evaluation process to obtain additional information for consideration by the Panel and ICCVAM.

The Panel was provided a comprehensive draft BRD prepared by NICEATM in conjunction with the PWG and ICCVAM. The draft BRD provided all available data and information related to the five *in vitro* pyrogen test methods. The five ECVAM submitted BRDs (one for each test method), the ECVAM response to PWG questions, and other supplemental information (i.e., key references and testing guidelines/regulations for pyrogenicity testing) were appended to the draft BRD. All of the information provided to the Panel was also made

publicly available, and public comments were requested via a *Federal Register (FR)* notice (Vol. 71, No. 238, pp. 74533-74534, 12/12/06). The *FR* notice also announced the public ICCVAM independent peer Panel review meeting scheduled for February 6, 2007.

The Panel was charged with:

- Reviewing the ICCVAM draft BRD for completeness and to identify any errors or omissions in the draft BRD
- Evaluating the information in the draft BRD to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM 2003¹) have been appropriately addressed
- Considering the ICCVAM draft test method recommendations for the following and commenting on the extent to which they are supported by the information in the draft BRD:
 - proposed test method uses
 - proposed recommended standardized protocols
 - proposed test method performance standards
 - proposed additional studies

At the Panel's public meeting on February 6, 2007, the Panel made recommendations for corrections and additions to the draft BRD and then discussed the current validation status of these five *in vitro* test methods. The Panel also commented on the ICCVAM draft test method recommendations for proposed test method uses, recommended standardized protocols, test method performance standards, and additional studies. The public was provided the opportunity to comment several times during the meeting. The Panel considered these comments as well as public comments submitted in advance of the meeting before concluding their deliberations.

The Panel gratefully acknowledges the efforts of NICEATM staff in coordinating the logistics of the peer review Panel meeting and in preparing materials for the review. The Panel also thanks Dr. Thomas Hartung (Head of ECVAM) for providing an overview of the test methods and for additional clarifications at the meeting. Finally, as Panel Chair, I want to thank each Panel member for their thoughtful and objective review of these test methods.

Karen Brown, Ph.D.
Chair, *In Vitro* Pyrogenicity Peer Review Panel
April 2007

¹ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03-4508. Research Triangle Park, NC. NIEHS. The guidelines can be obtained at http://iccvam.niehs.nih.gov/SuppDocs/SubGuidelines/SD_subg034508.htm.

EXECUTIVE SUMMARY

This report describes the conclusions and recommendations of the *In Vitro* Pyrogenicity Peer Panel ('Panel') regarding the validation status of five *in vitro* pyrogen test methods¹, and the ability of these test methods to individually serve as a substitute for the Rabbit Pyrogen Test (RPT) for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation. The test methods are:

- The Human Whole Blood (WB)/IL-1 *In Vitro* Pyrogen Test
- The Human WB/IL-1 *In Vitro* Pyrogen Test: Application of Cryopreserved Human WB
- The Human WB/IL-6 *In Vitro* Pyrogen Test
- The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test
- An Alternative *In Vitro* Pyrogen Test Using the Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6

Panel Recommendations for the ICCVAM Background Review Document

The Panel stated that, in general, the information presented in the ICCVAM draft Background Review Document (BRD) was sufficient for its purpose. Exceptions are included within the body of the Panel report. The Panel identified a number of sections where clarification or a more comprehensive explanation would improve the ICCVAM draft BRD. For example, the extent to which the RPT is currently performed when risk assessments and regulatory decisions are concerned only with the presence of endotoxin should be provided. Likewise, a more detailed review of the various mechanisms and processes thought to be involved in the actual induction of fever itself, and a more detailed description of the statistical approaches used to evaluate the resulting data would be helpful. The Panel stated that the rationale for the selected test substances was neither appropriate nor acceptable and they recommended the inclusion of non-endotoxin pyrogens, protein- and lipid-containing materials that are used parenterally, and 'classical' examples of biological products and medical devices. The Panel also requested that the formal validation statement from the ECVAM Scientific Advisory Committee (ESAC) (and the supporting documents) be appended to the ICCVAM BRD. The Panel agreed that a comprehensive summary of findings on overall conclusions about the usefulness and limitations of each of the *in vitro* pyrogen tests compared to the Bacterial Endotoxin Test (BET) or the RPT should be included in the ICCVAM final BRD.

With regard to animal welfare, the Panel suggested that the ICCVAM final BRD provide information on the number of rabbits used for pyrogenicity testing to permit an accurate assessment of the actual impact on animal use. The Panel recommended that the ICCVAM

¹These test methods are referred to in this report as *in vitro* pyrogen tests in order to maintain consistency with the designation provided by the test methods' submitter (ECVAM). However, the Panel noted that this designation may be inappropriate because the usefulness and limitations for these test methods have been defined only for their ability to detect bacterial endotoxin and not other pyrogens.

final BRD discuss the practice of, and the U.S. Federal restrictions on, the reuse of rabbits in pyrogenicity testing, as well as the availability and use of the recombinant clotting factor C (rFC) that could replace the need for horseshoe crab hemolymph. The Panel also felt that the lack of direct parallel testing in rabbits with the products tested in the validation study was a significant limitation to the study design.

The Panel concluded that the cost and logistical considerations involved in conducting a study using the *in vitro* test methods were incompletely stated. The Panel recommended that a more detailed cost comparison for conducting the RPT and the *in vitro* test methods be performed. The Panel also commented that both the cost and logistical problems associated with the need to harvest and use human blood in four of the test methods were understated.

Validation Status of the *In Vitro* Pyrogen Test Methods

The Panel agreed that the applicable validation criteria have been adequately addressed in the ICCVAM draft BRD in order to determine the usefulness and limitations of these test methods to serve as a substitute for the RPT, for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation. However, the Panel generally agreed that the performance of these test methods in terms of their reliability and relevance did not support this proposed use. A minority opinion (Dr. Peter Theran) suggested that the qualification in the above statement (i.e., that uses were subject to product specific validation) should allow for these test methods to be used for the specified purpose. A second minority opinion (Drs. Karen Brown, Albert Li, and Jon Richmond) expressed concern that it is not clear that the qualification included in the above statement would preclude the use of the *in vitro* test methods as replacements for the RPT in those circumstances where the BET is currently serving to replace the RPT.

Review of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Draft Recommendations for Test Method Usefulness and Limitations

The Panel concluded that the available data and demonstrated performance of these five *in vitro* test methods, in terms of their relevance² and reliability³, did not support the ICCVAM draft recommendations in terms of their usefulness and limitations. The Panel felt that the usefulness of these test methods for detecting Gram-negative endotoxin has not been properly assessed for concordance with the RPT or for relevance in comparison to the BET, and therefore, it was not possible to truly assess their usefulness and limitations.

One minority opinion stated (Dr. Peter Theran): This Panel has considered the failure to undertake additional RPTs a significant flaw in this validation study and therefore proposed that, in the future, similar validation studies should use the RPT to provide concordance data. I have no objection to the performance of *in vitro* tests in parallel with rabbit tests, which are already scheduled to be performed, in order to achieve concordance data. But, it is my

²The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the "accuracy" or "concordance" of a test method.

³A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and inter-laboratory reproducibility and intralaboratory repeatability.

opinion, that any recommendation for *de-novo* parallel RPT should be accompanied by a statement, as follows: “The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis).” The inclusion of this statement would reinforce the importance of the 3R’s and would serve as a reminder of U.S. Federal law.

Review of the ICCVAM Draft Recommendations for Test Method Standardized Protocols

The Panel agreed that the information provided in the ICCVAM draft BRD supported the ICCVAM draft recommended protocols for these five *in vitro* test methods, providing that the list of inadequacies⁴ identified by the Panel with respect to reliability and relevance are fully addressed.

Review of the ICCVAM Draft Recommendations for Test Method Performance Standards

The Panel did not support the statement that the available data and demonstrated performance in terms of relevance and reliability supported the ICCVAM draft recommendations for these *in vitro* test methods in terms of their performance standards. The Panel noted several inadequacies with regard to the essential test method components for each *in vitro* test method and agreed that the demonstrated performance of certain aspects of several of the assays, particularly in terms of relevance, yielded some concern. With regard to the minimum list of reference substances, the Panel agreed that if the intent of the proposal was to replace the RPT with one or more of the *in vitro* test methods under consideration, then the *in vitro* test methods must be validated for all classes of substances (e.g., pharmaceuticals, biologicals, and implants) and medical devices that are tested with the RPT.

The same minority opinion directed towards the issue of parallel testing using the RPT as detailed above was expressed.

Review of the ICCVAM Draft Recommendations for Future Studies

The Panel agreed that to better determine the relevance of these *in vitro* test methods, the proposed additional studies should be performed using the ICCVAM proposed protocols, taking into account the Panel's comments and recommendations. The Panel also agreed that if the intended use of the *in vitro* assays were only to detect Gram-negative endotoxin, it would seem critical to include parallel studies with the BET in any future validation efforts. However, if the intended use of the *in vitro* methods is to evaluate substances containing endotoxin that are unable to be evaluated with the BET, then the parallel testing studies should include the RPT. The Panel recognized that these test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.

⁴Based on the list of 20 separate inadequacies outlined in this report, three Panel members felt that this list would be better described as a list of "many and substantial" inadequacies.

The same minority opinion directed towards the issue of parallel testing using the RPT as detailed above was expressed.

OVERALL PEER REVIEW OUTCOMES

This international independent Peer Review Panel, consisting of 13 expert scientists from five different countries, provided comments and recommendations on the usefulness and limitations of five *in vitro* pyrogen test methods for the detection and quantification of Gram-negative endotoxin and on the ICCVAM draft test method recommendations on the use of these *in vitro* methods as partial replacements for the RPT. These remarks are summarized below.

- In general, the information presented in the ICCVAM draft BRD was sufficient for the purpose of determining the usefulness and limitations of these test methods for their proposed use and for adequately addressing the applicable validation criteria on the basis of the currently available evidence.
- The available data and demonstrated performance in terms of their reliability and relevance do not at this time support the ICCVAM draft proposed use for these test methods (i.e., as a partial substitute or replacement for the RPT, for the identification of Gram-negative endotoxin, on a case-by-case basis, subject to product specific validation). To better characterize the test methods and more clearly define their reliability and relevance, the Panel recommended that specific additional studies be performed using the ICCVAM proposed protocols, taking into account the Panel's comments and recommendations.
 - The lack of parallel testing in the *in vitro* tests and the RPT, and the resulting lack of concordance data, was considered to be a major limitation of the validation study design. For this reason, the Panel recommended that future studies include parallel testing. A minority opinion (Dr. Peter Theran) associated with parallel testing was expressed as follows: "The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis)".
- The available data and demonstrated performance in terms of their reliability and relevance does not support the ICCVAM draft performance standards for these *in vitro* test methods for regulatory purposes.
- The information provided in the ICCVAM draft BRD supports the ICCVAM draft recommended protocols for these five *in vitro* test methods, providing that the list of inadequacies⁵ identified by the Panel with respect to reliability and relevance are fully addressed.
- These test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.
- It is critical to recognize, despite concerns about the performance of these five *in vitro* test methods, that a formal process exists for materials regulated under 21 CFR 610.9

⁵Based on the list of 20 separate inadequacies outlined in this report, three Panel members felt that this list would be better described as a list of "many and substantial" inadequacies.

to qualify these *in vitro* methods for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation.

A. REVIEW OF THE VALIDATION STATUS OF *IN VITRO* PYROGEN TEST METHODS

1.0 INTRODUCTION AND RATIONALE FOR THE PROPOSED USE OF *IN VITRO* PYROGEN TEST METHODS¹

1.1 Introduction

1.1.1 Is the historical background provided for the *in vitro* pyrogen test methods and the rationale for their development adequate?

Yes, the Preface, the Executive Summary and **Section 1.1.1** of the ICCVAM draft Background Review Document (BRD) are all informative, clear, and concise with the following exceptions:

1. The action of pyrogens on circulating cells and the mechanism by which the pro-inflammatory cytokines produce pyrexia should be considered in **Section 1.1.1** instead of in **Section 1.3.2**).
2. The reduction in the use of animals to test medicinal products produced under current Good Manufacturing Practices (GMP) is an obvious goal. However, no information is provided on the current use of the Rabbit Pyrogen Test (RPT) or the bacterial endotoxin test (BET) (i.e., the approximate number of rabbits and horseshoe crabs used each year for pyrogen testing), or of anticipated trends in their use, or of the extent to which the RPT is currently used in contexts where risk assessments deem endotoxin to be the only relevant contaminant.
3. On lines 694-696 of the ICCVAM draft BRD (December 1, 2006), it is stated that the proposed *in vitro* tests were selected for their ability to replace the RPT. In the previous paragraph, it is stated that the RPT is capable of detecting both endotoxin and non-endotoxin pyrogens. Elsewhere, it is noted that these *in vitro* tests have not been validated for detecting non-endotoxin pyrogens. If the aim of testing these materials with the RPT is to detect a range of pyrogens, then these assays cannot, on the basis of information supplied in the validation dossier, completely replace the RPT.
4. A more detailed review of the various mechanisms and processes thought to be involved in the actual induction of fever itself, particularly in the case of drugs that are not administered intravenously, would have been useful. A number of reviews on this subject describe a far more complex picture than presented. These additional references include:
Netea et al. (2000) and Saper and Breder (1994).

¹These test methods are referred to in this report as *in vitro* pyrogen tests in order to maintain consistency with the designation provided by the test methods' submitter (ECVAM). However, the Panel noted that this designation may be inappropriate because the usefulness and limitations for these test methods have been defined only for their ability to detect bacterial endotoxin and not other pyrogens.

1.1.2 Is the previous review of the ECVAM validation studies adequately summarized?

Yes, the previous review of the ECVAM validation studies was adequately summarized. The questions resulting from the initial review have been answered and included in the ICCVAM draft BRD. However, it would have been better if the actual ESAC validation statement in full had been appended, as well as any documents used to support the ESAC conclusion. The ECVAM BRDs (though not the ESAC statement) contain inconsistent text relating to the possible practical uses of the novel tests that the validation tests were intended to support.

1.2 **Regulatory Rationale and Applicability**

1.2.1 Are the current regulatory testing requirements and ICCVAM prioritization criteria adequately discussed and up-to-date?

Yes, the current United States (U.S.) and European Union (EU) regulatory testing requirements are properly referenced and the relevant documents have been supplied. The previous product specific acceptance of peripheral blood mononuclear cell (PBMC) data by the U.S. Food and Drug Administration (FDA) is also mentioned in the Executive Summary.

Inclusion of the following information would have been useful:

1. It should be stated whether the acceptance of the PBMC data by the FDA was a replacement for the BET or the RPT. The document 21 CFR 610.9 provides for the use of alternative methods to test for pyrogenic substances as long as the use of these methods does not compromise the safety, purity or potency of the product. The 1987 FDA guideline on the validation of the BET as an end-product endotoxin test for human and animal parenteral drugs also sets forth acceptable conditions for the use of the test *in lieu* of the RPT. However, no mention is made of the fact that the European Directorate for the Quality of Medicines (EDQM) also has a working party of experts (apparently independent of ECVAM and ESAC) reviewing the whole area of *in vitro* pyrogens tests and their potential use.
2. The ICCVAM final BRD should discuss the availability and use of the rFC that could replace the need for horseshoe crab hemolymph.
3. The ICCVAM draft BRD gives few insights into how any recommendations, following acceptance by the relevant agencies, would be incorporated into U.S. Pharmacopeia (USP) and European Pharmacopeia (EP) test requirements.

Specific comments on the five ICCVAM prioritization criteria outlined in the ICCVAM draft BRD:

Criterion 1 (Applicability to regulatory testing needs and multiple agencies/programs): It is clear that the test methods are relevant to the end-product testing of a variety of healthcare products (for endotoxin) and that the FDA is the principal U.S. regulator for such products.

Criterion 2 (Warranted, based on extent of expected use or application and impact): It is clear from the documents that this criterion is only met with respect to the detection of endotoxin.

Criterion 3 (Potential to address any/all of the 3Rs): The tests have the potential to reduce or replace animal use and the associated morbidity and mortality. However, no information is provided in the ECVAM BRDs or in the ICCVAM draft BRD to permit the actual impact on animal use to be accurately assessed.

Criterion 4 (Potential to provide improved prediction): The documents indicate that the level of protection provided by each of the *in vitro* test methods is equivalent to that provided by the RPT. However, in the original ECVAM BRDs, it is recognized that sensitivity may have been underestimated and specificity overestimated as a consequence of having one of the spiked-sample points set at the regulatory limit. On lines 777-784 of the ICCVAM draft BRD (December 1, 2006), the statement that these methods would better predict the human pyrogenic response than the RPT because they use human cells is not supported by test results in the ICCVAM draft BRD. In contrast, it is stated on lines 1299-1303 of the ICCVAM draft BRD (December 1, 2006) that the pyrogenic response to endotoxin in rabbits and humans is “similar in both species. Based on these studies, the rabbit is considered to be predictive of the human response (and may often overpredict the response).”

Criterion 5 (Other advantages): The new test methods clearly take longer to produce definitive results. However, no animal facility is required. It was a surprise (in the absence of definitive cost information) that the novel tests were considered to be potentially more expensive than the RPT. Contract research organizations should be consulted on potential cost comparisons, as wide acceptance of these methods may in part be cost-dependent.

1.2.2 Is the description of the intended uses of the *in vitro* pyrogen tests complete?

These methods are proposed as partial replacements for the RPT. The RPT detects both endotoxin and non-endotoxin pyrogens, but the *in vitro* pyrogen tests have not been validated for non-endotoxin pyrogens. Therefore, they cannot be considered complete replacements for the RPT.

It is not clear when, or in which situations, the *in vitro* pyrogen test methods would be appropriate for use. The BET detects endotoxin in most cases and is used instead of the RPT for this purpose. The application of the *in vitro* test methods for the detection of endotoxin in sample types that cannot be measured in the BET is plausible; however, this proposed use would represent a very limited application for the *in vitro* pyrogen tests.

1.2.3 Are the similarities and endpoints measured by the proposed test methods and the reference (RPT) test method adequately described and discussed?

Yes, although the exact causes of the endpoint of the RPT (i.e., fever) are relatively complex and unclear, it has been known for many years that cytokines, especially those involved in the inflammatory response (i.e., IL-1, IL-6, and TNF) can induce febrile reactions. The development of tests based on the production of such cytokines from human white blood cells or cell lines appears to correlate well with the induction of fever in both the RPT and humans. However, the RPT detects a whole organ/body fever response; whereas, the proposed test methods detect only cytokine secretion. Evidence to suggest that detection of IL-1 or IL-6 is necessarily an indication of a febrile reaction is lacking. Additional information should be included in the ICCVAM final BRD on the relationship between IL-1

or IL-6 levels produced in cultures of monocytes and the development of fever in humans. The fact that the cytokine profiles for different endotoxins may vary between rabbits and humans should also be considered.

1.2.4 Is the description of the use of the proposed test methods in an overall strategy of hazard or safety assessment adequate?

Yes, the utility of the *in vitro* pyrogen methods as an addition to the current RPT, especially where non-endotoxin pyrogens are involved, has been clearly discussed. No specific claims are made for an immediate replacement of the RPT, although future studies may lead to such an event. The overall demonstration of the applicability of the methods to non-endotoxin detection is a stated goal. However, this goal does not appear to adequately match the methods employed since non-endotoxin standards were not used. One information gap (in the ICCVAM draft BRD and ECVAM BRDs) is the extent to which the RPT is currently performed when risk assessments and regulatory decisions are concerned only with the presence of endotoxin (that is clearly the intention when only the BET is used). Product-by-product validation will be required and the full extent of materials for which the new tests are not suited remains to be defined.

1.3 Scientific Basis for the *In Vitro* Pyrogen Test Methods

1.3.1 Is the purpose and mechanistic basis of the *in vitro* test method(s) adequately described and compared to known and/or suspected mechanisms/modes of action for fever production in humans?

Yes, the purpose and mechanistic basis of the *in vitro* test methods appears to be adequate while acknowledging that, at this point, the reference standard included in the validation study was Gram-negative endotoxin only. The mechanisms underlying fever induction, including the production of cytokines involved in the inflammatory cascade, appear to be important. The administration of such cytokines can directly induce fevers and their levels have been shown to dramatically increase during fevers. However, the known and suspected mechanisms/mode of action of fever may be far more complex than that described (see also response to **Section 1.1.1**).

The claim in **Section 1.3.1** to 'identify pyrogens' should perhaps be restated to 'detect pyrogens.'

1.3.2 Are the known similarities and differences of modes of action between the *in vitro* pyrogen test methods and the fever response in human and/or rabbits adequately considered?

Yes, an extensive literature search was performed that covered a wide range of cases illustrating the similarities and differences between the modes of action between the RPT, the *in vitro* pyrogen tests, and the induction of fever in humans. The correlation, or lack thereof, between the tests and human fever induction has been discussed in a scientifically valid manner. It should be noted that the RPT has served as a good predictor of human pyrogen response. Although there are false positives and false negatives associated with the RPT, it is not clear that these proposed *in vitro* assays provide better, similar, or worse results. A major concern is the lack of validation of these new assays directly compared to the RPT.

The mode of action is oversimplified. See response to **Section 1.1.1**, especially the reference to Netea et al. (2000) that provides an excellent review on the multiple-pathway mechanisms

that link cytokine responses (some of which are monitored by the proposed *in vitro* assays) and fever production. Furthermore, no description of the mode of action at either the molecular or cellular level is presented, which may prevent an adequate comparison between the methods. Specific questions that should be addressed include:

1. Are there any scientific data that compare IL-1 and IL-6 production and fever response between humans and rabbits?
2. Is the induction of IL-1 and IL-6 (or even fever) similar or different between endotoxin and other known TLR-4 ligands?
3. What is the mechanism of action for pyrogens that do not utilize TLR-4?
4. TLR-4-mediated IL-1 mRNA induction and the consequent release of mature IL-1 from cells by stimuli other than pyrogens are regulated by different molecular mechanisms. Are these mechanisms similar or different *in vitro* and *in vivo*, or between humans and rabbits?

1.3.3 Is the range of substances amenable to the *in vitro* pyrogen test methods, and are the limits of the test methods adequately characterized?

Yes, given what is known of materials with the potential to interfere with the test system supplemented by the need for product-by-product validation and the exclusion of interference. More work will have to be carried out to understand the types of materials that could be tested in these assays and how they would be handled (e.g., cell therapies and implants). However, it must be considered that a manufacturer of a medicinal product would have to validate the *in vitro* method they have selected specifically for their particular product before it would be acceptable to any regulatory authority. Thus, comprehensive testing of a wide variety of substances may not be necessary to introduce these tests into general use. Insufficient information exists at present to be confident that all types of materials that will demonstrate interference have been identified (e.g., materials that are cytotoxic, contain immunological adjuvants, or have antipyretic properties) but case-by-case evaluation provides the necessary safeguards. In addition, although the test methods have been shown to have the potential to identify non-Gram negative pyrogens, the validation study only presented detailed data and analysis with respect to the tests' potential to detect Gram-negative endotoxins (see also **Section 1.3.1**).

However, with respect to the limits of the test methods, no mention is made of the wide range of drugs that are toxic to blood cells or that induce a substantial pro-inflammatory response and consequently are not amenable to testing by these methods. Many pure, well-established non-endotoxin compounds have been shown to activate blood cells, including monocytic cells, to produce pro-inflammatory cytokines *in vitro* and *in vivo* (see suggested additional references [Ishii et al., 2005; Ishii and Akira, 2006] in **Section 12.0**).

On page 1-5, line 770 of the ICCVAM draft BRD (December 1, 2006) states, "Although the *in vitro* BET is performed using hemolymph (the equivalent of blood) drawn from *Limulus polyphemus* (horseshoe crabs), which are subsequently returned to the wild, there is some mortality associated with the procedure (which requires approximately 20% of the animal's total blood volume)". This concern has been largely solved with the commercial introduction of rFC, which was originally cloned from the horseshoe crab. This commercial product is currently being compared to the BET for submission for inclusion in the USP. A need for a

replacement for the RPT for early compound development testing and testing of biologics that have some propensity to harbor non-endotoxin pyrogens remains to be fulfilled. Thus, the goals of the overall effort need further refinement. Endotoxin is, of course, the important standard for validation purposes but non-endotoxin standards need to be characterized to further such a test for non-endotoxin testing; this concept is referred to on page 1-7, lines 821-822 of the ICCVAM draft BRD (December 1, 2006).

2.0 IN VITRO PYROGEN TEST METHOD PROTOCOL COMPONENTS

2.1 Overview of How the *In Vitro* Pyrogen Test Methods are Conducted

Are there gaps or missing information in the overview of how the tests are conducted?

This section seems adequate and complete. The overview of how the *in vitro* pyrogen tests are carried out is brief and to the point. The assays essentially expose human blood cells (either primary or cell line derived) to a test substance that may or may not induce cytokine release. Any cytokine release is subsequently detected with an immunoassay.

2.2 Description and Rationale for the Test Method Components for Proposed Standardized Protocols

Are the description and rationale for each of the following test method protocol components for the recommended versions of the *in vitro* test methods adequately described and appropriate? Should any protocol components be modified, and, if so, why? Are additional protocol components needed, and, if so, why?

2.2.1 Materials, equipment and supplies

Specific concerns with respect to human blood donors include: diurnal variation, genetic polymorphisms (i.e., in genes coding for Toll-like receptors [TLRs], cytokine receptors, response elements, etc.), and number of donors required.

The effect of components in the blood and their effects on the assay systems are not clear (i.e., the effect of variations in the number of monocytes in peripheral blood, which range from 2 to 10%, as well as the effect of neutrophil or lymphocyte presence on the cytokine response).

2.2.2 Endpoint(s) measured

The viability of the human blood cells should be monitored before and after incubation with the test samples. Cytotoxic substances should not be tested with these methods.

2.2.3 Duration of exposure

A fixed exposure time rather than a broad range of exposure times (e.g., 16 to 24 hours) should be defined.

2.2.4 Known limits of use

It is suggested that the *in vitro* pyrogen tests are suitable for the testing of medical devices and materials by direct contact rather than testing extracts. However, direct contact may not adequately permit the solubilization or leaching of potential pyrogens.

2.2.5 Nature of the response assessed

The nature of the response assessed is accurately summarized. However, a description of the blood cell types known to respond to pyrogens by producing IL-1 and/or IL-6 should be included.

2.2.6 Appropriate negative, vehicle, and positive controls and the basis for their selection

The ECVAM BRDs do not discuss why high quality Gram-positive material (Lipoteichoic acid [LTA]) available from the University of Konstanz was not also used as a 'model' pyrogen. The inclusion of such non-endotoxin positive controls would be useful in future validation studies to further characterize the usefulness and limitations of these methods for the detection of such substances.

2.2.7 Acceptable ranges of negative, vehicle, and positive control responses and the basis for the acceptable ranges, or procedures for establishing acceptable ranges

The ECVAM BRDs indicate that (refer to **Sections 6.1.1**), with hindsight, the use of an endotoxin spike solution at the threshold pyrogen dose (marking the pass/fail level for regulatory purposes) was not wise. See above (response to Criterion 4, **Section 1.2**) regarding possible relevance to determination of sensitivity and specificity of the novel test methods.

2.2.8 Nature of the data to be collected and the methods used for data collection

The description of the nature of the data to be collected and the methods used for data collection is accurate.

2.2.9 Type of media in which data are stored

The type of data storage media seems to fit the purpose. However, one printed version of the data should be stored.

2.2.10 Measures of variability

The description of the measures of variability reflects the current state of knowledge. Other relevant physiological variables may exist but the main sources of potential variation seem to have been addressed.

2.2.11 Statistical or nonstatistical methods used to analyze the resulting data

Generally adequate, but additional clarification is desired. It would seem appropriate to use a consistent approach across assays. For example, in some places, Dixon's test was used to identify outliers, while in others Grubb's test was used; the reasons and contexts for these differences are not apparent. However, it is accepted that minor problems arise with the calculation of sensitivity and specificity of the novel test methods from using a spike-point coincident with the regulatory limit.

The statement that "using an endotoxin curve, the endotoxin content of the product is calculated" is not true. The *in vitro* pyrogen test is not specific for Gram-negative endotoxin

and therefore, it is impossible to know whether the response measured is due to endotoxin or another pro-inflammatory response reactive substance in the sample.

2.2.12 Decision criteria and the basis for the prediction model used to classify a test substance as positive or negative for the presence of a pyrogenic material

The RPT data used to set the pass/fail criteria were produced in one rabbit strain in one laboratory and were not obtained concurrently within the validation study.

It is not clear that the criteria used to assign test results as positive or negative are based on the precise criteria set out in the USP. The significance of any deviations from these criteria is also not clear.

2.2.13 Information and data that will be included in the study report and availability of standard forms for data collection and submission

The descriptions provide a good overview of each test for the purposes of comparing and contrasting them with one another and with current methods.

2.3 Basis for Selection of Test Method Systems

Is the description of the basis for selection of the test method systems complete and appropriate?

A brief description of the advantages of each test method have been provided and are appropriate for considering the limitations of the existing tests for pyrogens, namely the RPT and the BET.

2.4 Proprietary Components

Are proprietary components appropriately identified (if applicable), and are the procedures adequate for ensuring their integrity from 'lot-to-lot' and over time?

The licensing procedure and availability of the Mono Mac 6 (MM6) cell line is unclear. Variations in the MM6 cell line (and primary cells) must be properly controlled. A direct comparison of the commercially available enzyme-linked immunosorbent assay (ELISA) kits should also be included in the ICCVAM final BRD.

2.5 Number of Replicates

Are the numbers of replicate and/or repeat experiments appropriate for each test method?

The appropriate number of donors from which to collect blood cells is unclear. Furthermore, some of the test methods permit pooling of blood donors while others do not. The rationale for these differences is unclear.

2.6 Modifications to the Test Method Protocols Based on ECVAM Validation Study Results

Are the protocol modifications based on ECVAM validation study results appropriate for each modified test method?

Yes, only minor modifications were made to two of the five assays, both to improve assay performance, and therefore the limited explanations are appropriate.

3.0 SUBSTANCES USED FOR THE VALIDATION OF *IN VITRO* PYROGEN TEST METHODS

3.1 Rationale for the Substances or Products Selected for Testing

Is the rationale for the selected test substances appropriate and acceptable?

No, the only rationale given for the choice of test substances is that they represent marketed parenteral pharmaceuticals that were readily available at reasonable cost. According to their USP monographs, seven of the ten test substances are currently tested in the BET, not in the RPT. No USP monographs exist for the remaining three because pyrogen testing is not required. The inclusion of test substances that may interfere with the *in vitro* responses should be tested.

Although the test materials spiked with endotoxin are described as having been initially pyrogen-free and having been approved for clinical use, all that can be said with confidence is that they did not contain a level of pyrogen above the permissible or tolerable limit. As a result, in describing the concentration of endotoxin in the spiked sample, it is more correct to state the minimum level of endotoxin they were known to contain rather than offering an absolute value.

Non-endotoxin pyrogens should be evaluated because these pyrogens must be tested in the RPT and they cannot be tested in the BET. The list of test substances should also include protein- and lipid-containing materials that are used parenterally. No 'classical' examples of biological products or medical devices were included; thus, the validation for either of these categories has not been provided.

Although it is stated that endotoxin was chosen as a model pyrogen, insufficient information exists in the ICCVAM draft BRD or in the supporting ECVAM BRDs to support this claim. The validation study documents, the ESAC validation statement and the ICCVAM draft BRD claim only that the test methods are suited for the detection/qualification of Gram-negative endotoxin for regulatory testing.

3.2 Number of Substances

Please comment on the adequacy of the number of substances used in the performance analyses.

The total number of substances included in the validation study is adequate only for validation of a specific class of products. Replacement of the RPT would require a much larger number of substances because of the wide range of product classes that would require testing. Moreover, the test substances should have represented each of the major classes of

products normally tested in the RPT (e.g., medical devices, biologicals, implants, and those substances known to interfere with the RPT, the BET, and/or the *in vitro* pyrogen tests) as positive controls for interference testing.

3.3 Identification and Description of Substances Tested

Are the test substances adequately identified and described?

The samples included in the validation process are adequately identified and described such that they could be readily obtained for future studies. However, more information on their purity and batch/lot numbers is needed in order to adequately demonstrate that the same substances were tested throughout the validation studies. In response to a request for additional information, ECVAM did provide the lot numbers used in the validation study, which demonstrated that they were identical. However, some differences in the lots tested in the catch-up validation study were noted (e.g., two of the ten substances had different lot numbers due to the lack of availability; one was a different substance with the same active ingredient).

3.4 Sample Coding Procedure

Were the coding procedures used in the validation studies appropriate?

The coding procedures were adequate for the assessment of relevance during the validation studies. However, the identity of the substances used in the reproducibility analyses was not blinded (although the spike concentrations were). A reason was not given.

4.0 *IN VIVO* REFERENCE DATA FOR THE ASSESSMENT OF TEST METHOD ACCURACY

Are the *in vivo* reference data used in the validation study appropriate to allow for adequate assessment of test method relevance² (accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of these *in vitro* pyrogen test methods as a partial replacement for the RPT, for materials which may be contaminated with gram-negative endotoxin, but which cannot be tested by the BET?

No, a summary of the reference data demonstrating whether substances that were shown to be pyrogenic in humans either passed or failed the RPT, BET or *in vitro* pyrogen tests would have been useful.

4.1 Description of the Protocol Used to Generate *In Vivo* Data

Is the RPT protocol used to generate reference data for the cited studies appropriate?

²The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the "accuracy" or "concordance" of a test method.

The RPT protocol and the pass-fail criteria used would not meet the current USP requirements. The significance of these deviations is not clear. The data are derived from a single study carried out at the Paul Ehrlich Institute (PEI) where historical controls tested over five years were accumulated and analyzed. The protocols used at the PEI were based on the EP monograph for the RPT, although this fact is not explicitly stated in the publications. Furthermore, the detailed protocol used by this laboratory was not provided.

4.2 Reference Data Used to Assess *In Vitro* Test Method Accuracy

Is the interpretation of the reference data used to assess *in vitro* test method accuracy correct? Is any other data or information needed to determine the accuracy of the test methods?

The reference data were previously and separately generated by one protocol, in one laboratory, using one strain of rabbit, and two sources of endotoxin. A second study, undertaken in Brazil, is cited. The response criteria of the Brazilian study do not match those of the PEI study. It is not clear why the Brazilian study was not relied upon for the validation study.

4.3 Availability of Original Records for the *In Vivo* Reference Data

Are there any concerns with the availability of the original reference data records as described?

The data were derived from a single study at the PEI and presented in graphical form. No additional data were available for analysis. Archived records have not been audited by ECVAM or ICCVAM.

4.4 *In Vivo* Data Quality

Are there any concerns with the RPT data quality?

The ECVAM documentation is not sufficiently specific and in the absence of the primary data, the quality of the RPT data is unknown. The ICCVAM draft BRD does not clearly indicate the GLP status of the laboratory or of the study. However, the PEI did not have formal GLP accreditation (refer to **Section 5.5**, ECVAM response to a request for additional information).

4.5 Availability and Use of Toxicity Information from the Species of Interest

Is the discussion of the availability of relevant pyrogenicity information for humans adequate and appropriate? Are there other sources of quality human data for pyrogenicity that should be considered? Would human data be compatible with regulatory needs (e.g., exposure duration, individual sensitivity)?

The available data are limited. However, the availability of information on clinical adverse events resulting from the administration of medical products producing pyrogenic effects, and the relevant pre-clinical test data, would be an excellent source of human data. See

suggested additional reference (McKinney et al. 2006). The data would reflect responses seen using appropriate human exposure; thus, it should be compatible with regulatory needs.

A discussion of relevant pyrogenicity information for humans is present in the ICCVAM draft BRD, but additional information is needed. An extensive literature on acute human pyrogenicity responses exists and this data should be better reviewed. Effects of longer exposure and individual sensitivity are available in Rylander (2002).

The data in the cited paper by Greisman and Hornick (1969)³ are not accurately described (page 4-6, lines 1299-1301 of the ICCVAM draft BRD [December 1, 2006]).

4.6 Information on the Relevance and Reliability of the *In Vivo* Test Methods

Is what is known about the relevance and reliability⁴ of the RPT adequately discussed and appropriately considered?

The appropriateness of the theoretical assumption model is unclear. It is not clear how the sensitivity and specificity values have been derived using this model. Therefore, reference to these values as accurate figures (particularly with respect to the 58% sensitivity) is a concern.

The theoretical sensitivity and specificity for the RPT that has been supplied does not seem to reflect its performance in practice or the regulatory decisions and level of patient safety that RPT data currently supports.

The 'correct' figures for the theoretical specificity of the RPT are confusing. It was stated to be 83% in **Section 4.6** of the ICCVAM draft BRD (December 1, 2006) but given as 88.3% in the ECVAM response to ICCVAM questions (page 24).

However, this difference has little bearing on the overall interpretation of the results.

5.0 Test Method Data and Results

5.1 Test Method Protocol

Are the *in vitro* test method protocols used to generate each set of data considered in the ICCVAM draft BRD appropriately described?

The following problems with all five *in vitro* test method protocols were noted:

1. Quality control (QC) testing of cell viability is not performed. Viability testing of the human cells before and after incubation should be performed.
2. No microscopic examination for anticipated levels of cell fragments and debris is described.
3. Substances should not be tested at cytotoxic concentrations by these methods.

³ Greisman SE, Hornick RB. 1969. Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin. Proc Soc Exp Bio Med 131(4):1154-1158.

⁴A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and inter-laboratory reproducibility and intralaboratory repeatability.

4. More detailed source information and the pyrogen status (i.e., pyrogen-free) should be required for all protocol components.
5. A description of the procedure used for donor recruitment and donor selection is not provided.
6. A description of the protocols used for preparation of blood samples for the cytokine assays is not found.

The following problems with specific test method protocols were noted:

1. In the Cryo WB IL-1 assay, the incubation of the test sample is performed in the presence of 10% DMSO (methods for its removal after thawing of the cryopreserved cell preparation and before its use are not described). DMSO is known to effect the detection of certain cytokines. In response to a request for additional information, ECVAM indicated that the DMSO is not removed.
2. A limit to the passage number should be defined for the MM6 cell cultures.
3. The use of the terms RPMI-M and RPMI-C (described in the ECVAM MM6/IL-6 Standard Operating Procedure [SOP]) is confusing in the ICCVAM protocol.
4. A typographical mistake appears in the ICCVAM MM6/IL-6 protocol (lines 285 and 286 of the ICCVAM draft BRD [December 1, 2006]) where 'FBS' is stated instead of 'PBS'.

5.2 Availability of Copies of Original Data Used to Evaluate Test Method Performance

Has the availability of the original data use in the test method performance evaluation been adequately described?

Yes, the availability of the original source data has been adequately described.

5.3 Description of the Statistical Approaches Used to Evaluate the Resulting Data

Are the statistical and non-statistical approaches used in each cited study to evaluate the *in vitro* test results appropriate? What other approaches could have been used?

The statistical approaches appear adequate. However, it is suggested that more emphasis should have been placed on a quantitative estimate of pyrogen concentrations rather than dichotomizing results based upon a hypothesis test. One would have expected to see *a priori* criteria for successful validation in terms of acceptable performance statistics.

The term 'correlation' appears to be used colloquially (e.g., lines 1365 and 1373 of the ICCVAM draft BRD [December 1, 2006]); a correlation is not a percentage. Therefore, 'correlation' should be replaced with 'association' everywhere, except when Pearson's correlation is being referenced.

Information on the identification and elimination of aberrant data from **Section 4.2** of the Trial Data report should be included in the ICCVAM final BRD.

5.4 Summary of Results

Is the summary of the results for each test method appropriate and adequate?

No data were presented to confirm that results in the *in vitro* tests reflect human physiological responses or that production of IL-1 or IL-6 *in vitro* correlates with pyrogenicity *in vivo*. A quantitative link between IL-1 and IL-6 concentrations and their donor-to-donor variation with physiological effects was not presented. It should be mentioned that according to Schindler et al. (2006)⁵, which describes the validation of the Cryo WB/IL-1 method, testing problems existed with many of the products included in the study (up to 9 of 10). This is evident by failure of the positive product control (PPC), which under normal circumstances would invalidate the test. Instead, when the PPC failed, the authors report that the saline control was used in place of the PPC and the experiment was still considered acceptable. This practice is unacceptable.

The lack of direct parallel testing in rabbits with the products tested in the validation study prevents an evaluation of actual physiological effects. It also would have been of assistance to the Panel if information had been provided to document that the use of human cells could partially replace the BET and RPT for the detection of substances that are pyrogenic in humans.

Some of the data (or lack thereof) indicate significant limitations of the *in vitro* assays. Specific examples are listed below:

1. In the ICCVAM draft BRD (December 1, 2006), page 2-7, line 989: The use of a single donor in the WB/IL-1 assay is inadequate.
2. In the ICCVAM draft BRD (December 1, 2006), page 2-10, line 1050: There are no data offered to document that the use of human cells will better reflect human physiological responses or that production of IL-1 or IL-6 *in vitro* correlates with pyrogenicity *in vivo*.
3. In the ICCVAM draft BRD (December 1, 2006), page 6-2, line 1456: 20 of 150 runs in the Cryo WB/IL-1 assay were not usable. Even then, the false positive rate of the remaining 120 assays was 18.6%.
4. In the ICCVAM draft BRD (December 1, 2006), page 6-4, line 1493: 1 of the 3 validation laboratories had a 50% false positive rate for the PBMC/IL-6 assay.
5. In the ICCVAM draft BRD (December 1, 2006), page 7-7, Table 7-4: Agreement across three validation laboratories was only 57% for the WB/IL-1 assay.

⁵ Schindler S, Spreitzer I, Löschner B, Hoffmann S, Hennes K, Halder M, Brügger P, Frey E, Hartung T, Montag T. 2006. International validation of pyrogen tests based on cryopreserved human primary blood cells. *J Immunol Methods* 316:42-51.

5.5 Use of Coded Chemicals and Compliance with GLP Guidelines

For each set of data for each test method, is whether coded substances were tested and whether experiments followed GLP Guidelines adequately documented?

The use of coded substances is adequately documented, but the rationale for not blinding the identity of the three substances tested in the reliability analyses is not known. The *in vitro* pyrogen test studies were conducted 'in the spirit of' GLP requirements. However, gaps and lapses in the information supplied by ECVAM would indicate that none of the testing laboratories were audited in real-time. In response to a request for additional information from ECVAM, it was stated that:

"The initial validation study has been carried out to large extent in laboratories such as National Control laboratories, which do not operate under GLP. It was, however, agreed to comply with the requirements of GLP, especially with regard to the creation and management of SOPs. The partner laboratories have received presentations on the requirements. No auditing was done but various quality checks and blinding mainly under the responsibility of ECVAM were included. In the catch-up validation two GLP laboratories and two National Control laboratories participated."

"Raw data: In both studies, the laboratories were asked to transfer readings into Excel sheets provided by the statistician. This was mostly done by directly inserting the ASCII files created by the plate reader. However, reader printouts are available and can be provided on request."

5.6 Lot-to-Lot Consistency of Test Substances

Is the information on the 'lot-to-lot' consistency of the test substances, the time frame of the various studies, and the laboratory in which the study or studies were conducted, adequately described?

Information on specific lots used in the validation studies was not provided in the ICCVAM draft BRD and therefore, lot-to-lot consistencies cannot be evaluated. Additional information has been received to demonstrate that the same lots were tested in the validation study, but there were lot differences in 2 of 10 substances used in the catch-up validation study. In addition, because one of the substances used in the original validation was no longer available, a different substance (with the same active ingredient) was used in the catch-up validation.

Unfortunately, little or no high concentration protein samples (e.g., Factor VIII concentrates or 5-25% human albumin samples), where lot-to-lot inconsistencies might be expected, were tested in the validation studies. This exclusion was explained to some extent by ECVAM in the responses that they provided to the ICCVAM/PWG questions. Interference testing for all sample types should be tested on multiple lots (also see the specific inadequacy [No. 10, lines 1361-1362] noted in the proposed test method standardized protocols).

6.0 RELEVANCE OF THE *IN VITRO* PYROGEN TEST METHODS

6.1 *In Vitro* Pyrogen Test Method Relevance

Has the relevance (e.g., accuracy/concordance, sensitivity, specificity, positive, and negative predictivity, false positive and false negative rates) of the *in vitro* test methods for detection of pyrogens, as defined by statutes in the United States Code (see Section 1), or for sterility testing defined by the U.S. Pharmacopeia or the International Standards Organization, been adequately evaluated? Are the discussions of the relevance of each *in vitro* test method and the reference test method appropriate and accurate?

In general, the evaluation of the relevance of the *in vitro* pyrogen tests appears to have been appropriately demonstrated and discussed, but limited by the ability to judge a positive versus negative response using a cut-off at 0.5 endotoxin units (EU)/mL. Furthermore, because only endotoxin-spiked samples were tested, relevance has been demonstrated only for the detection of bacterial endotoxin.

This section is entirely focused on comparisons between the *in vitro* pyrogen test methods since the RPT was not carried out in parallel, but rather estimates of the RPT performance were modeled statistically. The validity of this approach remains in question due to the nature of the RPT, where a definitive cut-off point does not exist, but was defined based on the results generated from the historical database. Therefore, no data exist with which to establish concordance with the RPT and thus, the discussion on concordance with the RPT is speculative.

Discrepancies between Table 6-1 and the accompanying text of the ICCVAM draft BRD (December 1, 2006) for the cryopreserved PBMC assay prevented assessment of this method.

6.2 Summary of the Performance Statistics for *In Vitro* Pyrogen Test Methods

Is the summary of the performance of the test methods adequately described? Are the strengths and limitations of each *in vitro* test method adequately identified?

A more critical description and explanation are needed (i.e., a failure of the prediction model or a failure of the assay to correctly detect the pyrogen concentration) for the cases where the test failed to correctly classify the pyrogen concentration.

The discussion of the strengths and limitations of each of the test methods should be expanded. Specific points include:

1. Inadequate performance is noted for: a) Cryo WB/IL-1 (false positive rate = 18.1%); b) WB/IL-1 (false negative rate = 27.3%); c) WB/IL-1 (false positive rate = 16.4%). High false positive rates are clearly a concern for manufacturers since lots may be unnecessarily withheld from release.
2. The high exclusion rate for individual runs in the case of the Cryo WB/IL-1 test (20% - 30% out of 150 runs) due to excessive variability among the four replicates, even with a relatively high coefficient of variation (CV) criteria (CV > 45%).

3. The low sensitivity (only 72%) for the WB/IL-1 assay, resulting in an extremely high false negative rate (27.3 %). High false negative rates would obviously be a major concern, as endotoxin-contaminated lots would be released.

Taken together, these statements could indicate that the WB/IL-1 assays (WB/IL-1 Cryo WB/IL-1, and WB/IL-1 96-well plate method) do not, in general, perform as well as the other assays that measure an IL-6 response.

It would have been very interesting to have had the opportunity to compare performance analysis data for the BET, since only endotoxin spiked samples were used in the validation and endotoxin testing is now the intended use for the *in vitro* pyrogen tests. Unfortunately, the BET was not performed in the validation so no direct comparison can be made between it and the new *in vitro* assays.

7.0 RELIABILITY OF THE *IN VITRO* PYROGEN TEST METHODS

7.1 Selection Rationale for the Substances Used to Evaluate the Reliability of *In Vitro* Pyrogen Test Methods

Is the selection rationale and the number and types of substances used to evaluate the reliability of the *in vitro* test methods (intralaboratory repeatability and intra- and inter-laboratory reproducibility) as well as the extent to which the chosen set of substances represent the range of possible test outcomes appropriate?

The use of a standard material such as the endotoxin control (WHO-LPS, 94/580) is a valid choice for conducting the experiments described since it is a well-characterized, well-documented material. However, the rationale for the selection of the drugs used in the studies for evaluating reproducibility versus sensitivity/specificity is not clear, except that they were manufactured under GMP, were licensed products, were reported not to be contaminated with unacceptable levels of endotoxin, and were all available at reasonable cost. It would have been more appropriate to evaluate reliability using a subset of the drugs used in the sensitivity/specificity studies.

7.2 Analysis of Repeatability and Reproducibility

Are the analyses and conclusions regarding the intralaboratory repeatability and reproducibility and the intra- and inter-laboratory reproducibility of each test method appropriate? Should other analyses be considered?

The experiments performed to evaluate intralaboratory repeatability and intra- and inter-laboratory reproducibility were overly complicated. However, the analysis based on 'positive or negative' calls suggests that the reliability of these *in vitro* test methods are generally acceptable both within and between laboratories, although a more critical description is needed to explain the lack of agreement among some test results.

It is interesting that the variability of the cell line-based MM6 assay is much reduced compared to that obtained for the whole blood assays, although this observation did not translate into an improved ability to assign a negative or positive status to a sample.

The following deficiencies were noted:

1. More discussion is needed about the use of a coefficient of variation (CV) analysis to evaluate the reliability of the *in vitro* test methods, including how an 'acceptable' CV was identified (e.g., 45% in the WB/IL-1 assay) and why the criteria for an acceptable CV was inconsistent among the different *in vitro* test methods.
2. It is not clear which statistical test(s) was used to detect outliers and whether the test(s) was based on original or log-transformed data. Furthermore, it is not clear how many data points were identified as outliers and how they were subsequently handled during data analysis. The information provided by ECVAM addressing these concerns should be integrated into the ICCVAM final BRD.
3. A quantitative assessment of the intra- and inter-laboratory variability would have been more informative than an assessment based on dichotomizing the test results. The assessment should have included estimates of the amount of inter- and intra-laboratory variability and the number of replicates needed to estimate the sources of variability. Consistent with general practice, acceptable levels of variability should have been identified *a priori*, and it should have been recognized that formal hypothesis testing is essential with the alternative hypothesis being no difference between groups.
4. Potential problems related to plate-to-plate variation and/or other plate design issues should be addressed in the ICCVAM final BRD.
5. The use of the term 'mean value calculated' needs to be clarified.
6. It is misleading to state that the test substances were spiked at four concentrations when two of the spikes are at the same concentration. The concentrations should be noted explicitly, even in summaries if this is their first reference.
7. The ICCVAM final BRD should state whether or not the data were log-transformed prior to analysis (as was stated in the ECVAM BRDs). Furthermore, in the ECVAM BRDs, the decision rationale for performing a log transformation versus a square-root transformation of the data should be provided. In all ECVAM BRDs, it is not clear whether all analyses used log-transformed data or if transformed data were used only for the *t*-test in the classification phase of the analysis (e.g., ECVAM BRD for WB/IL-1, page 25).
8. The ECVAM BRDs state that all data are log-transformed, but the y-axis on the graphs is labeled OD 450 (e.g., ECVAM BRD for Cryo WB/IL-1, Appendix D). The data should be log-transformed if this has not yet been done. The CV after transformation is of most interest; however, the figures appear to give data before the transformation indicating that the variance increases with the mean. Data after the transformation should also be plotted to show that the relationship of the mean and the variance is well suited to the log transformation. The analysis with respect to the transformation needs to be

clarified. The values on the x-axis are unreadable and need to be given in the legends or in the description that accompanies each figure.

9. The notation used in the *t*-test (e.g., the subscripts on the population and sample means) needs to be defined. In the standard two-sample *t*-test, the groups are assumed to be independent. However, it looks like one group is a collection of subgroups and the other group is one of these (i.e., the data from one group are used in the calculation of both means). This point needs to be clarified.

7.3 Historical Positive and Negative Control Data

Is the availability of historical negative and positive control data adequately considered?

The fact that the *in vitro* pyrogen test methods are not in routine use except for the two manufacturers cited (who are unlikely to provide what would be considered proprietary data) leads to a paucity of historical data.

8.0 TEST METHOD DATA QUALITY

8.1 Adherence to National and International GLP Guidelines

Is the extent of adherence to national and international GLP guidelines for all submitted *in vitro* and *in vivo* test data and the use of coded substances and coded testing adequately presented?

It is clear that SOPs exist and that protocols were developed for all *in vitro* experiments performed. However, the precise GLP status of the studies and the test laboratories is not clearly stated and the ICCVAM final BRD should be revised to clarify this information. The *in vivo* data are derived from a single published study.

8.2 Data Quality Audits

Are the results of any data quality audits, if conducted, adequately summarized?

From the information provided, it would seem that no audits were undertaken while the studies were in progress. However, the ECVAM BRDs state that 'deviations' were recorded but no further details or information is provided. A summary of the GLP deviations that occurred would have been useful for determining their overall significance to the experimental outcome.

8.3 Impact of Deviations from GLP Guidelines

Does the lack of an evaluation of the impact of deviations from GLP guidelines affect the data analysis?

This question cannot be answered, as no data have been provided on any deviations from GLP guidelines.

8.4 Availability of Laboratory Notebooks or Other Records

Is the availability of laboratory notebooks or other records for an independent audit adequately discussed?

Yes, the study authors state that all raw data are available for inspection and have been archived appropriately.

9.0 OTHER SCIENTIFIC REPORTS AND REVIEWS

9.1 Have Relevant Data Identified in Other Published or Unpublished Studies Conducted Using the *In Vitro* Test Methods Been Adequately Considered?

Although an extensive literature has been cited and discussed, no attempt at a comprehensive summary of findings or overall conclusions about the relevance of the *in vitro* pyrogen tests compared to the BET or the RPT, or the advantages/capabilities or disadvantages/limitations of the individual *in vitro* assays, has been presented in the ICCVAM draft BRD.

The following additional references should be included (see **Section 12.0** for full citations):

1. Marth and Kleinhappl (2002). The studies described here indicate the importance of monitoring multiple pro-inflammatory cytokine responses. In the specific case cited, the TNF- α pro-inflammatory cytokine response appeared to correlate best with fever.
2. Norata et al. (2005), van Deventer et al. (2000), von Aulock et al. (2003) are relatively new studies that evaluate the effects of genetic polymorphisms on TLR-4 responses.
3. Martis et al. (2005). This paper describes a situation where the PBMC/IL-6 assay was used to help resolve a non-febrile adverse drug reaction issue with a licensed product.

9.2 Are the Conclusions Published in Independent Peer-Reviewed Reports or Other Independent Scientific Reviews of the *In Vitro* Test Methods Adequately Discussed and Compared?

Yes, the conclusions are adequate for the published data.

The formal ESAC validation statement and other EU validation expert/panel process documents should be appended to the ICCVAM final BRD.

9.3 Are There Other Comparative *In Vitro* Test Method and RPT Data That Were Not Considered in the ICCVAM draft BRD, But are Available for Consideration?

It is known that manufacturers have parallel test result data for the BET and RPT for specific products, which unfortunately are not published or peer reviewed. As a consequence, a number of companies are now advocating that they should be permitted to use the BET as an alternative to the RPT to detect the presence of Gram-negative endotoxin on a case-by-case basis, such as for testing of established products with documented proof that safe, reliable and consistent GMP production and QC procedures are in place.

10.0 ANIMAL WELFARE CONSIDERATIONS (REFINEMENT, REDUCTION, AND REPLACEMENT)

10.1 How the Five *In Vitro* Pyrogen Test Methods Will Refine, Reduce, or Replace Animal Use

Is the extent to which the *in vitro* test methods will refine (reduce or eliminate pain or distress), reduce, or replace animal use in the RPT adequately described?

No numbers are included regarding the current number of rabbits used and/or killed with this test. These estimates would be helpful when assessing the potential impact of these *in vitro* tests. However, given that the proposed use for these test methods is very limited, it is not clear that their application would have a significant impact on animal numbers.

The ICCVAM final BRD should discuss the practice of, and the U.S. Federal restrictions on, the reuse of rabbits in pyrogenicity testing.

A discussion on the ethical cost of conducting concurrent RPT testing should be added.

10.2 Requirement for the Use of Animals

Is the discussion of the use of cultured human cells and the need for volunteers for donations of peripheral blood used in the *in vitro* test methods appropriate and adequate?

No, the licensing arrangements and the maintenance of the MM6 cell line are unclear.

The discussion that reduction of the use of animals (i.e., rabbits) will be associated with the increased use of another animal (i.e., humans) is inadequate.

11.0 PRACTICAL CONSIDERATIONS

11.1 Transferability of the *In Vitro* Pyrogen Test Methods

Are the following aspects of *in vitro* test method transferability, including an explanation of how this compares to the transferability of the RPT, adequately described with regard to the:

11.1.1 Facilities and major fixed equipment needs?

Yes, either a sterile tissue culture facility or a laboratory animal facility is needed.

11.1.2 General availability of other necessary equipment and supplies?

Yes, equipment and supplies for both *in vitro* and *in vivo* studies are routinely available. In general, the skills and kits required are available in most diagnostic and testing facilities.

The availability (in ready to use kit form), the convenience, and the lower costs of the BET will mitigate against widespread use of the *in vitro* pyrogen tests that are far more work intensive (e.g., cytokine and endotoxin standard curves must be established, tests must be performed in quadruplicate, multiple donors are required), less convenient (as yet only one of the assays is available in kit form), and probably associated with higher costs.

11.1.3 Nature of the drug substance tested?

Yes, the drug substances are adequately described. The overall requirements for the assays are comparable with most other types of *in vitro* QC diagnostic assays.

11.2 Personnel Training Considerations

Are the following aspects of the *in vitro* test method training adequately considered? Is the explanation of how this compares to the level of training required to conduct the RPT adequate with respect to:

11.2.1 The required level of training and expertise needed to conduct the test method?

Yes, the individual technical steps and competencies are common to many other laboratory activities.

11.2.2 Any training requirements needed for personnel to demonstrate proficiency and any laboratory proficiency criteria that should be met?

The training required for adequate conduct of biological assays cannot be overestimated. Aseptic tissue culture techniques are essential, as is the ability to accurately serially dilute material. It is necessary to maintain the MM6 cell line and functional and non-activated monocytes obtained from whole blood. Activation can be caused by physical disruption or contaminants. Competency in each of these techniques should be demonstrated prior to allowing personnel to carry out these tests on medicinal products intended for human use or for certification. It should also be noted that the required expertise needed does not typically reside in the laboratories that conduct the test (i.e., RPT) targeted for replacement by the proposed *in vitro* tests.

11.3 Cost Considerations

Is the cost involved in conducting a study using the *in vitro* test method, as compared to the cost of conducting the RPT, adequately evaluated, and is this considered to be cost-effective compared to the *in vivo* method?

No, the direct and indirect costs of operating an animal facility that would be needed to house rabbits are incompletely stated. The *in vitro* pyrogen tests would seem to be considerably more cost effective than the RPT. It would be interesting to see pricing costs from contract research organizations for both classes of tests, mindful that cost considerations will impact on the level of use.

11.4 Time Considerations

Is the amount of time needed to conduct a study using the *in vitro* test method as compared to the time it takes to conduct the RPT adequately evaluated, and is the *in vitro* test method considered to be time-effective compared to the *in vivo* method?

The *in vitro* pyrogen test methods require two days to complete (twice as long as the BET and RPT under normal circumstances). Furthermore, the *in vitro* pyrogen test methods are dependent on the availability of donors or blood supplies, which might further restrict the frequency to which these tests can be performed.

12.0 RECOMMENDED ADDITIONAL REFERENCES

Are all relevant publications referenced in the ICCVAM draft BRD? If not, what additional references should be included?

The following references should be included:

Barnett V, Lewis T. 1984. Outliers in Statistical Data, 3rd ed. In: Wiley Series in Probability and Mathematical Statistics. (V Barnett, T Lewis, eds). New York:John Wiley & Sons.

Brunson KW, Watson DW. 1974. Pyrogenic specificity of Streptococcal exotoxins, Staphylococcal enterotoxin, and Gram-negative endotoxin. *Inf Immun* 10(2):347-351.

Burrell R. 1994. Human responses to bacterial endotoxin. *Circ Shock* 43(3):137-153.

Dinareello CA. 2004. Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. *J Endotoxin Res* 10(4):201-222.

Dixon WJ. 1950. Analysis of extreme values. *Ann Math Stat* 21(4):488-506.

Gaines Das RE, Brügger P, Patel M, Mistry Y, Poole S. 2004. Monocyte activation test for pro-inflammatory and pyrogenic contaminants of parenteral drugs, test design and data analysis. *J Immunol Methods* 288(1-2):165-177.

Grubbs FE. 1950. Sample criteria for testing outlying observations. *Ann Math Stat* 21(1):27-58.

Hochstein HD, Fitzgerald EA, McMahon FG, Vargas R. 1994. Properties of US standard endotoxin (EC-5) in human male volunteers. *J Endotoxin Res* 1(1):52-56.

Ishii KJ, Coban C, Akira S. 2005. Manifold mechanisms of toll-like receptor-ligand recognition. 2005. *J Clin Immunol* 25(6):511-521.

Ishii KJ, Akira S. Innate immune recognition of, and regulation by, DNA. 2006. *Trends Immunol* 27(11):525-532.

Marth E, Kleinhappl B. 2002. Albumin is a necessary stabilizer of TBE-vaccine to avoid fever in children after vaccination. *Vaccine* 20(3-4):532-537.

Martich GD, Boujoukos AJ, Suffredini AF. 1993. Response of man to endotoxin. *Immunobiol* 187(3-5):403-416.

Martin MA, Roberts S. 2006. An evaluation of bootstrap methods for outlier detection in least squares regression. *J Appl Stat* 33(7):703-720.

Martis L, Patel M, Giertych J, Mongoven J, Tammime M, et al. 2005. Aseptic peritonitis due to peptidoglycan contamination of pharmacopoeia standard dialysis solution. *Lancet* 365:588-594.

McKinney BA, Reif DM, Rock MT, Edwards KM, Kingsmore SF, Moore JH, Crowe Jr JE. 2006. Cytokine expression patterns associated with systemic adverse events following smallpox vaccination. *J Inf Dis* 194(4):444-453.

Mullington J, Korth C, Hermann DM, Orth A, Galanos G, Holsboer F, Pollmächer T. 2000. Dose-dependent effects of endotoxin on human sleep. *Am J Physiol Regulatory Integrative Comp Physiol* 278(4):947-955.

Netea MG, Kallberg BJ, van der Meer JWM. 2000. Circulating cytokines as mediators of fever. *Clin Inf Dis* 31(Suppl 5):S178-S184.

Norata GD, Garlaschelli K, Ongari M, Raselli S, Gigore L, Benenuto F, Maggi FM, Catapano AL. 2005. Effect of Toll-like receptor 4 (TLR-4) variants on intima-media thickness and monocyte-derived macrophage response to LPS. *J Int Med* 258(1):21-27.

Rylander R. 2002. Endotoxin in the environment. *J Endo Res* 8(4):241-252.

Saper CB, Breder CD. 1994. The neurological basis of fever. *New England J Med* 330(26):1880-1886.

van Deventer SJH, Buller HR, ten Cate JW, Aarden LA, Hack EC, Sturk A. 1990. Experimental endotoxemia in humans: Analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. *Blood* 76(12):2520-2526.

van Deventer SJH. 2000. Cytokine and cytokine receptor polymorphisms in infectious disease. *Intensive Care Med* 26(Suppl 1):S98-S102.

von Aulock S, Schroder NWJ, Gueninzius K, Traub S, Hoffmann S, Graf K, Dimmeler S, Hartung T, Schumann RR, Hermann C. 2003. Heterozygous Toll-like receptor 4 polymorphism does not influence lipopolysaccharide-induced cytokine release in human whole blood. *J Inf Dis* 188(6):938-943.

13.0 Summary of Validation Status of the *In Vitro* Pyrogen Test Methods

Does the Panel agree that the applicable validation criteria have been adequately addressed in order to determine the usefulness and limitations of these *in vitro* test methods, to serve as a substitute for the RPT, for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation?

Yes, the information is adequate with which to make an informed decision.

Does the Panel agree that the performance of these test methods in terms of their reliability and relevance support the proposed use of these test methods (i.e., the detection of Gram-negative endotoxin in materials that are currently tested in the RPT, subject to product specific validation to demonstrate equivalency to the RPT)?

No, refer to the reasons indicated in the responses to **Sections 1.0 to 12.0**.

Minority Opinion # 1 (Drs. Karen Brown, Albert Li, and Jon Richmond): The qualification in the above statement 'subject to product specific validation' should allow for a vote of yes.

Minority Opinion #2 (Dr. Peter Theran): It is not clear that the qualification included in the above statement would preclude the use of the *in vitro* test methods as replacements for the RPT in those circumstances where the BET is currently serving to replace the RPT.

B REVIEW OF ICCVAM DRAFT TEST METHOD RECOMMENDATIONS

1.0 Proposed Test Method Usefulness and Limitations

Does the Panel agree that the available data and demonstrated performance in terms of relevance (i.e., accuracy/concordance, sensitivity, specificity, positive, and negative predictivity, false positive and false negative rates) and reliability (i.e., intralaboratory repeatability and intra- and inter-laboratory reproducibility) support the ICCVAM draft recommendations for these *in vitro* test methods in terms of the proposed test method usefulness and limitations⁶?

The Panel does not agree with this statement for the following reasons:

The usefulness of these *in vitro* test methods for detecting Gram-negative endotoxin has not been properly assessed for concordance with the RPT or for relevance in comparison to the BET. Therefore, it is not possible to truly assess their usefulness and limitations. It is regrettable that their ability to detect non-endotoxins could not be demonstrated and validated due to the limitations of the validation and performance evaluation studies conducted.

Test materials in pure form may directly promote the formation and release of cytokines and thus, they may not be suited to evaluation by the *in vitro* methods.

As much effort as possible should be placed on truly demonstrating that these assays can be reliably used to detect non-endotoxin pyrogens in actual manufacturing settings for a wide variety of products. Otherwise, these assays have little advantage over the already established and widely used BET.

Mechanisms exist for test method developers to qualify their method on a case-by-case basis (i.e., 21 CFR 610.9). Therefore, the use of any recommended method should be subject to product specific validation to demonstrate equivalence as regulated by 21 CFR 610.9.

Minority Opinion (Dr. Peter Theran): This Panel has considered the failure to undertake additional RPTs a significant flaw in this validation RPT study and therefore proposed that, in the future, similar validation studies should use the RPT to provide concordance data. I have no objection to the performance of *in vitro* tests in parallel with rabbit tests, which are already scheduled to be performed, in order to achieve concordance data. But, it is my opinion, that any recommendation for *de-novo* parallel RPT should be accompanied by a statement, as follows: “The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis).” The inclusion of this statement would reinforce the importance of the 3R’s and would serve as a reminder of U.S. Federal law.

2.0 Proposed Test Method Standardized Protocols

Does the Panel agree that the available data and demonstrated performance in terms of relevance (i.e., accuracy/concordance, sensitivity, specificity, positive, and negative

⁶The ICCVAM draft recommendations state that there is sufficient information, based on validation studies with a limited number of pharmaceuticals, to substantiate the use of these test methods for the detection of pyrogenicity mediated by Gram-negative endotoxin in materials that are currently tested in the RPT, subject to product specific validation to demonstrate equivalency.

predictivity, false positive and false negative rates) and reliability (i.e., intralaboratory repeatability and intra- and inter-laboratory reproducibility) support the ICCVAM draft recommendations for these *in vitro* test methods in terms of the proposed test method standardized protocols?

The Panel agrees with this statement, provided that the following list of inadequacies⁷ within the proposed standardized protocols are fully addressed:

1. Donor-to-donor inflammatory response variation is problematic and therefore multiple donors should be used and the number used appropriately justified.
2. Restricting the protocols to a 'limits' test design, based on the intravenous fever threshold, for all test materials independent of administration route could be considered inappropriate. The threshold concentration for intrathecally-administered materials would be lower because of the reduced permissible endotoxin limit associated with these types of products. The use of a 'benchmark reference lot comparison' test design would alleviate the necessity to use such strict permissible 'limits'. Interestingly, in the two known examples where *in vitro* pyrogen test data have been considered by the FDA for release testing (cited in the ICCVAM draft BRD), 'benchmark reference lot comparison' test design protocols have been used.
3. The protocols do not include sufficient descriptions of donor selection criteria (e.g., volunteer or paid, recruitment process, etc.) and conditions for venipuncture (e.g., qualified phlebotomists, number and frequency of venipunctures, etc.). In practice, the requirement for blood donors to have taken no medication and the recommended CO₂ concentrations are more stringent than the provisions suggested in the draft recommendations.
4. The protocols are inconsistent in their acceptance criteria with respect to the number of blood donors. The IL-6 primary cell assays require four donors to be used for each test with acceptance criteria applied to each donor. The IL-1 assays show equal variability between donors, but do not require these acceptance criteria.
5. The suggested dilution scheme for the initial endotoxin standard and for the subsequent dilutions should not be recommended. The initial dilution of the endotoxin standard in two of the assays uses 20 µL into 1980 µL. The margin of error with a micropipette is such that even the smallest error at this initial dilution could affect the whole assay and is often the cause of a substantial proportion of assay variability. To reduce this potential problem, it is recommended that alternative dilution schemes be developed based on the accuracy of the micropipettes.
6. The use of in-house ELISA assays should not be recommended due to poor transferability and the potential for poor interlaboratory reproducibility associated with these assays.

⁷Based on the list of 20 separate inadequacies outlined in this report, three Panel members felt that this list would be better described as a list of "many and substantial" inadequacies.

7. The protocols should clearly specify the need for resonication and/or vortexing of any reference endotoxin solution prior to each use.
8. To adequately test for interference, spiked test samples containing endotoxin must be pre-incubated for a specified time prior to addition to the blood cells.
9. The following should be included in the revised protocols: a consistent number of donors to be used for all test methods; the acceptable range of cytokine response for each test method; the rules and the rationale for exclusion of low and/or high responders.
10. Three separate lots should be included in the pre-qualification of any test material, similar to the protocol used for the BET.
11. The protocol for the MM6 cell line describes procedures that would be used for collecting blood from donors. This point obviously is not required for this particular protocol.
12. The ECVAM protocols are very complete as to sources for all solutions, equipment, etc. required for testing. The ICCVAM protocols are less specific. More specific details on all test method protocol components should be included.
13. Intellectual property issues, as identified in the ICCVAM draft BRD, should be addressed in the ICCVAM protocols.
14. To prevent inactivation of LPS binding protein, it should be specified that FBS is heat inactivated at 55°C.
15. The symbols for correlation coefficient (r and r^2) are interchanged inappropriately.
16. On pages 14 and 21, lines 298 and 450 respectively in the PBMC/IL-6 protocol, the basis for the definition of low responders must be justified.
17. On page 15, line 325 in the PBMC/IL-6 protocol, the performance of monocyte counts using a hemocytometer is inaccurate compared to modern flow cytometric methods.
18. If a hemocytometer is used, specifications for the number of replicate determinations (e.g., at least duplicate), the minimum number of cells counted, and the magnification used must be stated.
19. On page 14, line 295 in the WB/IL-1 protocol, the statement "not taken any drug" is not sufficiently inclusive. This statement must also specify no over-the-counter medications or recreational drugs.
20. On page 20, line 411 in the WB/IL-6 protocol, the statement "If necessary, . . . endotoxin concentration can be modified" is insufficient. The modification of endotoxin concentration must be defined.

3.0 Proposed Test Method Performance Standards

Does the Panel agree that the available data and demonstrated performance in terms of relevance (i.e., accuracy/concordance, sensitivity, specificity, positive, and negative predictivity, false positive and false negative rates) and reliability (i.e., intralaboratory repeatability and intra- and inter-laboratory reproducibility) support the ICCVAM draft recommendations for these *in vitro* test methods in terms of the proposed test method performance standards?

The Panel does not agree with this statement, based on the inadequacies within the proposed performance standards outlined below.

Essential Test Method Components

1. A uniform CV criterion should be defined, which is adequately stringent. The reported range of 20% - 45% is inappropriate.
2. The number of individual blood donors used and/or the number of donors to be included in a pool of multiple donors should be defined, if deemed appropriate.
3. The stringency by which the endotoxin curves are validated should be defined (either by using a four-parameter logistic model or by checking that the OD concentration values ascend in a sigmoidal manner).
4. The use of CVs or any other measure of variability should be appropriately justified. If the data have been log-transformed, then CVs are not informative.
5. The following issues may overestimate the performance of the test methods:
 - a) The nature of the prediction model used for dichotomizing the results;
 - b) Experimental design and data analysis that might lead to overestimation of the sensitivity of the tests;
 - c) The nature and interpretation of the *in vivo* data used in the study;
 - d) The nature and cause of incorrect results and the lack of agreement within and between laboratories;
 - e) Whether the tests accurately estimate the actual concentration of the pyrogen and whether results met some pre-defined criteria of success.
6. In **Section 2.3.3.1**, a 'significant increase' is not defined. In **Section 2.3.6**, consideration should be given to adding Quality Assurance data and known biological properties under the 'test substances and control substances' heading.

Accuracy and Reliability Values

The demonstrated performance of certain aspects of several of the assays, particularly in terms of accuracy or relevance, yields some concern. Two of the assays have false positive rates in excess of 16%, which essentially means that approximately 1 in every 6 production lots could be unnecessarily prevented from being released, a rate unlikely to be accepted by manufacturers. A number of these performance characteristic issues can probably be explained by the fact that some of the spike concentrations used were very close to the 'limit' concentration criterion set.

If the intended use of the *in vitro* assays were only to detect Gram-negative endotoxin, it would seem very important to compare their performance in parallel validation studies that should include the BET. If the intended use of the *in vitro* methods is to evaluate substances containing endotoxin that are unable to be evaluated with the BET, then the parallel testing studies should include the RPT. This type of comparison has neither been made from the RPT (2-way parallel testing was also not performed on the endotoxin-spiked sample sets included in the validation studies cited in the ICCVAM draft BRD) nor the BET standpoint. The last thing one wants to recommend is an inferior performing assay to the one that is already established; similar or superior is fine.

Minimum List of Reference Substances

If the intent of the proposal was to replace the RPT with one or more of the *in vitro* test methods under consideration, then the *in vitro* test methods must be validated for all classes of substances (e.g., pharmaceuticals, biologicals, and implants) and medical devices that can be tested with the RPT. Validation of the *in vitro* test methods with pyrogens (e.g., LTA, components of viruses and fungi) other than endotoxin also needs to be conducted.

Minority Opinion (Dr. Peter Theran): This Panel has considered the failure to undertake additional RPTs a significant flaw in this validation study and therefore proposed that, in the future, similar validation studies should use the RPT to provide concordance data. I have no objection to the performance of *in vitro* tests in parallel with rabbit tests, which are already scheduled to be performed, in order to achieve concordance data. But, it is my opinion, that any recommendation for *de-novo* parallel RPT should be accompanied by a statement, as follows: “The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis).” The inclusion of this statement would reinforce the importance of the 3R’s and would serve as a reminder of U.S. Federal law.

4.0 Proposed Additional Studies

Does the Panel agree that the available data and demonstrated performance in terms of relevance (i.e., accuracy/concordance, sensitivity, specificity, positive, and negative predictivity, false positive and false negative rates) and reliability (i.e., intralaboratory repeatability and intra- and inter-laboratory reproducibility) support the ICCVAM draft recommendations for these *in vitro* test methods in terms of the proposed additional studies?

The Panel agrees that to better determine the potential of these test methods, the proposed additional studies should be performed using the test methods described in the ICCVAM draft BRD, taking into account the comments and recommendations detailed previously. The Panel recognizes that these test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses. Wherever possible, either historical data from parallel studies conducted concurrently should be retrospectively evaluated or parallel testing should be conducted concurrently with RPT data generated for regulatory purposes.

The following additional recommendations are given:

1. A repository of test materials that have been identified clinically as pyrogenic would be invaluable for use in future validation studies and may allow such studies to be conducted without the further use of animals.
2. A 'limit' test design protocol and a 'benchmark reference lot comparison' test design protocol for each assay should be included.
3. Both endotoxin-spiked and non-endotoxin spiked samples should be included.
4. The non-endotoxin standards should be characterized as completely as possible prior to their use in any study and should satisfy the requirements set forth by ICCVAM for reference standards that are stated in the ICCVAM draft BRD.
5. The endotoxin-spike concentrations used for the performance assessment studies should not be so close to the positive test concentration limit, especially considering the relatively large enhancement and inhibition range permitted in the sample specific qualification investigations.
6. All aspects of the studies should be completely GLP compliant and importantly, the laboratories and results should be independently audited. This would include pre- and post-study audits of the laboratories.
7. The substances tested in the studies should also include products that have intrinsic pro-inflammatory properties.
8. A prospective study that includes 3-way parallel testing, with all of the *in vitro* assays (using both of the above mentioned protocol designs) being compared to the RPT and the BET, should be included to allow for complete concordance analysis and comparative performance assessment. These studies may be conducted with historical RPT data provided that the same substances (i.e., same lot) are tested in each method. Based on ethical and scientific rationale, the design of any side-by-side studies should be limited only to those that can gain more data than already available in the literature (i.e., data from parallel testing), most likely on the ability of the RPT and the *in vitro* pyrogen tests to detect non-endotoxin pyrogens.
9. Test substances that better represent all categories of sample types intended for testing by the methods (e.g., pharmaceuticals, biologicals, and medical devices) should be included. If relevant, extraction procedure protocols for the detection of pyrogens in medical device materials should be included.
10. The effects of direct administration of IL-1 and IL-6 to rabbits and the comparison of the resulting pyrogenic response with endotoxin-mediated pyrogenicity should be evaluated.
11. The correlation of IL-1 and IL-6 levels in the *in vitro* tests with levels produced in rabbits using similar doses of endotoxin should be evaluated.

The following statistical recommendations are noted:

1. For reliability assessments, formal hypothesis testing is essential with the alternative hypothesis being no different between groups.
2. For any additional studies, formal sample size calculations for equivalence testing should be made to determine that the required number of replicates needed to reject the null hypothesis (i.e., that there is a difference in reliability) at a given level of significance and power. If the study is not prospectively powered, the posterior power should be provided along with the observed significance level.
3. The proposed strategy for the Cryo WB/IL-1 test method is to retest if a test fails because of too much variability. The statistical properties of this multistage procedure should be characterized.

Minority Opinion (Dr. Peter Theran): This Panel has considered the failure to undertake additional RPTs a significant flaw in this validation study and therefore proposed that, in the future, similar validation studies should use the RPT to provide concordance data. I have no objection to the performance of *in vitro* tests in parallel with rabbit tests, which are already scheduled to be performed, in order to achieve concordance data. But, it is my opinion, that any recommendation for *de-novo* parallel RPT should be accompanied by a statement, as follows: “The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis).” The inclusion of this statement would reinforce the importance of the 3R’s and would serve as a reminder of U.S. Federal law.

C. OVERALL PEER REVIEW OUTCOMES

This international independent Peer Review Panel, consisting of 13 expert scientists from five different countries, provided comments and recommendations on the usefulness and limitations of five *in vitro* pyrogen test methods for the detection and quantification of Gram-negative endotoxin and on the ICCVAM draft test method recommendations on the use of these *in vitro* methods as partial replacements for the RPT. These remarks are summarized below.

- In general, the information presented in the ICCVAM draft BRD was sufficient for the purpose of determining the usefulness and limitations of these test methods for their proposed use and for adequately addressing the applicable validation criteria on the basis of the currently available evidence.
- The available data and demonstrated performance in terms of their reliability and relevance do not at this time support the ICCVAM draft proposed use for these test methods (i.e., as a partial substitute or replacement for the RPT, for the identification of Gram-negative endotoxin, on a case-by-case basis, subject to product specific validation). To better characterize the test methods and more clearly define their reliability and relevance, the Panel recommended that specific additional studies be performed using the ICCVAM proposed protocols, taking into account the Panel's comments and recommendations.
 - The lack of parallel testing in the *in vitro* tests and the RPT, and the resulting lack of concordance data, was considered to be a major limitation of the validation study design. For this reason, the Panel recommended that future studies include parallel testing. A minority opinion (Dr. Peter Theran) associated with parallel testing was expressed as follows: "The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis)".
- The available data and demonstrated performance in terms of their reliability and relevance does not support the ICCVAM draft performance standards for these *in vitro* test methods for regulatory purposes.
- The information provided in the ICCVAM draft BRD supports the ICCVAM draft recommended protocols for these five *in vitro* test methods, providing that the list of inadequacies⁸ identified by the Panel with respect to reliability and relevance are fully addressed.
- These test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.
- It is critical to recognize, despite concerns about the performance of these five *in vitro* test methods, that a formal process exists for materials regulated under 21 CFR 610.9 to qualify these *in vitro* methods for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation.

⁸Based on the list of 20 separate inadequacies outlined in this report, three Panel members felt that this list would be better described as a list of "many and substantial" inadequacies.

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Appendix A2
Summary Minutes from Peer Review Panel Meeting on
February 6, 2007

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Meeting Summary

**Independent Peer Review Panel Meeting
 Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of
 Pharmaceuticals and Other Products
 National Institutes of Health (NIH), Natcher Conference Center
 Bethesda, MD**

**February 6, 2007
 8:30 a.m. – 5:00 p.m.**

Panel Members:

Karen Brown	DRL Pharma and Pair O'Doc's Enterprises
Brian Crowe	Baxter Vaccine AG
Nancy Flournoy	University of Missouri-Columbia
Ihsan Gursel	Bilkent University
Ken Ishii	ERATO, Japan Science & Technology Agency, Osaka University
Jack Levin	University of California-San Francisco
Albert Li	<i>In Vitro</i> ADMET Laboratories
David Lovell	University of Surrey
Melvyn Lynn	Eisai Medical Research
Anthony Mire-Sluis	AMGEN, Inc.
Jon Richmond	UK Home Office
Peter Theran	MSPCA
Kevin Williams	Eli Lilly

ICCVAM and ICCVAM Pyrogenicity Working Group (PWG) Members:

Mustafa Akkoyunlu	FDA/CBER
Peter Amin	FDA/CBER
Kimberly Benton	FDA/CBER
Joseph George	FDA/CBER
David Hussong	FDA/OPS
Abigail Jacobs	FDA/CDER
Jodie Kulpa-Eddy (ICCVAM Vice Chair)	USDA/APHIS
Robert Mello	FDA/CDER
Richard McFarland (PWG Chair)	FDA/CBER
Penelope Rice	FDA/CFSAN
William Stokes	NIEHS
Raymond Tice	NIEHS
Daniela Verthelyi	FDA/CDER
Marilyn Wind (ICCVAM Chair)	CPSC
Jiaqin Yao	FDA/CDER

Public Attendees:

Allen Dearry	NIEHS
Basil Golding	FDA/CBER
Thomas Hartung	ECVAM
Coty Huang	FDA/CBER
Sue Leary	ARDF
Thomas Montag	ECVAM
Michael Myers	FDA/CVM
Steven Myers	Becton, Dickinson & Company
Seishiro Naito	NIID
Michael Scott	FDA/CVM
Kristie Stoick	PCRM
Michael Timm	University of Copenhagen
Rachel Waltman	USDA/APHIS

NICEATM Staff:

David Allen	ILS, Inc.
Elizabeth Lipscomb	ILS, Inc.
Linda Litchfield	ILS, Inc.
Debbie McCarley	NIEHS
James Truax	ILS, Inc.
Douglas Winters	ILS, Inc.

Abbreviations: APHIS = Animal and Plant Health Inspection Service; ARDF = Alternatives Research and Development Foundation; CBER = Center for Biologics Evaluation and Research; CDER = Center for Drug Evaluation and Research; CFSAN = Center for Food Safety and Applied Nutrition; CPSC = Consumer Product Safety Commission; CVM = Center for Veterinary Medicine; ECVAM = European Centre for the Validation of Alternative Methods; ERATO = Exploratory Research for Advanced Technology; FDA = U.S. Food and Drug Administration; ILS = Integrated Laboratory Services; MSPCA = Massachusetts Society for the Prevention of Cruelty to Animals; NIEHS = National Institute of Environmental Health Sciences; NIID = National Institute for Infectious Diseases; OPS = Office of Pharmaceutical Science; PCRM = Physicians Committee for Responsible Medicine; USDA = U.S. Department of Agriculture

Call to Order

Dr. Karen Brown (Panel Chair) called the meeting to order at 8:30 a.m. and introduced herself. She then asked all Peer Panel members, National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, members of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the ICCVAM Pyrogenicity Working Group (PWG) in attendance, the European Centre for the Validation of Alternative Methods (ECVAM) liaison to the PWG, and members of the public to state their name and affiliation for the record. Dr. Brown asked all individuals to identify themselves when they spoke and to use the provided microphones. Dr. Brown stated that three public comment sessions were scheduled during the meeting and she reminded individuals who wished to speak to register at the registration table. Dr. Brown emphasized that there was no need to repeat the same comments at each comment session.

Welcome from the ICCVAM Chair

Dr. Marilyn Wind, Consumer Product Safety Commission and Chair of ICCVAM, welcomed everyone to the Peer Review Panel meeting and thanked the Panel members for their participation. Dr. Wind stressed the importance of an independent scientific peer review to the ICCVAM test method evaluation process.

Welcome from the Director, NICEATM, and Conflict of Interest Statements

Dr. William Stokes, Director of NICEATM, welcomed everyone and reiterated Dr. Wind's appreciation to the participants for agreeing to serve on the Panel. Dr. Stokes stated that he would be serving as the Designated Federal Official for the public meeting. He stated this meeting was being held in accordance with the Federal Advisory Committee Act regulations and that the Panel was constituted under the NIH Special Emphasis Panel charter. Dr. Stokes read the conflict of interest statement and asked the Panel members to declare if they had any direct or indirect conflicts, and to recuse themselves from discussion and voting on any aspect of the meeting where there might be a conflict. None of the Panel members declared a conflict of interest.

Overview of the ICCVAM Test Method Evaluation Process

Dr. Stokes provided an overview of the ICCVAM test method evaluation process. He stated that the international Panel was made up of 13 scientists from five different countries (Austria, Japan, Turkey, United Kingdom, and United States). Dr. Stokes described that the purpose of the Panel was to assist ICCVAM by carrying out an independent scientific peer review of the information provided in the ICCVAM Background Review Document (BRD) on the validation studies of five *in vitro* test methods proposed for assessing the potential pyrogenicity of pharmaceuticals and other products. He stated that Panel members were experts selected and appointed by the National Institute of Environmental Health Sciences (NIEHS) to ensure sufficient scientific expertise to carry out a comprehensive review of these test methods.

Dr. Stokes listed the 15 ICCVAM member agencies and provided a brief review of ICCVAM's history. He summarized the ICCVAM Authorization Act of 2000 (available at: http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf) and detailed the purpose and duties of ICCVAM as mandated in the Act. He noted that one of ICCVAM's duties is to review and evaluate new, revised, and alternative test methods applicable to regulatory testing. He stated that all of the reports produced by NICEATM are available from the ICCVAM/NICEATM website or directly from NICEATM. Dr. Stokes pointed out that ICCVAM does not carry out research, development, or validation studies, but instead, facilitates these processes by convening scientific symposia, workshops, and expert Panel reviews such as this one.

Dr. Stokes then described the ICCVAM test method evaluation process, which begins with a test method nomination or submission. NICEATM conducts a prescreen evaluation to summarize the extent to which the proposed submission or nomination addresses the ICCVAM prioritization criteria. A report of this evaluation is then provided to ICCVAM, which in turn develops recommendations regarding the priority for evaluation. ICCVAM then seeks input on their recommendations from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public. Given sufficient regulatory applicability, sufficient data, resources, and priority, a method will move forward to a formal evaluation. A draft BRD is prepared by NICEATM in conjunction with an ICCVAM working group for the relevant toxicity testing area (e.g., pyrogenicity), which provides a

comprehensive review of all available data and information. ICCVAM then considers all of the available information and prepares draft recommendations for 1) proposed usefulness and limitations of the test methods, 2) test method protocol, 3) performance standards, and 4) future studies. The draft BRD is then made publicly available for review and comment. An independent peer review Panel is then convened to provide comments and recommendations on the draft BRD, public comments, and ICCVAM draft test method recommendations. A *Peer Review Panel Report* is published and considered by ICCVAM, along with public and SACATM comments, when their final recommendations are forwarded to the appropriate ICCVAM agencies.

Dr. Stokes reviewed the criteria for adequate validation. He stated that validation is defined by ICCVAM as the process by which the reliability and relevance of a procedure are established for a specific purpose, and that adequate validation is a prerequisite for consideration of a test method by U.S. Federal regulatory agencies. Dr. Stokes listed the ICCVAM acceptance criteria for test method validation and acceptance.

ICCVAM Charge to the Panel

Dr. Stokes reviewed the charge to the Panel, which was to: 1) review the draft BRD for completeness and identify any errors or omissions; 2) determine the extent to which each of the applicable criteria for validation and regulatory acceptance had been addressed for the proposed use; and 3) consider and provide comment on the extent to which the ICCVAM draft test method recommendations including the proposed use, recommended protocols, performance standards, and recommended additional studies are supported by the information provided in the BRD.

Dr. Stokes thanked the PWG, ICCVAM, and NICEATM for their work on this project, and he acknowledged the NICEATM staff for organizing the Panel meeting and preparing the materials being reviewed.

Overview of Pyrogenicity Testing Requirements and Current Pyrogenicity Testing Procedures

Dr. Richard McFarland, Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER) and Chair of the PWG, thanked the PWG members for their efforts in producing the draft BRD, and thanked the Panel members for their participation in the peer review process. Dr. McFarland discussed the scientific need for pyrogenicity testing and its relationship to the regulatory mandate for protection of public health. He discussed the need for risk management, especially the detection of endotoxin and non-endotoxin pyrogen-contaminated products, and he noted the need for classification and labeling of products as pyrogen-free (i.e., the product does not exceed established endotoxin limits). Dr. McFarland then summarized the U.S. and European legislation and statutory protocol requirements for pyrogen testing.

Overview of the Five *In Vitro* Pyrogen Test Method Protocols

Dr. Thomas Hartung, Head of ECVAM and invited test method expert, remarked that he has been closely involved in the ECVAM validation studies and as such recognized his considerable conflict of interest. Dr. Hartung summarized the disadvantages of the rabbit pyrogen test (RPT) and the bacterial endotoxins test (BET), and related these limitations to the development of the *in vitro* pyrogen test methods.

Dr. Hartung indicated that a typical *in vitro* pyrogen test method consists of two parts: 1) incubation of the test sample in a cellular cytokine release system (i.e., whole blood [WB], Mono Mac 6 [MM6] cells, Peripheral blood mononuclear cells [PBMC]); and 2) cytokine detection using a specific enzyme-linked immunosorbent assay (ELISA) (e.g., Interleukin [IL]-1 β or IL-6). He stated that the European Commission granted \$2.5 million for the validation of these novel test methods, but that this sum was only sufficient to cover "the basics". Dr. Hartung then made the following comments regarding the design of the ECVAM validation study:

- For the validation study, the endotoxin threshold was set at 0.5 Endotoxin Units (EU)/mL, based on the positive response of 50% of the most sensitive rabbit strain to 50 pg of endotoxin. A substance was considered pyrogen-free if the endotoxin level in an *in vitro* test method corresponded to less than 0.5 EU/mL. A positive product control (PPC) was used in a pretest to insure that there is no interference. Specific criteria were used to minimize assay variability (e.g., blood donors, coefficient of variation).
- In 1988, Dr. Stephen Poole described an IL-6 cytokine assay using isolated leukocytes. The PBMC test method evolved from this study and has subsequently been used by Novartis for U.S. Food and Drug Administration (FDA) release of one product (i.e., after product-specific validation and in conjunction with the rabbit pyrogen test).
- Two of the assays included in the validation exercise, WB/IL-1 β and WB/IL-6, utilize human WB. Many research studies have described using these test systems for routine pyrogen testing of up to 80 pharmaceutical products against a variety of pyrogens. A commercial kit has been developed using the WB/IL-1 β test system.
- A catch-up validation study was performed using the Cryo-WB/IL-1 β test method, which was not available during the original validation study. This assay utilizes cryopreserved WB pooled from several donors. Although the cells remain in diluted dimethyl sulfoxide, an effect on cell morphology or viability is not observed.

Overview of the Draft *In Vitro* Pyrogen Test Method Background Review Document (BRD)

Dr. David Allen, Integrated Laboratory Systems, Inc. (the NICEATM support contractor), presented an overview of the ICCVAM draft BRD. Dr. Allen indicated that five BRDs were submitted by ECVAM in June 2005. A *Federal Register* notice was used to request data from over 100 interested stakeholders, but no additional data were submitted. Following this request, a comprehensive ICCVAM draft BRD, which describes the current validation status of the five *in vitro* test methods based on U.S. Federal regulatory standards, was compiled and made available to the public on December 1, 2006.

Dr. Allen briefly summarized the performance characteristics of the *in vitro* test methods, which are detailed in the ICCVAM draft BRD (available at: <http://iccvam.niehs.nih.gov/methods/pyrogen/pyrodocs/pyroBRDUdocs/PyroBRD01Dec06.pdf>).

Dr. Allen noted that Dr. Marlies Halder, ECVAM liaison to the PWG, provided additional information requested by the Panel, including data audits, evidence of Good Laboratory Practice (GLP) compliance of testing laboratories, information on the protocol used for the historical RPT studies, and lot numbers of the test substances. He also stated that a request was made for the ECVAM Science Advisory Committee (ESAC) peer-review documents, but that these documents are not available to the public.

Peer Review Panel Evaluation:

Dr. Brown introduced the relevant Panel Group Leaders for each BRD Section: (Dr. Melvyn Lynn - Sections 1, 2, and 11; Dr. Jack Levin - Sections 3, 5, and 6; Dr. Anthony Mire-Sluis - Sections 7 and 8; Dr. Jon Richmond - Sections 4, 9, and 10). The Group Leaders presented the draft responses to the Evaluation Guidance Questions for consideration by the entire Panel. The Panel discussion and their recommended revisions to each section of the ICCVAM BRD are reflected in the *Independent Peer Review Panel Report: Five In Vitro Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products*, published in April 2007 (hereafter, the Panel report, available at: <http://iccvam.niehs.nih.gov/docs/pyrogen/PrRevPanFinRpt.pdf>).

Public Comments (Session 1)

Ms. Mary Lou Chapek - President and Chief Executive Officer of MBP Laboratories, Inc.

Dr. David Allen read the written comments submitted by Ms. Chapek to ICCVAM/NICEATM. Her comments are summarized as follows:

- Ms. Chapek expressed disappointment in the number of test methods reviewed by ICCVAM and accepted by federal agencies over the past 15 years. She commented that the pyrogenicity BRD and recommendations currently under discussion indicate a lack of focus. Ms. Chapek noted that substantial work remains to be done for validation of these test methods and she suggested the phased strategy outlined below.
- Phase I should concentrate on replacement of the BET, not the RPT. A large array of test substances compatible with the BET could be spiked with endotoxin to determine their accuracy and sensitivity and to determine the level of interference, if any, with each of these test systems.
- Phase II should consist of validation of one or two *in vitro* test methods for replacement of the RPT. Cell-based assays that do not depend on blood, which has an impractical limited time domain, would be preferable and could be compared directly to RPT data. The task would still be complex, but with a smaller focus. Phase II would also require evaluation and validation of all materials currently tested in the RPT, as well as the pyrogens detected in the RPT. Some of these standards would have to be developed. Although these studies may take years for completion, replacement of the RPT by one or two of the *in vitro* pyrogen tests in Phase II would constitute an achieved goal by ICCVAM.

Dr. Thomas Montag - Paul Ehrlich Institute (PEI) - Germany

Dr. Montag provided the following comments:

- He stated that the PEI is responsible for the quality and safety of biological drugs in general and that his laboratory has been involved in alternative pyrogen testing with Dr. Hartung for over 12 years. While the data is proprietary, he confirmed that he has used the WB/IL-1 β assay for approximately two years.
- Dr. Montag commented that donors are now pooled (up to 10 at a time) to minimize variability, especially for detection of non-endotoxin pyrogens. For the Cryo WB/IL-1 β pyrogen test, the blood is typically pretested for reactivity after pooling. In response to the PPC issue mentioned previously, he remarked that this was a design flaw that had been corrected in the ECVAM Standard Operating Procedure. He also stated that an expert Panel from the EDQM is now in the process of creating a draft of this alternative test method for publication.

Final Review of the BRD for Errors and Omissions

Dr. Brown asked the Panel to review the recommended revisions for each BRD section, taking into account the public comments, and to decide if additional changes are necessary. No changes were made to the draft recommendations based on the public comments.

Validation Status of the *In Vitro* Test Methods

Dr. Brown asked the Panel if they agreed that the applicable validation criteria had been adequately addressed in the ICCVAM BRD in order to determine usefulness and limitations of these *in vitro* test methods, to serve as a substitute for the RPT, for the identification of Gram-negative endotoxin on a case-by-case basis subject to product-specific validation.

The Panel agreed that the information was adequate with which to make an informed decision.

Dr. Brown asked the Panel if they agreed that the performance of these test methods in terms of their relevance and reliability support the proposed use for the detection of Gram-negative endotoxin in materials that are currently tested in the RPT, subject to product-specific validation to demonstrate equivalency to the RPT.

The Panel did not agree with this statement based on the reasons indicated in the responses to the questions related to Sections 1.0 to 12.0 of the ICCVAM BRD. Two minority opinions were expressed. Responses to these questions, and the associated minority opinions are detailed in the Panel Report.

Public Comments (Session 2)

Dr. David Hussong - FDA, Center for Drug Evaluation and Research (CDER)

Dr. Hussong commented that the Code of Federal Regulations (CFR), Section 211.167, states that if a drug is to be labeled as pyrogen-free, an appropriate test is required. The U.S. Pharmacopeia (USP) provides guidelines for the RPT and the BET. While the BET is not considered equivalent to the RPT, data from the BET is accepted. The USP states that use of alternative tests is permitted and that they may be used *in lieu* of the BET, provided that the alternative test uses a reference standard for comparison. It should be noted that the FDA CDER approves drugs, not test methods, but welcomes the use of alternative test methods.

Dr. Thomas Hartung - Head, ECVAM - Italy

Dr. Hartung stated that the *in vitro* pyrogen tests were designed to determine the threshold level of endotoxin in the most sensitive rabbit strain. This design was ambitious and consequently, resulted in the low sensitivity (58%) and specificity (83%) observed. It should be noted that some assays had values of 80% or 90% at this critical concentration and performed better than the RPT.

ICCVAM Draft Recommendations for *In Vitro* Pyrogen Test Methods Presentation of Draft ICCVAM Recommendations

Dr. Brown asked the Panel to evaluate the extent to which the ICCVAM draft recommendations are supported by the information and data provided in the ICCVAM draft BRD. Dr. Brown reminded the Panel that the purpose is not to approve or disapprove of the ICCVAM draft recommendations, but rather to comment on the extent to which they are supported by the information contained in the ICCVAM BRD. The Panel discussion and associated conclusions relevant to each of the ICCVAM recommendations are reflected in the Panel Report.

Public Comments (Session 3)

Ms. Kristie Stoick - Physicians Committee for Responsible Medicine

Ms. Stoick reviewed written comments that she previously submitted to ICCVAM/NICEATM. She stated that the pace of acceptance of alternative methods, such as these *in vitro* pyrogen tests, in the opinion of the animal protection community, is unacceptably slow. She continued to state that too much time is spent debating every scientific detail and that the ultimate goal is lost. She closed by asking ICCVAM to take into account her comments when considering the Panel's recommendations for the validation of these assays.

Final Review of the ICCVAM Draft Recommendations

Dr. Brown asked the Panel to review the ICCVAM draft recommendations, taking into account the public comments, and to decide if additional changes are necessary. No changes were made to the draft recommendations based on the public comments.

Concluding Remarks

Dr. Brown thanked the Panel and ICCVAM/NICEATM for their help. She expressed hope that this peer review process helped to establish a focus for ICCVAM and that the reduction in animal use would be the ultimate outcome. Dr. Stokes thanked the Panel for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes stated that the ICCVAM PWG and ICCVAM would consider these recommendations as they move forward with this process and the results of this meeting would culminate in a Peer Review Panel Report that would be released to the public toward the end of March for additional comment.

Adjournment

The meeting was adjourned at 5:47 p.m.

William S. Stokes, D.V.M.
NIEHS
P.O. Box 12233
MD-EC17
Research Triangle Park, NC 27709

Dear Dr. Stokes:

The Meeting Summary, Peer Review Panel Public Meeting, Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products, dated February 6, 2007, accurately summarizes the Peer Review Panel meeting of February 6, 2007, in Bethesda, MD.

Sincerely,



Signature

KAREN K. BROWN

Printed Name

3/4/08

Date

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Appendix A3
Pyrogenicity Peer Review Panel Biographies

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Karen Brown, Ph.D. (Panel Chair)

Dr. Brown received her Ph.D. in Microbiology and Biochemistry at Oklahoma State University in Stillwater. She is President, Pair O' Docs Enterprises, consulting with companies and with the U.S. Department of Agriculture Animal and Plant Health Inspection Service (APHIS) Center of Veterinary Biology on development of *in vitro* assays to replace animal tests for release of veterinary vaccines and is a Consultant, sharing the CEO position for MVP Laboratories. Dr. Brown's resume indicates her broad expertise in *in vitro* and *in vivo* pyrogen testing and thorough knowledge of regulatory requirements for drug and product development. Early in her career, Dr. Brown developed bench and supervisory experience working in Quality Control conducting animal testing, including the rabbit pyrogen test and *in vitro* Limu endotoxin testing. She initiated an *in vitro* development group at Bayer as Head of Biological Research and Development that specialized in developing and validating ELISAs for Limulus Amebocyte Lysate (LAL) testing and antigen quantitation for release of biological products. Dr. Brown remains involved in endotoxin testing by consulting with various companies to determine correlations in endotoxin levels in various veterinary products to reactions produced by some of these products when used to vaccinate animals. She has expertise in microbiology, veterinary medicine, vaccine and biologicals development and safety testing, *in vitro* methods development, and technical government relations (European Union [EU] and United States [U.S.]). Dr. Brown was Chairman of the *In Vitro* Working Group of the Veterinary Biologics Section of the Animal Health Institute (AHI) and APHIS liaison (regulatory) for registration of new vaccine and diagnostic products. Dr. Brown has conducted or managed research and development to register 44 new drug products, pharmaceuticals, vaccines, or diagnostic products or technologies and she has 44 publications and presentations and 23 U.S. patents. She is a member of the AHI, Veterinary Biologics Section, the Association of Veterinary Biological Companies, the American Society for Microbiology, and the U.S. Animal Health Association.

Brian Crowe, Ph.D.

Dr. Crowe received his Ph.D. in Microbiology from Trinity College in Dublin, Ireland. He is the Director of Immunology (Vaccines) at Baxter Vaccine AG in Austria and has responsibility for two research departments (Molecular Immunology and Humoral Immunology) and a quality control department (Biological Control) comprised of three quality control laboratories (Bacteriology, *in vitro* and *in vivo* testing). Dr. Crowe's resume demonstrates a significant and broad level of expertise in pyrogen test methodology and knowledge of laboratory, manufacturing, and validation procedures. Dr. Crowe has responsibility for general safety and toxicity testing and heads the Rabbit Pyrogen Testing and Endotoxin (LAL) Testing Units for Baxter Bioscience in Austria with testing rates of 3,000 to 26,000 samples per year. Dr. Crowe has extensive experience with high throughput screening, cytokine response assays, cytotoxicity testing, inflammatory response assays, complement testing, and other molecular, cellular, and humoral immunological response testing. He is also well versed in Good Manufacturing Practice and Good Laboratory Practice (GLP) standards and in issues of validation and audit requirements and procedures. Dr. Crowe has authored or coauthored 25 publications and 4 patents. His research interests are focused on bacterial and viral vaccines.

Nancy Flournoy, M.S., Ph.D.

Dr. Flournoy received a M.S. degree in Biostatistics from the University of California at Los Angeles, and a Ph.D. in Biomathematics from the University of Washington. She is Professor and Chair of the Department of Statistics at the University of Missouri-Columbia. Her research interests include adaptive designs, bioinformatics, chemometrics, clinical trials, and environmetrics. She has an extensive list of edited volumes and papers on statistical theory, statistical genetics and immunology, epidemiology in immune suppressed subjects, clinical trials for prevention and treatment of viral infection, transplantation biology and its effects on digestion, lungs, eyes, mouth, and central nervous system, optimization of statistical processing, and additional papers, interviews, and technical reports. She has editorial responsibilities for numerous statistical journals, serves on numerous advisory boards, and nominating committees. She is a member and past Chair of the Council of Sections of the American Statistical Association, and served in various other statistical, medical and toxicological societies or programs as Chair or as a member of the Board of Directors. She is a former member of the Scientific Advisory Committee on Alternative Toxicological Methods. She also served on the Expert Panels for the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) that evaluated the Revised Up-and-Down Procedure; the Current Validation Status of *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants; and Five *In Vitro* Pyrogen Test Methods.

Ihsan Gursel, MSc, Ph.D.

Dr. Gursel received his MSc. and Ph.D. degrees from the Middle East Technical University, Department of Biological Sciences in Ankara, Turkey. He is an Assistant Professor in the Department of Molecular Biology and Genetics at Bilkent University in Ankara. Dr. Gursel's resume indicates that he has significant experience studying the receptor family believed to mediate pyrogenic responses (i.e., Toll-like receptors [TLR]). Dr. Gursel's research interests include studies on the roll of TLR and TLR-ligand interactions in an innate immune response, gene expression and transcriptional profiling of immune cells via high throughput screening methods, design of controlled release systems for oligodeoxynucleotide targeting and delivery, and application of biodegradable natural polymers for biomaterials, tissue engineering, and drug delivery. Dr. Gursel has received numerous awards and grants to support his work and has authored or coauthored more than 45 publications, 7 patents, and has given 28 formal presentations related to his research. He has also refereed papers for the Journal of Leukocyte Biology, Immunopharmacology and Immunotoxicology, and Vaccine.

Ken Ishii, M.D., Ph.D.

Dr. Ishii received his M.D. and Ph.D. degrees from the School of Medicine at Yokohama City University in Kanagawa, Japan. He is a Group Leader for the Akira Innate Immunity Project at the Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Osaka University. Dr. Ishii's resume indicates that he has extensive regulatory experience that includes pyrogen testing requirements for pharmaceuticals. Dr. Ishii was formerly a Staff Scientist at the Food and Drug Administration (FDA) Center for Biologics and Evaluation Research (CBER). His work experience includes regulation of Investigational New Drug applications related to DNA-based vaccines and immunotherapy

using DNA vaccine and immunostimulatory DNA (CpG DNA). Dr. Ishii also has regulatory experience related to vaccines and immunotherapies against infectious diseases and allergy. He has authored or coauthored 58 publications in peer-reviewed journals and holds 17 patents.

Jack Levin, M.D.

Dr. Levin received an M.D. from the Yale University School of Medicine in New Haven, CT. He is an Independent Investigator at the Marine Biological Laboratory at Woods Hole, MA. Dr. Levin is also a Professor of Laboratory Medicine and Professor of Medicine at the University of California School of Medicine in San Francisco. He previously held various academic positions (e.g., Professor of Medicine) at Johns Hopkins Hospital in Baltimore and holds additional positions (e.g., Associate Member of the Cancer Research Institute at University of California at Santa Cruz, attending physician and Director of the Anticoagulation Clinic at the Veterans' Administration Medical Center in San Francisco). Dr. Levin is board-certified in Internal Medicine by the American Board of Internal Medicine. Dr. Levin's resume indicates that he has extensive experience studying the pyrogenic response and pyrogen testing (e.g., research in hemoglobin-lipopolysaccharide interactions and pioneered gel-clot LAL technology). Dr. Levin is a former editor-in-chief of the Journal of Endotoxin Research, a member of the American Society of Hematology (serving on various committees), a member of the Corporation, Marine Biological Laboratory, a Fellow of the American College of Physicians, a member of the American Society for Experimental Pathology, American Society for Clinical Investigation, the California Academy of Medicine, the International Endotoxin Society, and numerous other societies. Dr. Levin has co-organized nine international conferences and has 246 publications in peer-reviewed journals, book chapters, or edited series.

Albert Li, Ph.D., MBA

Dr. Li received his Ph.D. in Biomedical Sciences from the University of Tennessee, Oak Ridge and an Executive MBA from the University of Maryland University College in College Park. Dr. Li co-founded three companies to advance drug development. He is Chairman and CSO of ADMET Group, LLC; Founding Chairman, President, and CEO of *In Vitro* ADMET Laboratories in Rockville, MD; and Founding Chairman, President, and CEO of Advanced Pharmaceutical Sciences in Baltimore, MD. Dr. Li's resume indicates that he has a broad level of experience in validation of *in vitro* and alternative methods. Dr. Li has secured multiple research grants to advance a drug candidates from the preclinical laboratory through clinical trials, developed proprietary technology of interest to the pharmaceutical industry, and established a GLP laboratory for *in vitro* efficacy, metabolism, and toxicity testing. Dr. Li has published over 130 scientific papers, numerous books/special journal issues, and is frequently invited to speak in national and international conferences.

David Lovell, Ph.D., FIBiol, CBiol, F.S.S., CStat

Dr. Lovell received a Ph.D. from the Department of Human Genetics and Biometry, University College, London. He is currently Reader in Medical Statistics at the Postgraduate Medical School at the University of Surrey. Previously, he was Associate Director and Head of Biostatistics support to Clinical Pharmacogenomics at Pfizer Global Research and Development in Sandwich, Kent providing data management and statistical support to pharmacogenetics and genomics. He joined Pfizer in 1999 as the Biometrics Head of Clinical

Pharmacogenetics. Before joining Pfizer, Dr. Lovell was the Head of the Science Division at BIBRA International, Carshalton, which included Molecular Biology, Genetic Toxicology, Biostatistics and Computer Services. At BIBRA, Dr. Lovell managed the statistical and computing group providing specialized statistical support to BIBRA's Clinical Unit and contract research work. He conducted and managed research programs on genetics, statistics and quantitative risk assessment for the EU and United Kingdom (U.K.) Government Departments. His research interests at BIBRA were in the use of mathematical and statistical methods together with genetic models in the understanding of toxicological mechanisms and risk assessment problems. Dr. Lovell had previously been a Senior Research Officer with the U.K. Medical Research Council (MRC) Experimental Embryology and Teratology Unit, a visiting Postdoctoral Fellow at the National Institute of Environmental Health Sciences in North Carolina, U.S., a Geneticist at the MRC Laboratories, Carshalton and a Research Assistant in Cytogenetics at Birmingham University. He has acted as a consultant to a number of organizations, has considerable experience of working with Regulatory Authorities, has many publications related to his work and has wide experience of making presentations to a wide range of audiences. He is a member of the U.K. Government's advisory Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment and the Independent Scientific Advisory Committee for Medicines and Healthcare Products Regulatory Agency database research. He served on the NICEATM-ICCVAM Expert Panels that evaluated the Frog Embryo Teratogenesis Assay - *Xenopus*, *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, and Five *In Vitro* Pyrogen Test Methods.

Melvyn Lynn, M.S., Ph.D.

Dr. Lynn received M.S. and Ph.D. degrees in Microbiology from Rutgers University in New Brunswick, New Jersey. He is currently Senior Director and Global Head, Sepsis and Anti-Infectives Therapeutic Area at Eisai Inc. Dr. Lynn's expertise in the area of pyrogenicity is evidenced from his involvement in the clinical development of TLR4 antagonists and antimicrobials. Dr. Lynn directs global clinical development of a TLR-4 antagonist and antimicrobials and is head of a multifunctional, international project team, for which he regularly interacts with FDA and international regulatory agencies. Dr. Lynn has participated in global Standard Operating Procedure process development teams and served on the Eisai Global Clinical Development Global Development Board to address globalization of clinical development of drugs and clinical processes. Dr. Lynn has authored or coauthored 24 peer-reviewed publications, a review, two book chapters, a research letter, and 28 abstracts. Dr. Lynn has additional drug development experience during his tenure at the Bristol-Myers Squibb Company.

Anthony Mire-Sluis, Ph.D.

Dr. Mire-Sluis received his Ph.D. in Cell Biology and Biochemistry from the Department of Haematology at the Royal Free Hospital Medical School. He is Senior Director – Product Quality and External Affairs at AMGEN, Inc. with former positions as Head of the Cytokine Group at the National Institute for Biological Standards and Control, Director of Bioanalytical Sciences at Genentech, Inc., Head of Analytical Science and Standards in the Center for Biologics Evaluation and Research (CBER) at the FDA and Principal Advisor for Regulatory Science and Review in the Office of Biotechnology Products and Office of Pharmaceutical Sciences in the Center for Drug Evaluation and Research at the FDA. Dr. Mire-Sluis's resume demonstrates his expertise in regulatory science associated with pyrogen

testing with experience in product quality and development of biologicals, in immunology, and prior experience with the FDA. Dr. Mire-Sluis has managerial and product development experience including management of analytical and product quality departments of up to 75 staff (postdoctoral and technical levels). He is involved in strategic planning of development of biotechnology-derived products, including toxicology, assay development, and quality control. Dr. Mire-Sluis has expertise in the detection, measurement, and characterization of biological materials using immunological, molecular biological, and cell biological technology (cytokines, growth factors, enzymes, monoclonal antibodies). He is involved in high throughput screening technology, bioassay and immunoassay designs, risk assessment and process validation. He is a member of the World Health Organization consultative committee for therapeutic drug standardization, Chairman of the International Union of Immunological Societies Standardization Committee and of the human therapeutics committee of the International Association for Biologicals, a board member for the Journal of Immunological Methods, a member of the U.S. Pharmacopeia Biological Assay Statistical Analysis Expert Working Group and the Biological Assay Validation Expert Working Group. Dr. Mire-Sluis has authored almost 100 peer-reviewed publications.

Jonathan Richmond, BSc (Hons) Med.Sci., MB ChB, FRCSEd, FRMS

Dr. Richmond received a Bachelor of Science in Medical Science with Honors (B.Sc. [Hons] Med.Sci.) and Bachelor of Medicine and Bachelor of Surgery (MB ChB) degrees with Distinction in Medicine and Therapeutics from Edinburgh University. Presently, he is head of the Animals Scientific Procedures Division at the Home Office. He is a Fellow of the Royal College of Surgeons of Edinburgh (FRCSEd) and a Fellow of the Royal Society of Medicine (FRMS). Other appointments include convener of the U.K. interdepartmental group on the 3Rs, board member U.K. National Centre for the 3Rs, convener of the International Standards Organization Technical Corrigendum 194/Working Group 3 (*Biocompatibility of Medical Device Materials*), and member of related expert working groups. He is a former member of the EU Committee on Scientific and Technical Progress and past Chairman of the European Commission Technical Expert Working Group on ethical review. He served as chair of the peer review panel for the reduced murine local lymph node assay (LLNA) test protocol and prediction model for ESAC in 2007 and has been designated as an ESAC peer reviewer for ECVAM's performance standards for the standard LLNA. He served on the NICEATM-ICCVAM Expert Panel that evaluated Five *In Vitro* Pyrogen Test Methods. He has a variety of publications in peer-reviewed journals and national and international meetings, on the principles and practice of surgery, regulation of biomedical research, principles of humane research, bioethics, and public policy.

Peter Theran, V.M.D.

Dr. Theran holds a Doctor of Veterinary Medicine degree from the University of Pennsylvania. He has had many years of experience both as a veterinary internal medicine specialist at the Massachusetts Society for Prevention of Cruelty to Animals' Angell Memorial Animal Hospital in Boston, and as the director of Boston University Medical Center's Laboratory Animal Science Center. He presently serves on a number of government committees as an animal welfare member, and is a member of the Board of Directors of the Institute for *In Vitro* Sciences in Gaithersburg, MD and Chimp Haven in Shreveport, Louisiana. He served on the NICEATM-ICCVAM Expert Panels that evaluated the *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, and Five *In Vitro*

Pyrogen Test Methods. He is a former member of the Advisory Committee on Alternative Toxicological Methods and SACATM. He is presently working as a consultant.

Kevin Williams

Mr. Williams received a B.S. degree in Microbiology from Texas A&M University. He is a Microbiologist in the Quality Control Laboratory at Eli Lilly & Company. Mr. Williams' resume indicates that he is a well-noted expert in pyrogen testing (Bacterial Endotoxin Test [BET] and LAL) and validation and he has authored several books on endotoxins. His responsibilities include bacterial endotoxin testing and validation, automation of BET, depyrogenation validation, automated microbial identification system validation, validation of sterility tests, preservative effectiveness testing, microbial purity testing and validation, and bioburden testing and validation. Mr. Williams is a member of the LAL User Steering Committee, the Parenteral Drug Association, and the American Society for Microbiology. He has developed a method to calculate tolerance limits for excipients based on unit formula content of finished drug and developed novel methods of recovering endotoxin from parenteral drug packaging components. Mr. Williams served as editor of the textbook, "Microbial Contamination Control in Parenteral Manufacturing," and contributed a chapter on "Historical and Emerging Themes in Parenteral Manufacturing Control." He also edited the textbook "Endotoxins," and contributed chapters on endotoxin and contamination control.

Appendix B

Relevant Federal Pyrogenicity Regulations and Testing Guidelines

B1	U.S. Code of Federal Regulations (CFR).....	B-3
B1-1	21 CFR 211.167 - Current Good Manufacturing Practice for Finished Pharmaceuticals: Special Testing Requirements (April 1, 2007).....	B-5
B1-2	21 CFR 314.50(d)(1)(ii)(a) - Applications: Content and Format of an Application (April 1, 2007)	B-6
B1-3	21 CFR 610.9 - General Provisions: Equivalent Methods and Processes (April 1, 2007)	B-17
B1-4	21 CFR 610.13 - General Biological Products Standards: Purity (April 1, 2007)	B-19
B2	International Organization for Standardization - ISO 10993-11 - Biological Evaluation of Medical Devices - Part 11: Tests for Systemic Toxicity (First Edition 1993-12-15).....	B-21
B3	U.S. Pharmacopeia (USP) 30-NF25.....	B-23
B3-1	(85) - Bacterial Endotoxins Test	B-23
B3-2	(151) - Pyrogen Test	B-23
B3-3	(1041) - Biologics	B-23
B4	European Pharmacopeia 5.0	B-25
B4-1	2.6.8 - Pyrogens	B-25
B4-2	2.6.14 - Bacterial Endotoxins	B-25
B5	Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices (December 1987).....	B-27

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Appendix B1

U.S. Code of Federal Regulations (CFR)

- B1-1 21 CFR 211.167 - Current Good Manufacturing Practice for Finished Pharmaceuticals: Special Testing Requirements (April 1, 2007)**
- B1-2 21 CFR 314.50 (d)(1)(ii)(a) - Applications: Content and Format of an Application (April 1, 2007)**
- B1-3 21 CFR 610.9 - General Provisions: Equivalent Methods and Processes (April 1, 2007)**
- B1-4 21 CFR 610.13 - General Biological Products Standards: Purity (April 1, 2007)**

These documents are available at:

<http://www.access.gpo.gov/cgi-bin/cfrassemble.cgi?title=200442>

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Food and Drug Administration, HHS**§211.170****§211.167 Special testing requirements.**

(a) For each batch of drug product purporting to be sterile and/or pyrogen-free, there shall be appropriate laboratory testing to determine conformance to such requirements. The test procedures shall be in writing and shall be followed.

(b) For each batch of ophthalmic ointment, there shall be appropriate testing to determine conformance to specifications regarding the presence of foreign particles and harsh or abrasive substances. The test procedures shall be in writing and shall be followed.

(c) For each batch of controlled-release dosage form, there shall be appropriate laboratory testing to determine conformance to the specifications for the rate of release of each active ingredient. The test procedures shall be in writing and shall be followed.

§ 314.50

21 CFR Ch. I (4–1–07 Edition)

Subpart B—Applications

§ 314.50 Content and format of an application.

Applications and supplements to approved applications are required to be submitted in the form and contain the information, as appropriate for the particular submission, required under this section. Three copies of the application are required: An archival copy, a review copy, and a field copy. An application for a new chemical entity will generally contain an application form, an index, a summary, five or six technical sections, case report tabulations of patient data, case report forms, drug samples, and labeling, including, if applicable, any Medication Guide required under part 208 of this chapter. Other applications will generally contain only some of those items, and information will be limited to that needed to support the particular submission. These include an application of the type described in section 505(b)(2) of the act, an amendment, and a supplement. The application is required to contain reports of all investigations of the drug product sponsored by the applicant, and all other information about the drug pertinent to an evaluation of the application that is received or otherwise obtained by the applicant

from any source. FDA will maintain guidance documents on the format and content of applications to assist applicants in their preparation.

(a) *Application form.* The applicant shall submit a completed and signed application form that contains the following:

(1) The name and address of the applicant; the date of the application; the application number if previously issued (for example, if the application is a resubmission, an amendment, or a supplement); the name of the drug product, including its established, proprietary, code, and chemical names; the dosage form and strength; the route of administration; the identification numbers of all investigational new drug applications that are referenced in the application; the identification numbers of all drug master files and other applications under this part that are referenced in the application; and the drug product's proposed indications for use.

(2) A statement whether the submission is an original submission, a 505(b)(2) application, a resubmission, or a supplement to an application under § 314.70.

(3) A statement whether the applicant proposes to market the drug product as a prescription or an over-the-counter product.

(4) A check-list identifying what enclosures required under this section the applicant is submitting.

(5) The applicant, or the applicant's attorney, agent, or other authorized official shall sign the application. If the person signing the application does not reside or have a place of business within the United States, the application is required to contain the name and address of, and be countersigned by, an attorney, agent, or other authorized official who resides or maintains a place of business within the United States.

(b) *Index.* The archival copy of the application is required to contain a comprehensive index by volume number and page number to the summary under paragraph (c) of this section, the technical sections under paragraph (d) of this section, and the supporting information under paragraph (f) of this section.

Food and Drug Administration, HHS

§ 314.50

(c) *Summary.* (1) An application is required to contain a summary of the application in enough detail that the reader may gain a good general understanding of the data and information in the application, including an understanding of the quantitative aspects of the data. The summary is not required for supplements under §314.70. Resubmissions of an application should contain an updated summary, as appropriate. The summary should discuss all aspects of the application, and synthesize the information into a well-structured and unified document. The summary should be written at approximately the level of detail required for publication in, and meet the editorial standards generally applied by, refereed scientific and medical journals. In addition to the agency personnel reviewing the summary in the context of their review of the application, FDA may furnish the summary to FDA advisory committee members and agency officials whose duties require an understanding of the application. To the extent possible, data in the summary should be presented in tabular and graphic forms. FDA has prepared a guideline under §10.90(b) that provides information about how to prepare a summary. The summary required under this paragraph may be used by FDA or the applicant to prepare the Summary Basis of Approval document for public disclosure (under §314.430(e)(2)(ii)) when the application is approved.

(2) The summary is required to contain the following information:

(i) The proposed text of the labeling, including, if applicable, any Medication Guide required under part 208 of this chapter, for the drug, with annotations to the information in the summary and technical sections of the application that support the inclusion of each statement in the labeling, and, if the application is for a prescription drug, statements describing the reasons for omitting a section or subsection of the labeling format in §201.57 of this chapter.

(ii) A statement identifying the pharmacologic class of the drug and a discussion of the scientific rationale for the drug, its intended use, and the po-

tential clinical benefits of the drug product.

(iii) A brief description of the marketing history, if any, of the drug outside the United States, including a list of the countries in which the drug has been marketed, a list of any countries in which the drug has been withdrawn from marketing for any reason related to safety or effectiveness, and a list of countries in which applications for marketing are pending. The description is required to describe both marketing by the applicant and, if known, the marketing history of other persons.

(iv) A summary of the chemistry, manufacturing, and controls section of the application.

(v) A summary of the nonclinical pharmacology and toxicology section of the application.

(vi) A summary of the human pharmacokinetics and bioavailability section of the application.

(vii) A summary of the microbiology section of the application (for anti-infective drugs only).

(viii) A summary of the clinical data section of the application, including the results of statistical analyses of the clinical trials.

(ix) A concluding discussion that presents the benefit and risk considerations related to the drug, including a discussion of any proposed additional studies or surveillance the applicant intends to conduct postmarketing.

(d) *Technical sections.* The application is required to contain the technical sections described below. Each technical section is required to contain data and information in sufficient detail to permit the agency to make a knowledgeable judgment about whether to approve the application or whether grounds exist under section 505(d) of the act to refuse to approve the application. The required technical sections are as follows:

(1) *Chemistry, manufacturing, and controls section.* A section describing the composition, manufacture, and specification of the drug substance and the drug product, including the following:

(i) *Drug substance.* A full description of the drug substance including its physical and chemical characteristics and stability; the name and address of

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its manufacturer; the method of synthesis (or isolation) and purification of the drug substance; the process controls used during manufacture and packaging; and the specifications necessary to ensure the identity, strength, quality, and purity of the drug substance and the bioavailability of the drug products made from the substance, including, for example, tests, analytical procedures, and acceptance criteria relating to stability, sterility, particle size, and crystalline form. The application may provide additionally for the use of alternatives to meet any of these requirements, including alternative sources, process controls, and analytical procedures. Reference to the current edition of the U.S. Pharmacopeia and the National Formulary may satisfy relevant requirements in this paragraph.

(ii)(a) *Drug product.* A list of all components used in the manufacture of the drug product (regardless of whether they appear in the drug product) and a statement of the composition of the drug product; the specifications for each component; the name and address of each manufacturer of the drug product; a description of the manufacturing and packaging procedures and in-process controls for the drug product; the specifications necessary to ensure the identity, strength, quality, purity, potency, and bioavailability of the drug product, including, for example, tests, analytical procedures, and acceptance criteria relating to sterility, dissolution rate, container closure systems; and stability data with proposed expiration dating. The application may provide additionally for the use of alternatives to meet any of these requirements, including alternative components, manufacturing and packaging procedures, in-process controls, and analytical procedures. Reference to the current edition of the U.S. Pharmacopeia and the National Formulary may satisfy relevant requirements in this paragraph.

(b) Unless provided by paragraph (d)(1)(ii)(a) of this section, for each batch of the drug product used to conduct a bioavailability or bioequivalence study described in § 320.38 or § 320.63 of this chapter or used to conduct a primary stability study: The

batch production record; the specification for each component and for the drug product; the names and addresses of the sources of the active and noncompensial inactive components and of the container and closure system for the drug product; the name and address of each contract facility involved in the manufacture, processing, packaging, or testing of the drug product and identification of the operation performed by each contract facility; and the results of any test performed on the components used in the manufacture of the drug product as required by § 211.84(d) of this chapter and on the drug product as required by § 211.165 of this chapter.

(c) The proposed or actual master production record, including a description of the equipment, to be used for the manufacture of a commercial lot of the drug product or a comparably detailed description of the production process for a representative batch of the drug product.

(iii) *Environmental impact.* The application is required to contain either a claim for categorical exclusion under § 25.30 or 25.31 of this chapter or an environmental assessment under § 25.40 of this chapter.

(iv) The applicant may, at its option, submit a complete chemistry, manufacturing, and controls section 90 to 120 days before the anticipated submission of the remainder of the application. FDA will review such early submissions as resources permit.

(v) The applicant shall include a statement certifying that the field copy of the application has been provided to the applicant's home FDA district office.

(2) *Nonclinical pharmacology and toxicology section.* A section describing, with the aid of graphs and tables, animal and in vitro studies with drug, including the following:

(i) Studies of the pharmacological actions of the drug in relation to its proposed therapeutic indication and studies that otherwise define the pharmacologic properties of the drug or are pertinent to possible adverse effects.

(ii) Studies of the toxicological effects of the drug as they relate to the drug's intended clinical uses, including, as appropriate, studies assessing

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the drug's acute, subacute, and chronic toxicity; carcinogenicity; and studies of toxicities related to the drug's particular mode of administration or conditions of use.

(iii) Studies, as appropriate, of the effects of the drug on reproduction and on the developing fetus.

(iv) Any studies of the absorption, distribution, metabolism, and excretion of the drug in animals.

(v) For each nonclinical laboratory study subject to the good laboratory practice regulations under part 58 a statement that it was conducted in compliance with the good laboratory practice regulations in part 58, or, if the study was not conducted in compliance with those regulations, a brief statement of the reason for the non-compliance.

(3) *Human pharmacokinetics and bioavailability section.* A section describing the human pharmacokinetic data and human bioavailability data, or information supporting a waiver of the submission of in vivo bioavailability data under subpart B of part 320, including the following:

(i) A description of each of the bioavailability and pharmacokinetic studies of the drug in humans performed by or on behalf of the applicant that includes a description of the analytical procedures and statistical methods used in each study and a statement with respect to each study that it either was conducted in compliance with the institutional review board regulations in part 56, or was not subject to the regulations under § 56.104 or § 56.105, and that it was conducted in compliance with the informed consent regulations in part 50.

(ii) If the application describes in the chemistry, manufacturing, and controls section tests, analytical procedures, and acceptance criteria needed to assure the bioavailability of the drug product or drug substance, or both, a statement in this section of the rationale for establishing the tests, analytical procedures, and acceptance criteria, including data and information supporting the rationale.

(iii) A summarizing discussion and analysis of the pharmacokinetics and metabolism of the active ingredients

and the bioavailability or bioequivalence, or both, of the drug product.

(4) *Microbiology section.* If the drug is an anti-infective drug, a section describing the microbiology data, including the following:

(i) A description of the biochemical basis of the drug's action on microbial physiology.

(ii) A description of the antimicrobial spectra of the drug, including results of in vitro preclinical studies to demonstrate concentrations of the drug required for effective use.

(iii) A description of any known mechanisms of resistance to the drug, including results of any known epidemiologic studies to demonstrate prevalence of resistance factors.

(iv) A description of clinical microbiology laboratory procedures (for example, in vitro sensitivity discs) needed for effective use of the drug.

(5) *Clinical data section.* A section describing the clinical investigations of the drug, including the following:

(i) A description and analysis of each clinical pharmacology study of the drug, including a brief comparison of the results of the human studies with the animal pharmacology and toxicology data.

(ii) A description and analysis of each controlled clinical study pertinent to a proposed use of the drug, including the protocol and a description of the statistical analyses used to evaluate the study. If the study report is an interim analysis, this is to be noted and a projected completion date provided. Controlled clinical studies that have not been analyzed in detail for any reason (e.g., because they have been discontinued or are incomplete) are to be included in this section, including a copy of the protocol and a brief description of the results and status of the study.

(iii) A description of each uncontrolled clinical study, a summary of the results, and a brief statement explaining why the study is classified as uncontrolled.

(iv) A description and analysis of any other data or information relevant to an evaluation of the safety and effectiveness of the drug product obtained or otherwise received by the applicant from any source, foreign or domestic, including information derived from

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clinical investigations, including controlled and uncontrolled studies of uses of the drug other than those proposed in the application, commercial marketing experience, reports in the scientific literature, and unpublished scientific papers.

(v) An integrated summary of the data demonstrating substantial evidence of effectiveness for the claimed indications. Evidence is also required to support the dosage and administration section of the labeling, including support for the dosage and dose interval recommended. The effectiveness data shall be presented by gender, age, and racial subgroups and shall identify any modifications of dose or dose interval needed for specific subgroups. Effectiveness data from other subgroups of the population of patients treated, when appropriate, such as patients with renal failure or patients with different levels of severity of the disease, also shall be presented.

(vi) A summary and updates of safety information, as follows:

(a) The applicant shall submit an integrated summary of all available information about the safety of the drug product, including pertinent animal data, demonstrated or potential adverse effects of the drug, clinically significant drug/drug interactions, and other safety considerations, such as data from epidemiological studies of related drugs. The safety data shall be presented by gender, age, and racial subgroups. When appropriate, safety data from other subgroups of the population of patients treated also shall be presented, such as for patients with renal failure or patients with different levels of severity of the disease. A description of any statistical analyses performed in analyzing safety data should also be included, unless already included under paragraph (d)(5)(ii) of this section.

(b) The applicant shall, under section 505(i) of the act, update periodically its pending application with new safety information learned about the drug that may reasonably affect the statement of contraindications, warnings, precautions, and adverse reactions in the draft labeling and, if applicable, any Medication Guide required under part 208 of this chapter. These “safety up-

date reports” are required to include the same kinds of information (from clinical studies, animal studies, and other sources) and are required to be submitted in the same format as the integrated summary in paragraph (d)(5)(vi)(a) of this section. In addition, the reports are required to include the case report forms for each patient who died during a clinical study or who did not complete the study because of an adverse event (unless this requirement is waived). The applicant shall submit these reports (1) 4 months after the initial submission; (2) following receipt of an approvable letter; and (3) at other times as requested by FDA. Prior to the submission of the first such report, applicants are encouraged to consult with FDA regarding further details on its form and content.

(vii) If the drug has a potential for abuse, a description and analysis of studies or information related to abuse of the drug, including a proposal for scheduling under the Controlled Substances Act. A description of any studies related to overdose is also required, including information on dialysis, antidotes, or other treatments, if known.

(viii) An integrated summary of the benefits and risks of the drug, including a discussion of why the benefits exceed the risks under the conditions stated in the labeling.

(ix) A statement with respect to each clinical study involving human subjects that it either was conducted in compliance with the institutional review board regulations in part 56, or was not subject to the regulations under § 56.104 or § 56.105, and that it was conducted in compliance with the informed consent regulations in part 50.

(x) If a sponsor has transferred any obligations for the conduct of any clinical study to a contract research organization, a statement containing the name and address of the contract research organization, identification of the clinical study, and a listing of the obligations transferred. If all obligations governing the conduct of the study have been transferred, a general statement of this transfer—in lieu of a listing of the specific obligations transferred—may be submitted.

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(xi) If original subject records were audited or reviewed by the sponsor in the course of monitoring any clinical study to verify the accuracy of the case reports submitted to the sponsor, a list identifying each clinical study so audited or reviewed.

(6) *Statistical section.* A section describing the statistical evaluation of clinical data, including the following:

(i) A copy of the information submitted under paragraph (d)(5)(ii) of this section concerning the description and analysis of each controlled clinical study, and the documentation and supporting statistical analyses used in evaluating the controlled clinical studies.

(ii) A copy of the information submitted under paragraph (d)(5)(vi)(a) of this section concerning a summary of information about the safety of the drug product, and the documentation and supporting statistical analyses used in evaluating the safety information.

(7) *Pediatric use section.* A section describing the investigation of the drug for use in pediatric populations, including an integrated summary of the information (the clinical pharmacology studies, controlled clinical studies, or uncontrolled clinical studies, or other data or information) that is relevant to the safety and effectiveness and benefits and risks of the drug in pediatric populations for the claimed indications, a reference to the full descriptions of such studies provided under paragraphs (d)(3) and (d)(5) of this section, and information required to be submitted under §314.55.

(e) *Samples and labeling.* (1) Upon request from FDA, the applicant shall submit the samples described below to the places identified in the agency's request. FDA will generally ask applicants to submit samples directly to two or more agency laboratories that will perform all necessary tests on the samples and validate the applicant's analytical procedures.

(i) Four representative samples of the following, each sample in sufficient quantity to permit FDA to perform three times each test described in the application to determine whether the drug substance and the drug product

meet the specifications given in the application:

(a) The drug product proposed for marketing;

(b) The drug substance used in the drug product from which the samples of the drug product were taken; and

(c) Reference standards and blanks (except that reference standards recognized in an official compendium need not be submitted).

(ii) Samples of the finished market package, if requested by FDA.

(2) The applicant shall submit the following in the archival copy of the application:

(i) Three copies of the analytical procedures and related descriptive information contained in the chemistry, manufacturing, and controls section under paragraph (d)(1) of this section for the drug substance and the drug product that are necessary for FDA's laboratories to perform all necessary tests on the samples and to validate the applicant's analytical procedures. The related descriptive information includes a description of each sample; the proposed regulatory specifications for the drug; a detailed description of the methods of analysis; supporting data for accuracy, specificity, precision and ruggedness; and complete results of the applicant's tests on each sample.

(ii) Copies of the label and all labeling for the drug product (including, if applicable, any Medication Guide required under part 208 of this chapter) for the drug product (4 copies of draft labeling or 12 copies of final printed labeling).

(f) *Case report forms and tabulations.* The archival copy of the application is required to contain the following case report tabulations and case report forms:

(1) *Case report tabulations.* The application is required to contain tabulations of the data from each adequate and well-controlled study under §314.126 (Phase 2 and Phase 3 studies as described in §§312.21 (b) and (c) of this chapter), tabulations of the data from the earliest clinical pharmacology studies (Phase 1 studies as described in §312.21(a) of this chapter), and tabulations of the safety data from other clinical studies. Routine submission of other patient data from uncontrolled

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studies is not required. The tabulations are required to include the data on each patient in each study, except that the applicant may delete those tabulations which the agency agrees, in advance, are not pertinent to a review of the drug's safety or effectiveness. Upon request, FDA will discuss with the applicant in a "pre-NDA" conference those tabulations that may be appropriate for such deletion. Barring unforeseen circumstances, tabulations agreed to be deleted at such a conference will not be requested during the conduct of FDA's review of the application. If such unforeseen circumstances do occur, any request for deleted tabulations will be made by the director of the FDA division responsible for reviewing the application, in accordance with paragraph (f)(3) of this section.

(2) *Case report forms.* The application is required to contain copies of individual case report forms for each patient who died during a clinical study or who did not complete the study because of an adverse event, whether believed to be drug related or not, including patients receiving reference drugs or placebo. This requirement may be waived by FDA for specific studies if the case report forms are unnecessary for a proper review of the study.

(3) *Additional data.* The applicant shall submit to FDA additional case report forms and tabulations needed to conduct a proper review of the application, as requested by the director of the FDA division responsible for reviewing the application. The applicant's failure to submit information requested by FDA within 30 days after receipt of the request may result in the agency viewing any eventual submission as a major amendment under §314.60 and extending the review period as necessary. If desired by the applicant, the FDA division director will verify in writing any request for additional data that was made orally.

(4) Applicants are invited to meet with FDA before submitting an application to discuss the presentation and format of supporting information. If the applicant and FDA agree, the applicant may submit tabulations of patient data and case report forms in a form

other than hard copy, for example, on microfiche or computer tapes.

(g) *Other.* The following general requirements apply to the submission of information within the summary under paragraph (c) of this section and within the technical sections under paragraph (d) of this section.

(1) The applicant ordinarily is not required to resubmit information previously submitted, but may incorporate the information by reference. A reference to information submitted previously is required to identify the file by name, reference number, volume, and page number in the agency's records where the information can be found. A reference to information submitted to the agency by a person other than the applicant is required to contain a written statement that authorizes the reference and that is signed by the person who submitted the information.

(2) The applicant shall submit an accurate and complete English translation of each part of the application that is not in English. The applicant shall submit a copy of each original literature publication for which an English translation is submitted.

(3) If an applicant who submits a new drug application under section 505(b) of the act obtains a "right of reference or use," as defined under §314.3(b), to an investigation described in clause (A) of section 505(b)(1) of the act, the applicant shall include in its application a written statement signed by the owner of the data from each such investigation that the applicant may rely on in support of the approval of its application, and provide FDA access to, the underlying raw data that provide the basis for the report of the investigation submitted in its application.

(h) *Patent information.* The application is required to contain the patent information described under §314.53.

(i) *Patent certification—(1) Contents.* A 505(b)(2) application is required to contain the following:

(i) *Patents claiming drug, drug product, or method of use.* (A) Except as provided in paragraph (i)(2) of this section, a certification with respect to each patent issued by the United States Patent and Trademark Office that, in the opinion of the applicant and to the best of

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its knowledge, claims a drug (the drug product or drug substance that is a component of the drug product) on which investigations that are relied upon by the applicant for approval of its application were conducted or that claims an approved use for such drug and for which information is required to be filed under section 505(b) and (c) of the act and §314.53. For each such patent, the applicant shall provide the patent number and certify, in its opinion and to the best of its knowledge, one of the following circumstances:

(1) That the patent information has not been submitted to FDA. The applicant shall entitle such a certification “Paragraph I Certification”;

(2) That the patent has expired. The applicant shall entitle such a certification “Paragraph II Certification”;

(3) The date on which the patent will expire. The applicant shall entitle such a certification “Paragraph III Certification”; or

(4) That the patent is invalid, unenforceable, or will not be infringed by the manufacture, use, or sale of the drug product for which the application is submitted. The applicant shall entitle such a certification “Paragraph IV Certification”. This certification shall be submitted in the following form:

I, *(name of applicant)*, certify that Patent No. _____ *(is invalid, unenforceable, or will not be infringed by the manufacture, use, or sale of) (name of proposed drug product)* for which this application is submitted.

The certification shall be accompanied by a statement that the applicant will comply with the requirements under §314.52(a) with respect to providing a notice to each owner of the patent or their representatives and to the holder of the approved application for the drug product which is claimed by the patent or a use of which is claimed by the patent and with the requirements under §314.52(c) with respect to the content of the notice.

(B) If the drug on which investigations that are relied upon by the applicant were conducted is itself a licensed generic drug of a patented drug first approved under section 505(b) of the act, the appropriate patent certification under this section with respect to each patent that claims the first-ap-

proved patented drug or that claims an approved use for such a drug.

(ii) *No relevant patents.* If, in the opinion of the applicant and to the best of its knowledge, there are no patents described in paragraph (i)(1)(i) of this section, a certification in the following form:

In the opinion and to the best knowledge of *(name of applicant)*, there are no patents that claim the drug or drugs on which investigations that are relied upon in this application were conducted or that claim a use of such drug or drugs.

(iii) *Method of use patent.* (A) If information that is submitted under section 505(b) or (c) of the act and §314.53 is for a method of use patent, and the labeling for the drug product for which the applicant is seeking approval does not include any indications that are covered by the use patent, a statement explaining that the method of use patent does not claim any of the proposed indications.

(B) If the labeling of the drug product for which the applicant is seeking approval includes an indication that, according to the patent information submitted under section 505(b) or (c) of the act and §314.53 or in the opinion of the applicant, is claimed by a use patent, the applicant shall submit an applicable certification under paragraph (i)(1)(i) of this section.

(2) *Method of manufacturing patent.* An applicant is not required to make a certification with respect to any patent that claims only a method of manufacturing the drug product for which the applicant is seeking approval.

(3) *Licensing agreements.* If a 505(b)(2) application is for a drug or method of using a drug claimed by a patent and the applicant has a licensing agreement with the patent owner, the applicant shall submit a certification under paragraph (i)(1)(i)(A)(4) of this section (“Paragraph IV Certification”) as to that patent and a statement that it has been granted a patent license. If the patent owner consents to an immediate effective date upon approval of the 505(b)(2) application, the application shall contain a written statement from the patent owner that it has a licensing agreement with the applicant and that it consents to an immediate effective date.

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(4) *Late submission of patent information.* If a patent described in paragraph (i)(1)(i)(A) of this section is issued and the holder of the approved application for the patented drug does not submit the required information on the patent within 30 days of issuance of the patent, an applicant who submitted a 505(b)(2) application that, before the submission of the patent information, contained an appropriate patent certification is not required to submit an amended certification. An applicant whose 505(b)(2) application is filed after a late submission of patent information or whose 505(b)(2) application was previously filed but did not contain an appropriate patent certification at the time of the patent submission shall submit a certification under paragraph (i)(1)(i) or (i)(1)(ii) of this section or a statement under paragraph (i)(1)(iii) of this section as to that patent.

(5) *Disputed patent information.* If an applicant disputes the accuracy or relevance of patent information submitted to FDA, the applicant may seek a confirmation of the correctness of the patent information in accordance with the procedures under §314.53(f). Unless the patent information is withdrawn or changed, the applicant must submit an appropriate certification for each relevant patent.

(6) *Amended certifications.* A certification submitted under paragraphs (i)(1)(i) through (i)(1)(iii) of this section may be amended at any time before the effective date of the approval of the application. An applicant shall submit an amended certification as an amendment to a pending application or by letter to an approved application. If an applicant with a pending application voluntarily makes a patent certification for an untimely filed patent, the applicant may withdraw the patent certification for the untimely filed patent. Once an amendment or letter for the change in certification has been submitted, the application will no longer be considered to be one containing the prior certification.

(i) *After finding of infringement.* An applicant who has submitted a certification under paragraph (i)(1)(i)(A)(4) of this section and is sued for patent infringement within 45 days of the receipt of notice sent under §314.52 shall

amend the certification if a final judgment in the action is entered finding the patent to be infringed unless the final judgment also finds the patent to be invalid. In the amended certification, the applicant shall certify under paragraph (i)(1)(i)(A)(3) of this section that the patent will expire on a specific date.

(ii) *After removal of a patent from the list.* If a patent is removed from the list, any applicant with a pending application (including a tentatively approved application with a delayed effective date) who has made a certification with respect to such patent shall amend its certification. The applicant shall certify under paragraph (i)(1)(ii) of this section that no patents described in paragraph (i)(1)(i) of this section claim the drug or, if other relevant patents claim the drug, shall amend the certification to refer only to those relevant patents. In the amendment, the applicant shall state the reason for the change in certification (that the patent is or has been removed from the list). A patent that is the subject of a lawsuit under §314.107(c) shall not be removed from the list until FDA determines either that no delay in effective dates of approval is required under that section as a result of the lawsuit, that the patent has expired, or that any such period of delay in effective dates of approval is ended. An applicant shall submit an amended certification as an amendment to a pending application. Once an amendment for the change has been submitted, the application will no longer be considered to be one containing a certification under paragraph (i)(1)(i)(A)(4) of this section.

(iii) *Other amendments.* (A) Except as provided in paragraphs (i)(4) and (i)(6)(iii)(B) of this section, an applicant shall amend a submitted certification if, at any time before the effective date of the approval of the application, the applicant learns that the submitted certification is no longer accurate.

(B) An applicant is not required to amend a submitted certification when information on an otherwise applicable patent is submitted after the effective date of approval for the 505(b)(2) application.

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(j) *Claimed exclusivity.* A new drug product, upon approval, may be entitled to a period of marketing exclusivity under the provisions of §314.108. If an applicant believes its drug product is entitled to a period of exclusivity, it shall submit with the new drug application prior to approval the following information:

(1) A statement that the applicant is claiming exclusivity.

(2) A reference to the appropriate paragraph under §314.108 that supports its claim.

(3) If the applicant claims exclusivity under §314.108(b)(2), information to show that, to the best of its knowledge or belief, a drug has not previously been approved under section 505(b) of the act containing any active moiety in the drug for which the applicant is seeking approval.

(4) If the applicant claims exclusivity under §314.108(b)(4) or (b)(5), the following information to show that the application contains “new clinical investigations” that are “essential to approval of the application or supplement” and were “conducted or sponsored by the applicant:”

(i) “*New clinical investigations.*” A certification that to the best of the applicant’s knowledge each of the clinical investigations included in the application meets the definition of “new clinical investigation” set forth in §314.108(a).

(ii) “*Essential to approval.*” A list of all published studies or publicly available reports of clinical investigations known to the applicant through a literature search that are relevant to the conditions for which the applicant is seeking approval, a certification that the applicant has thoroughly searched the scientific literature and, to the best of the applicant’s knowledge, the list is complete and accurate and, in the applicant’s opinion, such published studies or publicly available reports do not provide a sufficient basis for the approval of the conditions for which the applicant is seeking approval without reference to the new clinical investigation(s) in the application, and an explanation as to why the studies or reports are insufficient.

(iii) “*Conducted or sponsored by.*” If the applicant was the sponsor named in

the Form FDA-1571 for an investigational new drug application (IND) under which the new clinical investigation(s) that is essential to the approval of its application was conducted, identification of the IND by number. If the applicant was not the sponsor of the IND under which the clinical investigation(s) was conducted, a certification that the applicant or its predecessor in interest provided substantial support for the clinical investigation(s) that is essential to the approval of its application, and information supporting the certification. To demonstrate “substantial support,” an applicant must either provide a certified statement from a certified public accountant that the applicant provided 50 percent or more of the cost of conducting the study or provide an explanation of why FDA should consider the applicant to have conducted or sponsored the study if the applicant’s financial contribution to the study is less than 50 percent or the applicant did not sponsor the investigational new drug. A predecessor in interest is an entity, e.g., a corporation, that the applicant has taken over, merged with, or purchased, or from which the applicant has purchased all rights to the drug. Purchase of non-exclusive rights to a clinical investigation after it is completed is not sufficient to satisfy this definition.

(k) *Financial certification or disclosure statement.* The application shall contain a financial certification or disclosure statement or both as required by part 54 of this chapter.

(l) *Format of an original application—*

(1) *Archival copy.* The applicant must submit a complete archival copy of the application that contains the information required under paragraphs (a) through (f) of this section. FDA will maintain the archival copy during the review of the application to permit individual reviewers to refer to information that is not contained in their particular technical sections of the application, to give other agency personnel access to the application for official business, and to maintain in one place a complete copy of the application. Except as required by paragraph (1)(1)(i) of this section, applicants may submit the archival copy on paper or in electronic format provided that electronic

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submissions are made in accordance with part 11 of this chapter.

(i) *Labeling*. The content of labeling required under § 201.100(d)(3) of this chapter (commonly referred to as the package insert or professional labeling), including all text, tables, and figures, must be submitted to the agency in electronic format as described in paragraph (l)(5) of this section. This requirement is in addition to the requirements of paragraph (e)(2)(ii) of this section that copies of the formatted label and all labeling be submitted. Submissions under this paragraph must be made in accordance with part 11 of this chapter, except for the requirements of § 11.10(a), (c) through (h), and (k), and the corresponding requirements of § 11.30.

(ii) [Reserved]

(2) *Review copy*. The applicant must submit a review copy of the application. Each of the technical sections, described in paragraphs (d)(1) through (d)(6) of this section, in the review copy is required to be separately bound with a copy of the application form required under paragraph (a) of this section and a copy of the summary required under paragraph (c) of this section.

(3) *Field copy*. The applicant must submit a field copy of the application that contains the technical section described in paragraph (d)(1) of this section, a copy of the application form required under paragraph (a) of this section, a copy of the summary required under paragraph (c) of this section, and a certification that the field copy is a true copy of the technical section described in paragraph (d)(1) of this section contained in the archival and review copies of the application.

(4) *Binding folders*. The applicant may obtain from FDA sufficient folders to bind the archival, the review, and the field copies of the application.

(5) *Electronic format submissions*. Electronic format submissions must be in a form that FDA can process, review, and archive. FDA will periodically issue guidance on how to provide the electronic submission (e.g., method of transmission, media, file formats, preparation and organization of files).

[50 FR 7493, Feb. 22, 1985]

EDITORIAL NOTE: For FEDERAL REGISTER citations affecting § 314.50, see the List of CFR

Sections Affected, which appears in the Finding Aids section of the printed volume and on GPO Access.

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Subpart B—General Provisions

§ 610.9 Equivalent methods and processes.

Modification of any particular test method or manufacturing process or the conditions under which it is conducted as required in this part or in the

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additional standards for specific biological products in parts 620 through 680 of this chapter shall be permitted only under the following conditions:

(a) The applicant presents evidence, in the form of a license application, or a supplement to the application submitted in accordance with §601.12(b) or (c), demonstrating that the modification will provide assurances of the safety, purity, potency, and effectiveness of the biological product equal to or greater than the assurances provided by the method or process specified in the general standards or additional standards for the biological product; and

(b) Approval of the modification is received in writing from the Director, Center for Biologics Evaluation and Research or the Director, Center for Drug Evaluation and Research.

[62 FR 39903, July 24, 1997, as amended at 70 FR 14984, Mar. 24, 2005]

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Products shall be free of extraneous material except that which is unavoidable in the manufacturing process described in the approved biologics license application. In addition, products shall be tested as provided in paragraphs (a) and (b) of this section.

(a)(1) *Test for residual moisture.* Each lot of dried product shall be tested for residual moisture and shall meet and not exceed established limits as specified by an approved method on file in the biologics license application. The test for residual moisture may be exempted by the Director, Center for Biologics Evaluation and Research or the Director, Center for Drug Evaluation and Research, when deemed not necessary for the continued safety, purity, and potency of the product.

(2) *Records.* Appropriate records for residual moisture under paragraph (a)(1) of this section shall be prepared and maintained as required by the applicable provisions of §§ 211.188 and 211.194 of this chapter.

(b) *Test for pyrogenic substances.* Each lot of final containers of any product intended for use by injection shall be

§ 610.14

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tested for pyrogenic substances by intravenous injection into rabbits as provided in paragraphs (b) (1) and (2) of this section: *Provided*, That notwithstanding any other provision of Subchapter F of this chapter, the test for pyrogenic substances is not required for the following products: Products containing formed blood elements; Cryoprecipitate; Plasma; Source Plasma; Normal Horse Serum; bacterial, viral, and rickettsial vaccines and antigens; toxoids; toxins; allergenic extracts; venoms; diagnostic substances and trivalent organic arsenicals.

(1) *Test dose*. The test dose for each rabbit shall be at least 3 milliliters per kilogram of body weight of the rabbit and also shall be at least equivalent proportionately, on a body weight basis, to the maximum single human dose recommended, but need not exceed 10 milliliters per kilogram of body weight of the rabbit, except that: (i) Regardless of the human dose recommended, the test dose per kilogram of body weight of each rabbit shall be at least 1 milliliter for immune globulins derived from human blood; (ii) for Streptokinase, the test dose shall be at least equivalent proportionately, on a body weight basis, to the maximum single human dose recommended.

(2) *Test procedure, results, and interpretation; standards to be met*. The test for pyrogenic substances shall be performed according to the requirements specified in United States Pharmacopeia XX.

(3) *Retest*. If the lot fails to meet the test requirements prescribed in paragraph (b)(2) of this section, the test may be repeated once using five other rabbits. The temperature rises recorded for all eight rabbits used in testing shall be included in determining whether the requirements are met. The lot meets the requirements for absence of pyrogens if not more than three of the eight rabbits show individual rises in temperature of 0.6 °C or more, and if the sum of the eight individual max-

imum temperature rises does not exceed 3.7 °C.

[38 FR 32056, Nov. 20, 1973, as amended at 40 FR 29710, July 15, 1975; 41 FR 10429, Mar. 11, 1976; 41 FR 41424, Sept. 22, 1976; 44 FR 40289, July 10, 1979; 46 FR 62845, Dec. 29, 1981; 49 FR 15187, Apr. 18, 1984; 50 FR 4134, Jan. 29, 1985; 55 FR 28381, July 11, 1990; 64 FR 56453, Oct. 20, 1999; 67 FR 9587, Mar. 4, 2002; 70 FR 14985, Mar. 24, 2005]

Appendix B2

International Organization for Standardization

ISO 10993-11 - Biological Evaluation of Medical Devices - Part 11: Tests for Systemic Toxicity (First Edition 1993-12-15)

This document is available for purchase at:

<http://www.iso.org/iso/store.htm>

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Appendix B3

U.S. Pharmacopeia (USP) 30-NF25

- B3-1 (85) - Bacterial Endotoxins Test**
- B3-2 (151) - Pyrogen Test**
- B3-3 (1041) - Biologics**

These documents provide a description of the respective biological test and are available for purchase at:

<http://www.usp.org/products>

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Appendix B4

European Pharmacopeia 5.0

- B4-1 2.6.8 - Pyrogens**
- B4-2 2.6.14 - Bacterial Endotoxins**

These documents provide a description of the respective biological test and are available for purchase at:

http://www.edqm.eu/site/Online_Publications-581.html

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Appendix B5

Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices (December 1987)

This document is available at:

<http://www.fda.gov/cder/guidance/old005fn.pdf>

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GUIDELINE ON
VALIDATION OF THE LIMULUS AMEBOCYTE LYSATE TEST
AS AN END-PRODUCT ENDOTOXIN TEST FOR HUMAN
AND ANIMAL PARENTERAL DRUGS, BIOLOGICAL PRODUCTS, AND
MEDICAL DEVICES

December 1987

Prepared by: Center for Drug Evaluation and Research
Center for Biologic Evaluation and Research
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Maintained by: Division of Manufacturing and Product Quality (HFN-320)
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INTRODUCTION

This guideline sets forth acceptable conditions for use of the Limulus Amebocyte Lysate test. It also describes procedures for using this methodology as an end-product endotoxin test for human injectable drugs (including biological products), animal injectable drugs, and medical devices. The procedures may be used in lieu of the rabbit pyrogen test.

For the purpose of this guideline, the terms "lysate" or "lysate reagent" refer only to Limulus Amebocyte Lysate licensed by the Center for Biologic Evaluation and Research. The term "official test" means that a test is referenced in a United States Pharmacopeia drug monograph, a New Drug Application, New Animal Drug Application or a Biological License.

I. BACKGROUND

In a notice of January 12, 1973 (38 FR 1404), FDA announced that Limulus Amebocyte Lysate (LAL), derived from circulating blood cells (amebocytes) of the horseshoe crab, (Limulus polyphemus), is a biological product. As such, it is subject to licensing requirements as provided in section 351 of the Public Health Service Act (42 U.S.C. 262). Since 1973, LAL has proved to be a sensitive indicator of the presence of bacterial endotoxins (pyrogens). Because of this demonstrated sensitivity, LAL can be of value in preventing the administration or use of products which may produce fever, shock, and death if administered to or used in humans or animals when bacterial endotoxins are present.

When the January 12, 1973 notice was published, available data and experience with LAL were not adequate to support its adoption as the final pyrogen test in place of the rabbit pyrogen test, which had been accepted and recognized for many years. In order to establish a data base and gain experience with the use of LAL, that notice permitted the introduction of LAL into the marketplace without a license. This was upon the condition that its use be limited to the in-process testing of drugs and other products, that the decision to use it be reached voluntarily by affected firms, and the labeling on LAL state that the test was not suitable as a replacement for the rabbit pyrogen test.

Since that time, production techniques have been greatly improved and standardized so that they consistently yield LAL with an endotoxin sensitivity over 100 times greater than originally obtained. Moreover, it is widely recognized that the LAL test is faster, more economical, and requires a smaller volume of product than does the rabbit pyrogen test. In addition, the procedure is less labor intensive than the rabbit test, making it possible to perform many tests in a single day.

In a notice published in the Federal Register of November 4, 1977 (42 FR 57749), FDA described conditions for the use of LAL as an end-product test for endotoxins in human biological products and medical devices. The notice stated further that the application of LAL testing to human drug products would be the subject of a future Federal Register publication.

The then Bureau of Medical Devices, now FDA's Center for Devices and Radiologic Health (CDRH), issued recommended procedures for the use of LAL testing as an end-product endotoxin test on March 26, 1979. These procedures were revised as a result of the comments received from interested parties .

As a direct result of CDRH's experience in approving petitions for the use of the LAL test in place of the rabbit pyrogen test, several procedures for using the LAL test have evolved and have been adopted for devices.

In the FEDERAL REGISTER of January 18, 1980 (45 FR 3668), FDA announced the availability of a draft guideline that set forth procedures for use of the LAL test as an end-product testing method for endotoxins in human and animal injectable drug products. This draft guideline was made

available to interested parties to permit manufacturers, especially those who had used the LAL test in parallel with the rabbit pyrogen test, to submit data that could be considered in the preparation of any final guideline.

In response to comments received on the January 18 draft guideline, FDA made several significant changes (i.e. Endotoxin limits changed and deletion of section on Absence of Non-endotoxin Pyrogenic Substances), and many minor editorial changes. The agency also determined that a single document should be made available covering all FDA regulated products that may be subject to LAL testing. Primarily because of the addition of biological products and medical devices to the guideline, the agency made, in the FEDERAL REGISTER of March 29, 1983 (43 FR 13096), another draft of the guideline available for public comment.

Based on the comments received on the March 29 draft guideline, FDA has made several changes in this final guideline. The comments used in support of these changes may be viewed at FDA's Dockets Management Branch, Room 4-62, 5600 Fishers Lane, Rockville, MD between 9 am and 4 pm Monday through Friday. Briefly, the significant changes made are:

- A. Inclusion of validation criteria for the chromogenic, endpoint-turbidimetric and kinetic-turbidimetric LAL techniques.
- B. Any technique (gel-clot, chromogenic or turbidimetric) can be used in testing a product for endotoxin. However, if a gel-clot lysate is used in a different technique the results must be interpreted using the criteria for the technique being used.
- C. Elimination of the requirement to test the sensitivity of a rabbit pyrogen testing colony.
- D. The Center for Devices and Radiological Health (CDRH) has adopted the USP Endotoxin Reference Standard and revised the limit expressions from ng/mL to EU/mL. The new limit for medical devices is 0.5 EU/mL except for devices in contact with cerebrospinal fluid for which the limit is 0.06 EU/mL. These limits for devices are equivalent to those for drugs for a 70 Kg man when consideration is given to the following:
 1. In the worst case situation, all endotoxin present in the combined rinsings of 10 devices could have come from just one device. A wide variation in bioburden is common to some devices.
 2. Published FDA studies indicate that less than half of added endotoxin is recovered from devices using a non-pyrogenic water rinse.
- E. The Center for Drug Evaluation and Research (CDER) has added a listing of the maximum doses per Kg per hour and the corresponding endotoxin limits for most of the aqueous injectable drugs and biologics currently on the market. This listing was added to promote uniformity among companies making the same product.

II. LEGAL EFFECT OF THE GUIDELINE

This guideline is issued under section 10.90(b) (21 CFR 10.90(b)) of FDA's administrative regulations, which provides for use of guidelines to outline procedures or standards of general applicability that are acceptable to FDA for a subject matter within its statutory authority. Although guidelines are not legal requirements, a person who follows an agency guideline may be assured that the procedures or standards will be acceptable to FDA. The following guideline has been developed to inform manufacturers of human drugs (including biologicals), animal drugs, and medical devices of procedures FDA considers necessary to validate the use of LAL as an end-product endotoxin test. A manufacturer who adheres to the guideline would be considered in compliance with relevant provisions of the applicable FDA current good manufacturing practice regulations (CGMP) for drugs and devices and other applicable requirements. As provided in 21 CFR 10.90(b), persons who use methods and techniques not provided in the guideline should be able to adequately assure, through validation, that the method or technique they use is adequate to detect the endotoxin limit for the product.

III. REGULATORY PROVISIONS THAT PERMIT INITIATION OF END-PRODUCT TESTING WITH LAL

The regulatory provisions that a firm must meet before using the LAL test as an end-product test are not the same for all categories of products because of the different applicable statutory provisions and regulations. These provisions are as follows:

- A. Human Drugs subject to New Drug Applications (NDAs) or Abbreviated New Drug Applications (ANDAs), Antibiotic Drug Applications, and animal drugs subject to New Animal Drug Applications (NADAs), and Abbreviated New Animal Drug Application.

For these classes of drugs, manufacturers are to submit a supplemental application to provide for LAL testing. However, under 21 CFR 314.70(c) for drugs for human use and 21 CFR 514.8(d)(3) for drugs for animal use various changes may be made before FDA approval. Under these sections changes in testing of a human or animal drug that give increased assurance that the drug will have the characteristics of purity it purports or is represented to possess should be placed into effect at the earliest possible time. Therefore, if a firm validates the LAL test for a particular drug product covered by a new drug application by the procedures in this guideline using a LAL reagent licensed by the Center for Biologic Evaluation and Research (OBER) for the technique being used, the change may be made concurrently with the submission of the supplement providing for it. The supplement should contain initial quality control data, inhibition/enhancement data and the endotoxin limit for the drug product.

- B. Biological products for human use.

Under 21 CFR 601.12 significant changes in the manufacturing methods of biological products are required to be reported to the agency and may not become effective until approved by the Director, OBER. Therefore, a manufacturer of a biological product shall obtain an approved amendment to its product license before changing to the use of LAL in an end-product test, irrespective of the validation procedure used.

- C. Drugs not subject to premarket approval.

A manufacturer of an injectable drug for human or animal use that is not subject to premarket approval would be able to use the LAL test as an end-product test for endotoxins without submitting any information to the agency. CGMPs require the manufacturer to have data on file to validate the use of the LAL test for each product for which it is being used.

- D. Medical Devices.

On the basis of extensive experience in review of LAL data on devices since November 1977, CDRH believes that the LAL test,

when validated according to this guideline, is at least equivalent to the rabbit pyrogen test as an end-product test for medical devices. A manufacturer labeling a device as non-pyrogenic must validate the LAL test for that device in the test laboratory to be used for end-product testing before using the LAL test as an end-product endotoxin test for any device.

The data discussed under Section V of this guideline may be expressed graphically or in tabular form and should be on file at the manufacturing site; no preclearance prior to use of the LAL test as an end-product test is required if it is used according to this FDA guideline. Voluntary submission of LAL validation and inhibition data obtained following issuance of this guideline will be accepted for CDRH review and comment.

When a manufacturer plans to use LAL test procedures that deviate significantly from the LAL guideline, a premarket notification under section 510(k) of the Federal Food, Drug, and Cosmetic Act (the Act) or a Premarket Approval Application (PMA) supplement under section 515 of the Act should be submitted. Significant deviations would include-- but not necessarily be limited to-- higher endotoxin concentration release criteria, sampling from fewer than three lots for inhibition/enhancement testing, lesser sensitivity to endotoxin, rabbit retest when the LAL method shows endotoxin above the recommended allowable endotoxin dose, and a device rinsing protocol resulting in greater dilution of endotoxin than that recommended in this guideline.

CDRH will also consider submissions in the form of a premarket notification or PMA supplement for another deviation from this draft guideline; process control of endotoxin contamination with reduced end-product testing, i.e., a decrease in the number of devices per lot undergoing end-product testing. The manufacturer must demonstrate adequate control of the production process by the use of routine checks for endotoxin at key stages of production except where it has been shown that no possibility of contamination exists.

To facilitate subsequent PMA review, providers of investigational devices subject to 21 CFR part 812 or 813 are encouraged to use this guideline when a non-pyrogenic device is to be manufactured.

IV. HUMAN AND ANIMAL DRUGS AND BIOLOGICAL PRODUCTS

GENERAL REQUIREMENT

Manufacturers shall use an LAL reagent licensed by CBER in all validation, in-process, and end-product LAL tests.

A. VALIDATION OF THE LAL TEST

Validation of the LAL test as an endotoxin test for the release of human and animal drugs includes the following: (1) initial qualification of the laboratory, and (2) inhibition and enhancement tests.

1. INITIAL QUALIFICATION OF THE LABORATORY

Various methodologies have been described for the detection of endotoxin, using limulus amoebocyte lysate. Currently, commercially available licensed lysates use the gel clot, chromogenic, endpoint-turbidimetric or kinetic-turbidimetric techniques. Other methods which have been reported show potential for increasing further the sensitivity of the LAL method.

Manufacturers should assess the variability of the testing laboratory before any official tests are performed. Each analyst using a single lot of LAL and a single lot of endotoxin should perform the test for confirmation of labeled LAL reagent sensitivity or of performance criteria. Appendix A gives the procedures and test criteria for the current licensed techniques.

2. INHIBITION AND ENHANCEMENT TESTING

The degree of product inhibition or enhancement of the LAL procedure should be determined for each drug formulation before the LAL test is used to assess the endotoxin content of any drug. All validation tests should be performed on undiluted drug product or on an appropriate dilution. Dilutions should not exceed the Maximum Valid Dilution (MVD) (see Appendix D). At least three production batches of each finished product should be tested for inhibition and enhancement.

a) GEL-CLOT TECHNIQUE

Inhibition/enhancement testing should be conducted according to the directions in the preparatory section of the USP Bacterial Endotoxins Test (see Appendix B). Briefly, the method involves taking a drug concentration containing varying concentrations of a standard endotoxin that bracket the sensitivity of the lysate and comparing it to a series of the same endotoxin concentrations in water alone. The drug product is "spiked" with endotoxin and then diluted with additional drug product (so that the drug concentration remains constant) to the same endotoxin concentrations in

water. Results of endotoxin determination in water and the drug product should fall within plus/minus a twofold dilution of the labeled sensitivity. If the undiluted drug product shows inhibition, the drug product can be diluted, not to exceed the MVD, with the same diluent that will be used in the release testing and the above procedure repeated. Negative controls (diluent plus lysate) should be included in all inhibition/enhancement testing.

b) CHROMOGENIC AND ENDPOINT-TURBIDIMETRIC TECHNIQUES

In inhibition/enhancement testing by these techniques, a drug concentration containing 4 lambda concentration of the RSE or CSE (lambda is equal to the lowest endotoxin concentration used to generate the standard curve) is tested in duplicate according to the lysate manufacturer's methodology. The standard curve for these techniques shall consist of at least four RSE or CSE concentrations in water that extend over the desired range. If the desired range is greater than one log, additional standards concentrations should be included. The standard curve must meet the criteria for linearity as outlined in Appendix A(2). The detected amount of endotoxin in the spiked drug must be within plus or minus 25% of the 4 lambda concentration for the drug concentration to be considered to neither enhance nor inhibit the assay. If the undiluted drug product shows inhibition, the drug product can be diluted, not to exceed the MVD, and the test repeated.

An alternate procedure may be performed as described above except the RSE/CSE standard curve is prepared in LAL negative drug product, i.e. no detectable endotoxin, instead of LAL negative water. The standard curve must meet the test for linearity, i.e. r equal to or greater than 0.980, and in addition the difference between the O.D. readings for the lowest and highest endotoxin concentrations must be greater than 0.4 and less than 1.5 O.D. units. If the standard curve does not meet these criteria, the drug product cannot be tested by the alternate procedure.

c) KINETIC-TURBIDIMETRIC TECHNIQUE

In inhibition/enhancement testing by this technique, a drug concentration containing 4 lambda concentration of the RSE or CSE (lambda is equal to the lowest endotoxin concentration used to generate the standard curve) is tested in duplicate according to the lysate manufacturer's methodology. The standard curve shall consist of at least four RSE or CSE concentrations. If the desired range is greater than one log, additional standard concentrations should be included. The standard curve must meet the criteria outlined in Appendix A(3). The calculated mean amount of endotoxin in the spiked drug product, when referenced to the standard curve, must be within plus or minus 25% to be considered to neither enhance nor inhibit the assay. If the undiluted drug product shows

inhibition or enhancement, the drug product can be diluted, not to exceed the MVD, and the test repeated.

An alternate procedure may be performed whereby the RSE/CSE standard curve is prepared in drug product or product dilution instead of water. The drug product cannot have a background endotoxin concentration of more than 10% (estimated by extrapolation of the regression line) of the lambda concentration (lambda equals the lowest concentration used to generate the standard curve). The standard curve must meet the test for linearity, i.e. r equal to or less than -0.980 , and in addition the slope of the regression must be less than -0.1 and greater than -1.0 . If the standard curve does not meet these criteria, the drug product cannot be tested by the alternate procedure.

In those instances when the drug is manufactured in various concentrations of active ingredient while the other components of the formulation remain constant, only the highest and lowest concentration need be tested. If there is a significant difference, i.e. greater than twofold, between the inhibition endpoints or if the drug concentration, per mL, in the test solutions is different, then each remaining concentrations should be tested. If the drug product shows inhibition or enhancement at the MVD, when tested by the procedures in the above sections, and is amenable to rabbit testing, then the rabbit test will still be the appropriate test for that drug. If the inhibiting or enhancing substances can be neutralized without affecting the sensitivity of the test or if the LAL test is more sensitive than the rabbit pyrogen test the LAL test can be used. For those drugs not amenable to rabbit pyrogen testing, the manufacturer should determine the smallest quantity of endotoxin that can be detected. This data should be submitted to the appropriate FDA Office for review.

The inhibition/enhancement tests must be repeated on one unit of the product if the lysate manufacturer is changed. If the lysate technique is changed, the inhibition and enhancement tests must be repeated using three batches. When the manufacturing process, the product formulation, the source of a particular ingredient of the drug formulation, or lysate lot is changed, the positive product control can be used to reverify the validity of the LAL test for the product. Firms that are obtaining an ingredient from a new manufacturer are encouraged to include as part of their vendor qualification the rabbit pyrogen test to determine that the ingredient does not contain non-endotoxin pyrogens.

B. Routine Testing of Drugs by the LAL Test.

End-product testing is to be based on data from the inhibition/enhancement testing as outlined in Section A(2). Samples, standards, positive product controls and negative controls should be tested at least in duplicate.

For the gel-clot technique, an endotoxin standard series does not have to be run with each set of tests if consistency of standard endpoints has been demonstrated in the test laboratory. It should be run at least once a day with the first set of tests and repeated if there is any change in lysate lot, endotoxin lot or test conditions during the day. An endotoxin standard series should be run when confirming end-product contamination. Positive product controls (two lambda concentration of standard endotoxin in product) must be positive. If your test protocols state that you are using the USP Bacterial Endotoxin Test, remember that it requires a standard series to be run with each test. The above deviation must be noted in your test protocol.

For the chromogenic and endpoint-turbidimetric techniques, an endotoxin standard series does not have to be run with each set of tests if consistency of standard curves has been demonstrated in the test laboratory. It should be run at least once a day with the first set of tests and repeated if there is any change in lysate lot, endotoxin lot or test conditions during the day. However, at least duplicates of a 4 lambda standard concentration in water and in each product (positive product control) must be included with each run of samples. The mean endotoxin concentration of the standard must be within plus/minus 25% of the actual concentration and the positive product control must meet the same criteria after subtraction of any endogenous endotoxin. An endotoxin standard series should be run when confirming end-product contamination. If the alternate procedure is used, a standard in product series must be conducted each time the product is tested.

For the kinetic-turbidimetric test, it is not necessary to run a standard curve each day or when confirming end product contamination if consistency of standard curves has been demonstrated in the test laboratory.. However, at least duplicates of a 4 lambda standard concentration in water and in each product (positive product control) must be included with each run of samples. The mean endotoxin concentration of the standard when calculated using an archived standard curve (See Appendix C), must be within plus/minus 25% of the actual concentration and the positive product control must meet the same criteria after subtraction of any endogenous endotoxin. If the alternate procedure is used, a standard in product series must be conducted each time the product is tested.

Before a new lot of lysate is used, the labeled sensitivity of the lysate or the performance criteria should be confirmed by the laboratory, using the procedures in Appendix A.

The sampling technique selected and the number of units to be tested should be based on the manufacturing procedures and the batch size. A minimum of three units, representing the beginning, middle, and end, should be tested from a lot. These units can be run individually or pooled. If the units are pooled and any endotoxin is detected, repeat testing can be performed. The LAL test may be repeated no more than twice. The first repeat consists of twice the initial number of replicates of the sample in question to examine the possibility that extrinsic contamination occurred in the initial

assay procedure. On pooled samples, if any endotoxin is detected in the first repeat, proceed to second repeat. The second repeat consists of an additional 10 units tested individually. None of the 10 units tested in the second repeat may contain endotoxin in excess of the limit concentration for the drug product.

The following should be considered the endotoxin limit for all parenteral drugs to meet if the LAL test is to be used as an end-product endotoxin test:

1. K/M: For any parenteral drug except those administered intrathecally, the endotoxin limit for endotoxin is defined as K/M, which equals the amount of endotoxin (EU) allowed per ng or mL of product. K is equal to 5.0 EU/Kg. (SEE appendix D for definition of M).

For parenteral drugs that have an intrathecal route of administration, K is equal to 0.2 EU/Kg.

Drugs exempted from the above endotoxin limits are:

1. Compendial drugs for which other endotoxin limits have been established.
2. Non-compendial drugs covered by new drug applications, antibiotic drug applications, new animal drug applications, and biological product licenses where different limits have been approved by the agency.
3. Investigational drugs or biologicals for which an IND or INAD exemption has been filed and approved.
4. Drugs or biologicals which cannot be tested by the LAL method.

A batch which fails a validated LAL release test should not be retested by the rabbit test and released if it passes. Due to the high variability and lack of reproducibility of the rabbit test as an endotoxin assay procedure, we do not consider it an appropriate retest procedure for LAL failures.

V. MEDICAL DEVICES

General Requirements

The CDRH has reviewed the results of the "HIMA Collaborative Study for the Pyrogenicity Evaluation of a Reference Endotoxin by the USP Rabbit Test." This study recommends 0.1 ng/mL (10 mL/kg) of E. coli 055:B5 endotoxin from Difco Laboratories as the level of endotoxin which should be detectable in the LAL test when used for end-product testing of medical devices. This sensitivity (0.1 ng/mL given 10 mL/kg) is sufficient for LAL testing and for retest of devices in rabbits. According to recent collaborative studies in the rabbit pyrogen and LAL tests, one nanogram of E. coli 055:B5 endotoxin is similar in potency to 5 EU of the USP Endotoxin Reference Standard. The endotoxin limit for medical devices has been converted to EU and is now 0.5 EU/mL using the rinse volume recommended in Section 2 below. Liquid devices should be more appropriately validated and tested according to the requirements for drugs by taking the maximum human dose per kilogram of body weight per hour into consideration (See Section IV,B).

Manufacturers may retest LAL test failures with the LAL test or a USP rabbit pyrogen test. If the endotoxin level in a device eluate has been quantitated by LAL at 0.5 EU/mL endotoxin or greater, then retest in rabbits is not appropriate. Medical devices that contact cerebrospinal fluid should have less than 0.06 EU/mL of endotoxin. These values correspond to those set by the CDER for intrathecal drugs.

Manufacturers shall use an LAL reagent licensed by OBRR in all validation, in-process, and end-product LAL tests.

A. Validation of the LAL Test

1. Sensitivity: Data demonstrating the sensitivity and reproducibility of the LAL test.
2. Inhibition/Enhancement Testing: Each product line of devices utilizing different materials or methods of manufacture should be checked for inhibition or enhancement of the LAL test.

Further explanation of the above points is given as follows:

1. SENSITIVITY

A manufacturer must be able to demonstrate a sensitivity of at least 0.5 EU/mL. The level of endotoxin selected as the pass/fail point for evaluating pyrogenicity of products using the LAL test must be equivalent to or below this level. Manufacturers may use another endotoxin if a reproducible correlation between it and the USP Reference Endotoxin Standard has been demonstrated in their laboratory (see appendix C).

The sensitivity of the LAL technique used should be determined by the procedures and criteria in Appendix A. Routine performance of the LAL test should include standards (run in duplicate) and a negative control. An endotoxin standard series is useful for checking lysate sensitivity and the competence of the technician, and for identifying other problems such as the contamination of glassware.

The stability of the endotoxin standards and appropriate storage conditions should also be considered; dilute endotoxin solutions are not as stable as more concentrated solutions under certain conditions.

2. INHIBITION AND ENHANCEMENT TESTING

Lack of product inhibition or enhancement of the LAL test should be shown for each type of device before use of the LAL test. Possible inhibition of different chemical components of similar devices should be considered. A manufacturer may logically divide its device products into groups of products according to common chemical formulation; and may then qualify only a representative product from each such group. Ideally, the product chosen from each group would be the one with the largest surface area contacting body or fluid for administration to a patient.

At least three production lots of each product type should be tested for inhibition. In general, use of the sampling technique selected should result in a random sampling of a finished production lot. CDRH recommends testing 2 devices for lot sizes under 30, 3 devices for lot sizes 30-100, and 3 percent of lots above size 100, up to a maximum of 10 devices per lot.

The process of preparing an eluate/extract for pyrogen or inhibition/enhancement testing may vary for each device. Some medical devices can be flushed, some may have to be immersed in the non-pyrogenic rinse solution, while others may be tested by disassembling or by cutting the device into pieces prior to extraction by immersion. In general, for devices being flushed, the non-pyrogenic rinse solution should be held in the fluid pathway for one hour at room temperature (above 18° C); effluents should be combined. If a device is to undergo extraction, a minimum extraction time should be 15 minutes at 37° C, one hour at room temperature (above 18° C) or other demonstrated equivalent conditions.

Guidelines for rinse volumes include the following:

- a. Each of the 10 test units should be rinsed with 40 mL of non-pyrogenic water.

- b. For unusually small or large devices, the surface area of the device which comes in contact with the patient may be used as an adjustment factor in selecting the rinsing or extracting volume. The endotoxin limit can be adjusted accordingly.

The rinsing scheme should not result in a greater dilution of endotoxin than used in USP rabbit pyrogen testing of transfusion and infusion assemblies. For inhibition/enhancement testing, both the rinsing/extraction solution and the device eluate/extract should be tested as prescribed below under the specific technique being used.

a) GEL-CLOT TECHNIQUE

In inhibition/enhancement testing, a device eluate/extract containing varying concentrations of a standard endotoxin that bracket the sensitivity of the lysate is compared with a series of the same endotoxin concentrations in water alone. The device eluate/extract is "spiked" with endotoxin and then diluted with additional eluate/extract to the same endotoxin concentrations as in the water series. Results of endotoxin determination in water and the device product eluate/extract should fall within plus/minus a twofold dilution of the labeled sensitivity. If the device eluate/extract shows inhibition, the gel-clot technique cannot be used to test the device. Negative controls (diluent plus lysate) should be included in all inhibition/enhancement testing.

b) CHROMOGENIC AND ENDPOINT-TURBIDIMETRIC TECHNIQUES

In inhibition/enhancement testing by these techniques, a device eluate/extract containing 4 lambda concentration of the RSE or CSE (lambda is equal to the lowest endotoxin concentration used to generate the standard curve) is tested in duplicate according to the lysate manufacturer's methodology. The standard curve for these techniques shall consist of at least four RSE or CSE concentrations in water that extend over the desired range. If the desired range is greater than one log, additional standard concentrations should be included. The standard curve must meet the criteria for linearity as outlined in Appendix A(2). The detected amount of endotoxin in the spiked eluate/extract must be within plus or minus 25% of the 4 lambda concentration for the device to be considered to neither enhance nor inhibit the assay. If the device eluate/extract shows inhibition, the device cannot be tested by this technique.

An alternate procedure may be performed as described above except the RSE/CSE standard curve is prepared in LAL negative device eluate/extract, i.e. no detectable endotoxin, instead of LAL negative water. The standard curve must meet the test for linearity, i.e. r equal to or greater than 0.980, and in addition the difference between

the O.D. readings for the lowest and highest endotoxin concentrations must be greater than 0.4 and less than 1.5 O.D. units. If the standard curve does not meet these criteria the device cannot be tested by the alternate procedure.



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

July 15, 1991

Food and Drug Administration
Rockville, MD 20857

Interim Guidance for Human and Veterinary Drug Products and Biologicals

KINETIC LAL TECHNIQUES

Until we update the guideline the following guidance and the lysate manufacturers approved procedures can be used. The kinetic LAL techniques should be done according to the lysate manufacturers recommended procedures, i.e., sample/lysate ratio, incubation temperature and times, measurement wavelength, etc. Instrumentation other than the one recommended by lysate manufacturer can be used. The performance characteristics (slope, γ -intercept and correlation coefficient), for the lysate lot, sent by the manufacturer will not be valid. New performance characteristic have to be established for each lot by performing the procedures outlined in Appendix A.

INHIBITION/ENHANCEMENT TESTING

In inhibition/enhancement testing of a product by kinetic techniques, test a drug concentration containing a quantity of the RSE or CSE between 0.1 and 0.5 EU/mL or 1.0 and 5.0 EU/mL depending on its Pass/Fail Cutoff (PFC) in duplicate according to the lysate manufacturer's methodology. The 4 lambda spike procedure, in the current guideline, is still valid and can be used in the kinetic techniques. This procedure should be used with caution if lambda is less than 0.01 EU/mL.

The Pass/Fail Cutoff equals the endotoxin limit of the product solution (EU/mL) times the potency of the product divided by the product dilution used for the test. For PFCs less than or equal to 1.0 EU/mL the endotoxin spike should be between 0.1 and 0.5 EU/mL, otherwise the endotoxin spike should be between 1.0 and 5.0 EU/mL.

The standard curve shall consist of at least three RSE or CSE concentrations. Additional standards should be included to bracket each log increase in the range of the standard curve so that there is at least one standard per log increment of the range. The standard curve must meet the criteria outlined in Appendix A. The calculated mean amount of endotoxin when referenced to the standard curve, minus any measurable endogenous endotoxin in the spiked drug product, must be within plus or minus 50% of the known spike concentration to be considered to neither enhance or inhibit the assay. If there is no measurable endogenous endotoxin in the product the value will usually be equal to or less than plus or minus 25% of the standard curve value. If the undiluted drug product shows inhibition or enhancement, the drug product can be diluted, not exceeding the MVD, and test repeated.

An alternate procedure may be used, in which the RSE/CSE standard is prepared in drug product or product dilution instead of water. The drug product (at the concentration used to prepare the standard curve), cannot have an endotoxin concentration greater than the lowest concentration used to generate the product standard curve, when referenced against a standard curve prepared in water. The product standard curve must meet the test for linearity, i.e., r equal to or greater than the absolute value of 0.980, and slope of the regression line must be less than -0.1 and greater than -1.0. If the standard curve does not meet these criteria, the drug product cannot be tested by the alternate procedure.

ROUTINE TESTING

The standard curve shall consist of at least three RSE or CSE concentrations in duplicate. Additional standards should be included to bracket each log increase in the range of the standard curve so that there is at least one standard per log increment of the range. The standard curve must meet the criteria outlined in Appendix A. For the kinetic techniques, it is not necessary to run a standard

curve each day if consistency of standard curves is shown in your test laboratory. Determine consistency by regression analysis of the data points from the standard curves generated over three consecutive test days (minimum of three curves). If the coefficient of correlation, r , meets the criteria in Appendix A then consistency is proven and the curve becomes the "archived curve." If r does not meet the criteria then consistency in your laboratory has not been shown and you cannot use an archived curve in routine testing. The archived curve is only valid for a lysate/endotoxin lot combination. If you use an archived standard curve, at least duplicates of a standard endotoxin concentration, equal to the mid-point on a log basis, between the endotoxin concentration of the highest and lowest standards in the standard curve, in water must be included with each run of samples. The mean endotoxin concentration of this standard control must be within plus/minus 25% of the standard curve concentration when calculated using the archived standard curve. Independent of using an endotoxin standard curve, at least duplicates of a standard endotoxin in each product or product dilution (positive product control), equal to either 0.1 - 0.5 or 1.0 - 5.0 EU/mL depending on its PFC or 4 lambda, must be included with each run of samples. The mean endotoxin concentration of the positive product control when referenced to the standard curve must be within plus/ minus 50% of the known concentration after subtraction of any endogenous endotoxin. An endotoxin standard series should be run when retesting to determine if end-product endotoxin contamination exceeds product limit. If you use the alternate procedure, a standard curve prepared in product must be conducted with each product test.

APPENDIX A

Using a RSE or CSE of known potency, in endotoxin units, assay at least 3 concentrations in triplicate that extend over the desired endotoxin range. Additional standards should be included to bracket each log increase in the range of the standard curve so that there is at least one standard per log increment of the range. Do regression - correlation analysis on the log Reaction Time versus the log of the endotoxin concentration for each replicate. DO NOT AVERAGE THE REACTION TIMES OF REPLICATES OF EACH STANDARD BEFORE PERFORMING REGRESSION-CORRELATION ANALYSIS.

The coefficient of correlation, r , shall be greater than or equal to the absolute value of 0.980. If r is less than the absolute value of 0.980 the cause of the non-linearity should be determined and test repeated.

VI. APPENDICES

APPENDIX B

BACTERIAL ENDOTOXINS TEST

United States Pharmacopeia XXI/National Formulary XVI
and
First Supplement to USP XXI/NF XVI

(85) BACTERIAL ENDOTOXINS TEST

This chapter provides a test for estimating the concentration of bacterial endotoxins that may be present in or on the sample of the article(s) to which the test is applied using Limulus Amebocyte Lysate (LAL) which has been obtained from aqueous extracts of the circulating amoebocytes of the horseshoe crab, *Limulus polyphemus*, and which has been prepared and characterized for use as a LAL reagent for gel-clot formation.

Where the test is conducted as a limit test, the specimen is determined to be positive or negative to the test judged against the endotoxin concentration specified in the individual monograph. Where the test is conducted as an assay of the concentration of endotoxin, with calculation of confidence limits of the result obtained, the specimen is judged to comply with the requirements if the result does not exceed (a) the concentration limit specified in the individual monograph, and (b) the specified confidence limits for the assay. In either case the determination of the reaction end-point is made with dilutions from the material under test in direct comparison with parallel dilutions of a reference endotoxin, and quantities of endotoxin are expressed in defined Endotoxin Units.

Since LAL reagents have also been formulated to be used for turbidimetric (including kinetic assays) or colorimetric readings,

such tests may be used if shown to comply with the requirements for alternative methods. These tests require the establishment of a standard regression curve and the endotoxin content of the test material is determined by interpolation from the curve. The procedures include incubation for a pre-selected time of reacting endotoxin and control solutions with LAL Reagent and reading of the spectrophotometric light absorbance at suitable wavelengths. In the case of the turbidimetric procedure the reading is made immediately at the end of the incubation period, or in the kinetic assays, the absorbance is measured throughout the reaction period and rate values are determined from those readings. In the colorimetric procedure the reaction is arrested at the end of the pre-selected time by the addition of an appropriate amount of acetic acid solution, prior to the readings. A possible advantage in the mathematical treatment of results, if the test be otherwise validated and the assay suitably designed, could be the application of tests of assay validity and the calculation of the confidence interval and limits of potency from the internal evidence of each assay itself (see *Design and Analysis of Biological Assays* (111)).

Reference Standard and Control Standard Endotoxins

The reference standard endotoxin (RSE) is the USP Endotoxin Reference Standard which has a defined potency of 10,000 USP Endotoxin Units (EU) per vial. Constitute the entire contents of 1 vial of the RSE with 5 mL of LAL Reagent Water,¹ vortex for not less than 20 minutes,¹ and use this concentrate for making appropriate serial dilutions. Preserve the concentrate in a refrigerator, for making subsequent dilutions, for not more than 14 days. Allow it to reach room temperature, if applicable, and vortex it vigorously for not less than 5 minutes before use. Vortex each dilution for not less than 1 minute before proceeding to make the next dilution.¹ Do not use stored dilutions. A control standard endotoxin (CSE) is an endotoxin preparation other than the RSE that has been standardized against the RSE. If a CSE is a preparation not already adequately characterized, its evaluation should include characterizing parameters both for endotoxin quality and performance (such as reaction in the rabbit), and for suitability of the material to serve as a reference (such as uniformity and stability). Detailed procedures for its weighing and/or constitution and use to assure consistency in performance should also be included.¹ Standardization of a CSE against the RSE using a LAL Reagent for the gel-clot procedure may be effected by assaying a minimum of 4 vials of the CSE or 4 corresponding aliquots, where applicable, of the bulk CSE and 1 vial of the RSE, as directed under *Test Procedure*, but using 4 replicate reaction tubes at each level

¹ LAL Reagent Water—Sterile Water for Injection or other water that shows no reaction with the specific LAL Reagent with which it is to be used, at the limit of sensitivity of such reagent.

of the dilution series for the RSE and 4 replicate reaction tubes similarly for each vial or aliquot of the CSE. If all of the dilutions for the 4 vials or aliquots of the CSE cannot be accommodated with the dilutions for the 1 vial of the RSE on the same rack for incubation, additional racks may be used for accommodating some of the replicate dilutions for the CSE, but all of the racks containing the dilutions of the RSE and the CSE are incubated as a block. However, in such cases, the replicate dilution series from the 1 vial of the RSE are accommodated together on a single rack and the replicate dilution series from any one of the 4 vials or aliquots of the CSE are not divided between racks. The antilog of the difference between the mean \log_{10} end-point of the RSE and the mean \log_{10} end-point of the CSE is the standardized potency of the CSE which then is to be converted to and expressed in Units per ng under stated drying conditions for the CSE, or in Units per container, whichever is appropriate. Standardize each new lot of CSE prior to use in the test. Calibration of a CSE in terms of the RSE must be with the specific lot of LAL Reagent and the test procedure with which it is to be used. Subsequent lots of LAL Reagent from the same source and with similar characteristics need only checking of the potency ratio. The inclusion of one or more dilution series made from the RSE when the CSE is used for testing will enable observation of whether or not the relative potency shown by the latter remains within the determined confidence limits.¹ A large lot of a CSE may, however, be characterized by a collaborative assay of a suitable design to provide a representative relative potency and the within-laboratory and between-laboratory variance.¹

A suitable CSE has a potency of not less than 2 Endotoxin Units per ng and not more than 50 Endotoxin Units per ng, where in bulk form, under adopted uniform drying conditions, e.g., to a particular low moisture content and other specified conditions of use, and a potency within a corresponding range where filled in vials of a homogeneous lot.

Preparatory Testing

Use a LAL reagent of confirmed label or determined sensitivity. In addition, where there is to be a change in lot of CSE, LAL Reagent or another reagent, conduct tests of a prior satisfactory lot of CSE, LAL and/or other reagent in parallel on changeover. Treat any containers or utensils employed so as to destroy extraneous surface endotoxins that may be present, such as by heating in an oven at 250° or above for sufficient time.²

The validity of test results for bacterial endotoxins requires an adequate demonstration that specimens of the article, or of solutions, washings, or extracts thereof to which the test is to be applied do not of themselves inhibit or enhance the reaction or otherwise interfere with the test. Validation is accomplished by testing untreated specimens or appropriate dilutions thereof, concomitantly with and without known and demonstrable added amounts of RSE or a CSE, and comparing the results obtained. Appropriate negative controls are included. Validation must be repeated if the LAL Reagent source or the method of manufacture or formulation of the article is changed.

Test for confirmation of labeled LAL Reagent sensitivity—Confirm¹ the labeled sensitivity of the particular LAL reagent with the RSE (or CSE) using not less than 4 replicate vials, under conditions shown to achieve an acceptable variability of the test, viz., the antilog of the geometric mean \log_{10} lysate gel-clot sensitivity is within 0.5λ to 2.0λ , where λ is the labeled sensitivity in Endotoxin Units per mL.¹ The RSE (or CSE) concentrations selected in

confirming the LAL reagent label potency should bracket the stated sensitivity of the LAL reagent. Confirm the labeled sensitivity of each new lot of LAL reagent prior to use in the test.

Inhibition or Enhancement Test—Conduct assays with standard endotoxin, of untreated specimens in which there is no endogenous endotoxin detectable, and of the same specimens to which endotoxin has been added, as directed under *Test Procedure*, but using not less than 4 replicate reaction tubes at each level of the dilution series for each untreated specimen and for each specimen to which endotoxin has been added. Record the end-points (E , in Units per mL) observed in the replicates. Take the logarithms (e) of the end-points, and compute the geometric means of the log end-points for the RSE (or CSE), for the untreated specimens and for specimens containing endotoxin by the formula $\Sigma e/f$, in which Σe is the sum of the log end-points of the dilution series used and

² For a test for validity of procedure for inactivation of endotoxins, see "Dry-heat Sterilization" under *Sterilization and Sterility Assurance of Compendial Articles* (1211). Use a LAL Reagent having a sensitivity of not less than 0.15 Endotoxin Unit per mL.

f is the number of replicate end-points in each case. Compute the amount of endotoxin in the specimen to which endotoxin has been added. The test is valid for the article if this result is within twofold of the known added amount of endotoxin. Alternatively, if the test has been appropriately set up, the test is valid for the article if the geometric mean end-point dilution for the specimen to which endotoxin has been added is within one 2-fold dilution of the corresponding geometric mean end-point dilution of the standard endotoxin.

If the result obtained for the specimens to which endotoxin has been added is outside the specified limit, the article is unsuitable for the *Bacterial Endotoxins Test*, or, in the case of Injections or solutions for parenteral administration, it may be rendered suitable by diluting specimens appropriately.

Repeat the test for inhibition or enhancement using specimens diluted by a factor not exceeding that given by the formula x/λ (see *Maximum Valid Dilution*, below). Use the least dilution sufficient to overcome the inhibition or enhancement of the known added endotoxin, for subsequent assays of endotoxin in test specimens.

If endogenous endotoxin is detectable in the untreated specimens under the conditions of the test, the article is unsuitable for the *Inhibition or Enhancement Test*, or, it may be rendered suitable by removing the endotoxin present by ultra-filtration, or by appropriate dilution. Dilute the untreated specimen (as constituted, where applicable, for administration or use), to a level not exceeding the maximum valid dilution, at which no endotoxin is detectable. Repeat the test for *Inhibition or Enhancement* using the specimens at those dilutions.

Test Procedure

In preparing for and applying the test, observe precautions in handling the specimens in order to avoid gross microbial contamination. Washings or rinsings of devices must be with LAL Reagent Water in volumes appropriate to their use and, where applicable, of the surface area which comes into contact with body tissues or fluids. Use such washings or rinsings if the extracting fluid has been in contact with the relevant pathway or surface for not less than 1 hour at controlled room temperature (15° to 30°). Such extracts may be combined, where appropriate. The ultimate rinse or wash volume is such as to result in possible dilution of any contained endotoxin to a level not less than that suitable for use in the *Pyrogen Test* (151) under *Transfusion and Infusion Assemblies* (161).

For validating the test for an article, for endotoxin limit tests or assays, or for special purposes where so specified, testing of specimens is conducted quantitatively to determine response end-points for gel-clot readings. Usually graded strengths of the specimen and standard endotoxin are made by multifold dilutions. Select dilutions so that they correspond to a geometric series in which each step is greater than the next lower by a constant ratio. Do not store diluted endotoxin, because of loss of activity by adsorption. In the absence of supporting data to the contrary, negative and positive controls are incorporated in the test.

Use not less than 2 replicate reaction tubes at each level of the dilution series for each specimen under test. Whether the test is employed as a limit test or as a quantitative assay, a standard endotoxin dilution series involving not less than 2 replicate reaction tubes is conducted in parallel. A set of standard endotoxin dilution series is included for each block of tubes, which may consist of a number of racks for incubation together, provided the environmental conditions within blocks are uniform.

Preparation—Since the form and amount per container of

standard endotoxin and of LAL reagent may vary, constitution and/or dilution of contents should be as directed in the labeling. The pH of the test mixture of the specimen and the LAL Reagent is in the range 6.0 to 7.5 unless specifically directed otherwise in the individual monograph. The pH may be adjusted by the addition of sterile, endotoxin-free sodium hydroxide or hydrochloric acid or suitable buffers to the specimen prior to testing.

Maximum Valid Dilution (MVD)—The Maximum Valid Dilution is appropriate to Injections or to solutions for parenteral administration in the form constituted or diluted for administration, or where applicable, to the amount of drug by weight if the volume of the dosage form for administration could be varied. Where the endotoxin limit concentration is specified in the individual monograph in terms of volume (in EU per mL), divide the limit by λ , which is the labeled sensitivity (in EU per mL) of the lysate employed in the assay, to obtain the MVD factor. Where the endotoxin limit concentration is specified in the individual monograph in terms of weight or of Units of active drug (in EU per mg or in EU per Unit), multiply the limit by the concentration (in mg per mL or in Units per mL) of the drug in the solution tested or of the drug constituted according to the label instructions, whichever is applicable, and divide the product of the multiplication by λ , to obtain the MVD factor. The MVD factor so obtained is the limit dilution factor for the preparation for the test to be valid.

Procedure—To 10- X 75-mm test tubes add aliquots of the appropriately constituted LAL reagent, and the specified volumes of specimens, endotoxin standard, negative controls, and a positive product control consisting of the article, or of solutions, washings or extracts thereof to which the RSE (or a standardized CSE) has been added at a concentration of endotoxin of 2λ for that LAL reagent (see under *Test for confirmation of labeled LAL Reagent sensitivity*). Swirl each gently to mix, and place in an incubating device such as a water bath or heating block, accurately recording the time at which the tubes are so placed. Incubate each tube, undisturbed, for 60 ± 2 minutes at $37 \pm 1^{\circ}$, and carefully remove it for observation. A positive reaction is characterized by the formation of a firm gel that remains when inverted through 180° . Record such a result as positive (+). A negative result is characterized by the absence of such a gel or by the formation of a viscous gel that does not maintain its integrity. Record such a result as negative (-). Handle the tubes with care, and avoid subjecting them to unwanted vibrations, or false negative observations may result. The test is invalid if the positive product control or the endotoxin standard does not show the end-point concentration to be within ± 1 twofold dilutions from the label claim sensitivity of the LAL Reagent or if any negative control shows a gel-clot end-point.

Calculation and Interpretation

Calculation—Calculate the concentration of endotoxin (in Units per mL or in Units per g or mg) in or on the article under test by the formula pS/U , in which S is the antilog of the geometric mean \log_{10} of the end-points, expressed in Endotoxin Units (EU) per mL for the Standard Endotoxin. U is the antilog of $\Sigma e/f$, where e is the \log_{10} of the end-point dilution factors, expressed in decimal fractions. f is the number of replicate reaction tubes read at the end-point level for the specimen under test, and p is the correction factor for those cases where a specimen of the article cannot be taken directly into test but is processed as an extract, solution, or washing.

Where the test is conducted as an assay with sufficient replication to provide a suitable number of independent results, calculate for each replicate assay the concentration of endotoxin in or on the article under test from the antilog of the geometric mean log end-point ratios. Calculate the mean and the confidence limits from the replicate logarithmic values of all the obtained assay results by a suitable statistical method (see *Calculation of Potency from a Single Assay* (111)).

Interpretation—The article meets the requirements of the test if the concentration of endotoxin does not exceed that specified in the individual monograph, and where so specified in the individual monograph or in this chapter, the confidence limits of the assay do not exceed those specified.

APPENDIX C

DETERMINATION OF THE RELATIONSHIP BETWEEN THE
CONTROL STANDARD ENDOTOXIN (CSE) AND THE REFERENCE
STANDARD ENDOTOXIN (RSE)

If a manufacturer chooses to use an endotoxin preparation (CSE) other than the United States Pharmacopeia Reference Standard Endotoxin (RSE), the CSE will have to be standardized against the RSE. If the CSE is not a commercial preparation which has been adequately characterized, it should be studied and fully characterized as to uniformity, stability of the preparation, etc. The relationship of the CSE to the RSE should be determined prior to use of a new lot, sensitivity, or manufacturer of the LAL or a new lot source or manufacturer of the CSE.

A. GEL-CLOT TECHNIQUE

The following is an example of a procedure to determine the relationship of the CSE to the RSE:

At least 4 samples (vials) for the lot of CSE should be assayed. State in ng/mL the endpoint for the CSE and in EU/mL of the RSE. The values obtained should be the geometric mean of the endpoints using a minimum of 4 replicates.

Example: LAL endpoints for the RSE and CSE are as follows:

RSE = 0.3 EU/mL
CSE = 0.018 ng/mL

The EUs per ng of CSE are calculated as follows:

$$\frac{\text{RSE}}{\text{CSE}} = \frac{0.3 \text{ EU/mL}}{0.018 \text{ ng/mL}} = 16.7 \text{ EU/ng}$$

This indicates that 0.018 ng of the CSE is equal to 0.3 EU of the RSE. Thus, the CSE contains 16.7 EU/ng.

B. CHROMOGENIC AND ENDPOINT-TURBIDIMETRIC TECHNIQUES

At least 4 samples (vials) for the lot of CSE should be assayed. In addition to a water blank, assay dilutions of RSE which fall in the linear range and dilutions of the CSE. Linear regression analysis is performed on the absorbance values of the RSE standards (y-axis) versus their respective endotoxin concentrations (x-axis). Calculate the EU/ng of the CSE by inserting the average CSE O.D. readings for each concentration which falls in the RSE standard range into the RSE straight line equation. The resulting CSE values (in EU) are then divided by their corresponding concentrations (in ng/mL). These values are then averaged to obtain the potency of the CSE lot.

EXAMPLE:

RSE Standard Curve

	Concentration	O.D.
RSE (EU/mL)	0.1	0.11
	0.25	0.26
	0.5	0.49
	1.0	1.06

y-intercept = -0.008 slope = 1.056 r = 0.999

Straight Line Equation (Y) = -0.008 + (1.056 * X)

CSE Standard Curve

CSE Conc. (ng/mL)	AVERAGE O.D.	Corresponding RSE (EU/mL)	EU/ng (RSE/CSE)
0.01	0.12	0.119	11.9
0.025	0.31	0.301	12.0
0.05	0.60	0.626	12.5
0.1	1.23	1.291	12.9

Mean EU/ng = 12.3

G. KINETIC-TURBIDIMETRIC TECHNIQUE

In order to assign EUs to a CSE, the following should be performed on 4 vials from the same CSE lot.

Twofold dilutions of the RSE should be made in the range of 1.0 EU/mL to 0.03 EU/mL. Determine the Time of Reaction (T) for at least duplicates of each standard concentration. Construct a standard curve (Log₁₀ T versus Log₁₀ endotoxin concentration (E)). Calculate the mean T for 1.0 and 0.03 EU/mL. These T's define the RSE standard range.

For each of the four vials of CSE make twofold dilutions such that the T values for at least 3 concentrations of the CSE are within the RSE standard range. Determine the T values for at least duplicates of each endotoxin concentration. Calculate the EU/ng of CSE by inserting the log mean CSE T values for each endotoxin concentration which falls in the RSE standard range into the RSE straight line equation. The resulting CSE values (in EU) are then divided by their corresponding concentrations (in ng/mL). These values are averaged to obtain the potency of the CSE lot.

EXAMPLE:

RSE Standard Curve

Straight Line Equation (Y) = 3.03 + (-0.181 * X)
 RSE Standard Range = 1037 - 2235 seconds (17.3-37.3 minutes)

CSE Standard Curve

Vial	Endotoxin Concentration(ng/mL)					
	0.1	0.05	0.025	0.0125	0.006	0.003
1	1018.8	1114	1218.6	1402.7	1548.7	1740.7
2	990.7	1090.6	1249.8	1406.4	1586.0	1780.0
3	998.2	1116.8	1227.8	1411.0	1554.1	1800.9
4	1003.4	1086.1	1198.5	1415.6	1593.9	1781.0

Note: Each T in the above table is expressed in seconds and represents the mean of at least duplicate determinations.

Mean T (sec.)	1002.8*	1101.9	1223.7	1408.9	1570.7	1775.7
Log mean T	3.001	3.042	3.088	3.149	3.196	3.249

Calculations:

Solving for EU/mL equivalent by substituting onset times generated with CSE (ng/mL) into the above RSE standard line equation, $X = (Y - 3.03)/-0.181$ where Y = log mean onset time and X = log EU/ml equivalent.

CSE Endo. Conc. (ng/mL)	Log Mean T	EU/mL Equivalent (RSE Std. Line)		EU/ng
		Log	Antilog	
0.1*	3.001	0.16	1.45	14.5
0.05	3.042	-0.066	0.859	17.2
0.025	3.088	-0.32	0.479	19.2
0.0125	3.149	-0.657	0.22	17.6
0.006	3.196	-0.917	0.121	20.2
0.003	3.249	-1.210	0.062	20.6

Mean EU/ng = 19.0 (SD = 1.52)

* Outside the RSE standard range - not used in calculation of mean.

The values for the y-intercept and slope of the four CSE curves used for the EU/ng determination may be stored for use in routine testing (archived standard curve) instead of running a series of standards each day. Using the EU/ng conversion factor, CSE standards within the range of the RSE curve can be made up in endotoxin units. Standards outside this range require the use of RSE and a new RSE standard curve. If CSE standards outside the RSE standard range are required the EU/ng conversion factor must be determined for the new range as described above.

APPENDIX D

MAXIMUM VALID DILUTION

To determine how much the product can be diluted and still be able to detect the limit endotoxin concentration, the following two methods will determine the Maximum Valid Dilution:

METHOD I

This method is used when there is an official USP limit or when the limits listed in Appendix E are used.

$$MVD = \frac{\text{Endotoxin Limit} \times \text{Potency of Product}}{\lambda}$$

For drugs administered on a weight-per-kilogram basis, the potency is expressed as mg or units/mL and for drugs administered on a volume-per-kilogram basis, the potency is equal to 1.0 mL/mL.

METHOD II

This method is used when there is no official USP limit and the limits listed in Appendix E are not used.

Step 1. Minimum Valid Concentration (MVC)

$$MVC = \frac{\lambda M}{K}$$

Where:

λ = GEL CLOT: Labeled sensitivity-EU/mL.
 CHROMOGENIC, TURBIDIMETRIC and KINETIC-TURBIDIMETRIC:
 The lowest point used in the standard curve.

M = Rabbit Dose or Maximum Human Dose/Kg of body weight that would be administered in a single one hour period, whichever is larger. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/Kg at the product expiration date or time. Use 70 Kg as the weight of the average human when calculating the maximum human dose per Kg. Also, if the pediatric dose/Kg is higher than the adult dose then it shall be the dose used in the formula.

K = 5.0 EU/Kg for parenteral drugs except those administered intrathecally; 0.2 EU/Kg for intrathecal drugs

APPENDIX D (cont.)

Step 2. Maximum Valid Dilution (MVD)

$$MVD = \frac{\text{Potency of Product}}{MVC}$$

For drugs administered on a weight-per-kilogram basis, the potency is expressed as mg or units/mL, and for drugs administered on a volume-per-kilogram, the potency is equal to 1.0 mL/mL.

METHOD I EXAMPLES

Endotoxin Limit Expressed by Weight:

Product: Cyclophosphamide Injection
 Potency: 20 mg/mL
 Lysate Sensitivity (λ): 0.065 EU/mL
 Endotoxin Limit (Appendix E): 0.17 EU/mg

$$MVD = \frac{0.17 \text{ EU/mg} \times 20 \text{ mg/ml}}{0.065 \text{ EU/mL}} = \frac{3.4}{0.065} = 1:52.3 \text{ or } 1:52$$

Endotoxin Limit Expressed by Volume:

Product: 5% Dextrose Injection
 Lysate Sensitivity (λ): 0.065 EU/mL
 Endotoxin Limit (Appendix E): 0.5 EU/mL

$$MVD = \frac{0.5 \text{ EU/mL} \times 1 \text{ mL/mL}}{0.065 \text{ EU/mL}} = \frac{0.5}{0.065} = 1: 7.7$$

METHOD II EXAMPLES

PARENTERAL DRUGS EXCEPT INTRATHECAL

Drug Administered on a Weight-per-Kilogram Basis

Product: Cyclophosphamide Injection
 Potency: 20 mg/mL
 Maximum Dose/Kg (M): 30 mg/Kg
 Lysate Sensitivity (λ): 0.065 EU/mL

$$MVC = \frac{\lambda \times M}{K} = \frac{0.065 \text{ EU/mL} \times 30 \text{ mg/Kg}}{5.0 \text{ EU/Kg}} = 0.390 \text{ mg/mL}$$

$$MVD = \frac{\text{Potency of Product}}{MVC} = \frac{20 \text{ mg/mL}}{0.390 \text{ mg/mL}} = 1:51.2 \text{ or } 1:51$$

APPENDIX D (cont.)

Drug Administered on a Volume-per-Kilogram Basis

Product: 5% Dextrose in Water
 Maximum Dose/Kg (M): 10.0 mL/Kg
 Lysate Sensitivity (λ): 0.065 EU/mL

$$MVC = \frac{\lambda M}{K} = \frac{0.065 \text{ EU/mL} \times 10.0 \text{ mL/Kg}}{5.0 \text{ EU/Kg}} = 0.13 \text{ mL/mL}$$

$$MVD = \frac{\text{Potency of Product}}{MVC} = \frac{1.0 \text{ mL/mL}}{0.13 \text{ mL/mL}} = 1:7.7$$

INTRATHECAL DRUGS

Drug Administered on a Weight-per-Kilogram Basis

Product: Gentamicin Sulfate
 Potency: 2.0 mg/mL
 Maximum Dose/Kg (M): 0.11 mg/Kg
 Lysate Sensitivity (λ): 0.1 EU/mL

$$MVC = \frac{\lambda M}{K} = \frac{0.1 \text{ EU/mL} \times 0.11 \text{ mg/Kg}}{0.2 \text{ EU/Kg}} = 0.055 \text{ mg/mL}$$

$$MVD = \frac{\text{Potency of Product}}{MVC} = \frac{2.0 \text{ mg/mL}}{0.055 \text{ mg/mL}} = 1:36.4$$

Drug Administered on a Volume-per-Kilogram Basis

Product: Lidocaine Hydrochloride Injection
 Maximum Dose/Kg (M): 0.057 mL/Kg
 Lysate Sensitivity (λ): 0.1 EU/mL

$$MVC = \frac{\lambda M}{K} = \frac{0.1 \text{ EU/mL} \times 0.057 \text{ mL/Kg}}{0.2 \text{ EU/Kg}} = 0.0285 \text{ mL/mL}$$

$$MVD = \frac{\text{Potency of Product}}{MVC} = \frac{1.0 \text{ mL/mL}}{0.0285 \text{ mL/mL}} = 1:35.0$$

APPENDIX E

April ,1992

MAXIMUM DOSE AND ENDOTOXIN LIMIT TABLE

Drug Name	Dose (M)	Endotoxin Limit (EU/mg,ml,units of product)
-A-		
Acetic Acid Irrigation	10.00 mL	0.50 +
Acetazolamide Sodium	10.00 mg	0.50 +
Acetylcysteine Injection	150.00 mg	0.03
Acyclovir Sodium	30.00 mg	0.17
Adenosine Phosphate	0.71 mg	7.04
Albumin,Normal Human Serum (25%)	3.00 mL	1.67
Albumin,Normal Human Serum (20%)	3.75 mL	1.33
Albumin,Normal Human Serum (5%)	10.00 mL	0.50
Albuterol Sulfate	0.008 mg	625.00
Alcohol and Dextrose Injection	1.79 mL	2.70
Alfentanil Hydrochloride	250.00 mcg	0.02
Alkaloids of Belladonna	0.007 mg	714.29
Alpha ₁ -Proteinase Inhibitor	60.00 mg	0.08
Alphaprodine HCl Injection	0.60 mg	8.33
Alprostadil (Postaglandin)	6.00 mcg	0.83
Alteplase	1.25 mg	4.00
Amdinocillin	10.00 mg	0.50 +
Amikacin Sulfate Injection	15.00 mg	0.33 +
Amino Acid Injection	25.00 mg	0.20
Amino Acids and Electrolytes	25.00 mg	0.20
Essential Amino Acids and Dextrose	25.00 mg	0.20
Aminocaproic Acid Injection	100.00 mg	0.05
Aminohippurate Sodium Injection	125.00 mg	0.04 +
Aminophylline Injection	5.00 mg	1.00
Amitriptyline HCl Injection	0.42 mg	12.00
Ammonia N 13 Injection	7.00 mL	25.00 +
Ammonium Chloride Injection	2.90 mEq Cl	1.72
Amobarbital Sodium	14.30 mg	0.40 +
Amoxicillin, Sterile and Suspension	20.00 mg	0.25 +

Amphotericin B for Injection	1.00 mg	5.00
*Amphotericin B for Injection	0.01 mg	20.00
Ampicillin Sodium	33.30 mg	0.15 +
Ampicillin and Sulbactam	28.60 mg	0.17
Amrinone Lactate	10.00 mg	0.50
Anileridine	0.70 mg	7.20 +
Anticoagulant Heparin Solution	2.00 mL	2.50
Anticoagulant, Citrate Dextrose Sol.	--- mL	5.56
Anticoagulant, Citrate Phosphate Dextrose	--- mL	5.56
Anticoagulant, Citrate Phosphate Dextrose Adenine Solution	--- mL	5.56
Antihemophilic Factor	10.00 units	0.50
Antihemophilic Plasma(1 hr. at 56-57oC)	3.00 mL	1.67
Antirabies Serum	3.00 mL	1.67
Antitoxin (Gas Gangrene)	3.00 mL	1.67
Antivenom	3.00 mL	1.67
Apommorphine HCl Tablets for Injection	0.09 mg	55.56
Arginine HCl Injection	500.00 mg	0.01
Ascorbic Acid	4.20 mg	1.20 +
Asparaginase for Injection	1000.00 IU	0.01 #
Atracurium Besylate	0.50 mg	10.00
Atropine Sulfate	0.09 mg	55.60 +
Aurothioglucose Suspension	0.70 mg	7.14
Azathioprine Sodium for Injection	5.00 mg	1.00 +
Azlocillin	75.00 mg	0.07 +
Aztreonam for Injection	28.60 mg	0.17 +

-B-

Bacitracin	500.00 units	0.01 +
Bacitracin Zinc	500.00 units	0.01
Benzquinamide HCl	1.00 mg	5.00
Benztropine Mesylate Injection	0.09 mg	55.60 +
Benzylpenicilloyl Polylysine	0.004 mL	1250.00
Betamethasone Acetate and Betamethasone Sodium Phosphate	0.17 mg	29.20 +
Betamethasone Sodium Phosphate	0.17 mg	29.20 +
Betazole HCl Injection	2.86 mg	1.75
Bethanechol Chloride	0.20 mg	25.00
Biperiden Lactate Injection	0.06 mg	83.30 +

Bleomycin Sulfate	0.50 unit	10.00 #+
Bretylium Tosylate Injection	25.00 mg	0.20
Bretylium Tosylate in Dextrose	25.00 mg	0.20
Brompheniramine Maleate Injection	0.14 mg	35.71
Bumetanide	0.01 mg	500.00 +
Bupivacaine Hydrochloride Injection	2.50 mg	2.50 +
Bupivacaine Hydrochloride and Epinephrine Injection	3.20 mg	1.60 +
Bupivacaine HCl and Dextrose	0.11 mg	1.80 +
Buprenorphine HCl	0.004 mg	1250.00
Butorphanol Tartrate	0.057 mg	88.00 +

-C-

Caffeine Citrated	20.00 mg	0.25
Caffeine and Sodium Benzoate	7.14 mg	0.70 +
Calcitonin - Human	0.007 mg	714.30
Calcitonin - Salmon	4.00 IU	1.25
Calcitriol	0.05 mcg	100.00
Calcium Ascorbate	14.30 mg	0.35
Calcium Chloride	25.00 mg	0.20 +
Calcium Disodium Edetate	35.00 mg	0.143
Calcium Gluceptate Injection	15.70 mg	0.32 +
Calcium Gluconate	28.60 mg	0.17 +
Calcium Glycerophosphate and Calcium lactate	1.43 mg	3.50
Calcium Levulinate	0.14 mL	35.70 +
Capreomycin Sulfate	14.30 mg	0.35 +
Carbazochrome Salicylate	0.14 mg	34.96
Carbenicillin Disodium	100.00 mg	0.05 +
Carboplatin	9.26 mg	0.54 #
Carboprost Tromethamine	0.007 mcg	714.30 +
Carmustine for Injection	5.14 mg	1.00 #
Cefamandole Nafate and Sodium	33.30 mg	0.15 +
Cefazolin Sodium	33.30 mg	0.15 +
Cefmetazole Sodium	--- mg	0.20 +
Cefonicid Sodium	14.30 mg	0.35 +
Cefoperazone Sodium	28.57 mg	0.20 +
Ceforanide	20.00 mg	0.25 +
Cefotaxime Sodium	28.50 mg	0.20 +
Cefotetan Disodium	28.60 mg	0.17

Cefoxitin Sodium	40.00 mg	0.13 +
Ceftazidime	50.00 mg	0.10 +
Ceftizoxime Sodium	50.00 mg	0.10 +
Ceftriaxone Sodium	28.60 mg	0.20 +
Cefuroxime Sodium	50.00 mg	0.10 +
Cephacetrile Sodium for Injection	80.00 mg	0.06
Cephaloridine	14.30 mg	0.35
Cephalothin Sodium Injection	60.00 mg	0.08 +
Cephapirin Sodium	28.60 mg	0.17 +
Cephradine for Injection	25.00 mg	0.20 +
Cerulitide diethylamine	0.30 mcg	16.67
Chloramphenicol Sodium Succinate	25.00 mg	0.20 +
Chlordiazepoxide HCl	1.40 mg	3.57 +
Chloroprocaine HCl	11.43 mg	0.45
Cholecystokinin	1.00 IDU	5.00
Chorionic Gonadotropin	142.90 units	0.03 +
Chlormerodrin Hg197 Injection	7.00 mL	25.00 +
Chlormerodrin Hg203 Injection	7.00 mL	25.00 +
Chlormerodrine	1.40 mg	3.57
Chloroquine HCl Injection	7.50 mg	0.70 +
Chloroprocaine HCl	20.00 mg	0.25
Chloroprocaine HCl - Epinephrine	20.00 mg	0.25
Chlorothiazide Sodium	15.00 mg	0.30 +
Chlorpheniramine Maleate	0.57 mg	8.80 +
Chlorpromazine HCl	0.72 mg	6.90 +
Chlorprothixene Injection	0.72 mg	6.90 +
Chlortetracycline HCl	5.00 mg	1.00 +
Chromate Sodium Cr51 Injection	7.00 mL	25.00 +
Chromic Chloride Injection	0.30 ug	16.70
Chromic Phosphate P32 Suspension	7.00 mL	25.00 +
Chymopapain	42.90 pKat	0.12
Chymotrypsin	4.30 units	1.16
Sterile Cilastatin Sodium	--- mg	0.23 +
Cimetidine HCl Injection	10.00 mg	0.50
Cisplatin for Injection	2.57 mg	1.90 #
Citric Acid, Magnesium Oxide, & Sodium Carbonate Irrigation	--- mL	0.50 +
Citrate, Phosphate, Dextrose, Adenine	0.90 mL	5.56
Clindamycin Phosphate Injection	8.60 mg	0.58 +
Cloxacillin	12.50 mg	0.40 +
Codeine Phosphate Injection	0.86 mg	5.80 +

Colchicine Injection	0.03 mg	166.70 +
Colistimethate Sodium	2.50 mg	2.00 +
Conjugated Estrogens	0.36 mg	13.89
Corticotropin, Gel, Zinc & Re.	1.60 units	3.10 +
Cortisone Acetate	5.00 mg	1.00
Cosyntropin	3.57 mcg	1.40
Cryptenamine Acetate	1.86 CSR units	2.69
Cupric Chloride Injection	0.02 mg	250.00 +
Cupric Sulfate Injection	0.02 mg	250.00 +
Cyanocobalamine and Repository	14.30 mcg	0.40 +
Cyclizine Lactate	1.00 mg	5.00
Cyclophosphamide	30.00 mg	0.20 #+
Cyclosporine Injection and Conc.	0.12 mL	42.00 +
Cysteine HCl	7.14 mg	0.70 +
Cytarabine	3.00 mg	0.07 +
*Cytarabine	1.93 mg	0.10 #

-D-

Dacarbazine for Injection	9.60 mg	0.52 #
Dactinomycin for Injection	0.05 mg	100.00 #+
Dantrolene Sodium	10.00 mg	0.50
Daunorubicin HCl	1.16 mg	4.30 #
Decamethonium Bromide	0.043 mg	116.30
Deferoxamine Mesylate	15.00 mg	0.33
Dehydrocholate Sodium Injection	150.00 mg	0.04
Deslanoside	0.03 mg	167.00
Desmopressin Acetate	0.30 mcg	16.70
Desoxycorticosterone Acetate	0.07 mg	71.40 +
Desoxycorticosterone Pivalate Sus.	1.80 mg	2.78 +
Dexamethasone Acetate Suspension	0.23 mg	21.74 +
Dexamethasone Sodium Phosphate	0.16 mg	31.30 +
Dexpanthenol	7.10 mg	0.70
Dextran 40	5.00 mL	1.00
Dextran 40 in Sodium Chloride	5.00 mL	1.00
Dextran 70	10.00 mL	0.50
Dextrose < 5%	10.00 mL	0.50
Dextrose- 5%-70%	0.50 gm	10.00
Dextrose and Sodium Chloride	0.50 gm	10.00
Dezocine	0.29 mg	17.24

Diatrizoate Meglumine Injection	60%	1.00 mL	5.00
	30%	4.40 mL	1.10
Diatrizoate Meglumine			
and Diatrizoate Sodium	66% - 10%	2.30 mL	2.17
	60% - 30%	1.40 mL	3.57
	52% - 8%	2.80 mL	1.80
	50% - 25%	2.80 mL	1.80
	34.3% - 35%	2.80 mL	1.80
	28.5% - 29.1%	2.80 mL	1.80
Diatrizoate Meglumine			
and Iodipamide Meglumine		0.11 mL	45.45
Diatrizoate Sodium	50%	1.00 mL	5.00
	25%	4.00 mL	1.25
	20%	0.90 mL	5.56
Diazepam Injection		0.43 mg	11.60 +
Diazoxide Injection		10.00 mg	0.50 +
*Dibucaine		0.14 mg	35.70 +
Dibucaine HCl and Dextrose		0.07 mg	71.43
Dicloxacillin Sodium		0.29 mg	16.70 +
Dicyclomine HCl Injection		0.29 mg	17.20 +
Diethylstilbestrol Injection		7.14 mg	0.70 +
Diethylstilbestrol Diphosphate		7.14 mg	0.70 +
Digitoxin Injection		0.045 mg	111.00 +
Digoxin Injection		0.025 mg	200.00 +
Digoxin Immune Fab		5.00 mg	1.00
Dihydroergotamine Mesylate		0.014 mg	357.00
Dihydroergotamine Mesylate, Heparin			
Sodium & Lidocaine HCl		1667.00 units	0.003 +
		(Heparin)	
Dihydrostreptomycin Sulfate		10.00 mg	0.50 +
Dihydrotachysterol		0.03 mg	166.67
Diluent for Meningococcal Vaccine		5.00 mL	1.00
Dimenhydrinate Injection		1.25 mg	4.00
Dimethyl Sulfoxide Irrigation		10.00 mL	0.50 +
Dimercaprol		5.00 mg	1.00
Dinoprost Tromethamine		0.57 mg	8.77
Diphenhydramine HCl Injection		1.50 mg	3.40 +
Diphenidol		0.30 mg	16.67
Diphtheria Antitoxin, Pur. Conc. (equine)		3.00 mL	1.67
Dipyridamole		0.14 mg	37.70
Dobutamine HCl		0.90 mg	5.56

Dopamine HCl	0.30 mg	16.67 +
Dopamine HCl in Dextrose	0.30 mg	16.67 +
Doxapram HCl Injection	1.50 mg	3.30 +
Doxorubicin HCl for Injection	1.93 mg	2.20 #+
Doxycycline Hyclate for Injection	4.40 mg	1.14 +
Dromostanolone Propionate	1.40 mg	3.57
Droperidol	0.14 mg	35.70
Dyphylline Injection	7.10 mg	0.70 +

-E-

Edetate Calcium Disodium	50.00 mg	0.10 +
Edetate Disodium	25.00 mg	0.20
Edrophonium Chloride Injection	0.60 mg	8.33 +
Electrolyte Solutions- LVP	10.00 mL	0.50
Multiple Electrolytes Type 1 & 2	--.-- mL	0.50
Multiple Electrolytes and Invert Sugar Type 1,2, and 3	--.-- mL	0.50
Multiple Electrolytes and Dextrose Type 1,2,3,and 4	--.-- mL	0.50
Emetine HCl	0.93 mg	5.40 +
Enalaprilat	0.018 mg	280.00
Ephedrine Sulfate Injection	3.00 mg	1.70 +
Epinephrine Injection	0.014 mg	357.00 +
Epinephrine Suspension	0.025 mg	200.00
Ergocalciferol (D2)	142.80 units	0.035
Ergoloid Mesylates	0.004 mg	1250.00
Ergonovine Maleate	6.00 mcg	0.80
Ergotamine Tartrate	0.014 mg	357.00 +
Erythromycin Gluceptate/Lactobionate	5.00 mg	1.00 +
Esmolol	0.50 mg	10.00
Estradiol (aqueous)	0.02 mg	250.00 +
Estrogens (Combined) Aqueous	0.026 mg	192.31
	Estrone	
Estrogens Conjugated	0.36 mg	14.00
Estrogenic Substances or Estrogens	0.057 mg	88.00
Estrone Aqueous Suspension	0.057 mg	88.00 +
Ethacrynate Sodium	1.00 mg	5.00 +
Ethamivan Injection	1.40 mg	3.60
Ethylnorepinephrine HCl Injection	0.029 mg	172.40 +

Etidocaine HCl	5.50 mg	0.90
Etidocaine HCl and Epinephrine	5.50 mg	0.08
Etidronate Disodium	7.50 mg	0.67
Etomidate Injection	0.60 mg	8.35
Etoposide Injection	2.57 mg	1.95 #
Evans Blue Injection	0.36 mg	14.00 +

-F-

Factor IX	50.00 units	0.10
Famotidine	0.30 mg	16.67
Fat Emulsion	(10%) 3.20 mL	1.56
	(20%) 1.60 mL	3.13
Fentanyl Citrate	0.10 mg	50.00
Fentanyl Citrate and Droperidol	0.004 mg	1250.00
	Fentanyl	
Ferrous Citrate Fe59 Injection	7.00 mL	25.00 +
Fibrinogen	30.00 mg	0.17
Fibrinogen, Dried	30.00 mg	0.17
Fibrinolysin and Desoxyribonuclease	1.00 units	5.00
Flurodopa F 18 Injection	7.00 mL	25.00 +
Floxuridine	0.60 mg	8.33 #
Fluorodeoxyglucose F18	7.00 mL	25.00
Fluorescein Sodium Injection	10.70 mg	0.47
Fluorouracil Injection	15.00 mg	0.33 #
Fluphenazine HCl	0.03 mg	166.67
Flupenthixol Decanoate	0.57 mg	8.77
Folate Sodium	0.01 mg	500.00
Folic Acid Injection	0.014 mg	357.10 +
Fructose	10.00 mL	0.50 +
Fructose and Sodium Chloride	10.00 mL	0.50 +
Furosemide Injection	1.40 mg	3.60 +

-G-

Gallamine Triethiodide	1.00 mg	5.00 +
Gallium Citrate Ga67 Injection	7.00 mL	25.00 +
Gentamicin Sulfate	3.00 mg	1.70 +
*Gentamicin Sulfate	0.11 mg	45.46
Globulins (Humans)	1.00 mL	5.00
Glucagon for Injection	0.04 units	125.00

Glycine Irrigation	--- mL	0.50 +
Glycopyrrolate	0.009 mg	555.50 +
Gold Au198 Injection	7.00 mL	25.00 +
Gold Sodium Thiomalate Injection	1.00 mg	5.00
Gonadorelin HCl	1.40 mcg	3.60

-H-

Haloperidol, Decanoate and Lactate	0.07 mg	71.40 +
Hemin for Injection	4.00 mg	1.25
Heparin Sodium and Calcium	143.00 units	0.03
Heparin Sodium Injection	143.00 units	0.03
Heparin Lock Flush Solution	10.00 mL	0.50
Heparin and Sodium Chloride	10.00 mL	0.50
Hetacillin Potassium	--- mg	0.30 +
Hetastarch	20.00 mL	0.25
Hexafluorenum Bromide Injection	0.60 mg	8.35
Histamine Phosphate	0.04 mg	125.00 +
Hyaluronate Sodium	0.071 mg	70.42
Hyaluronidase Injection and for Injection	2.14 units	2.30 +
Hydralazine HCl Injection	3.50 mg	1.45 +
Hydrocortisone Suspension	4.00 mg	1.25 +
Hydrocortisone Acetate	1.07 mg	4.67
Hydrocortisone Sodium Phosphate	4.00 mg	1.25 +
Hydrocortisone Sodium succinate	4.00 mg	1.25 +
Hydromorphone HCL	0.057 mg	88.00 +
Hydroxocobalamin	14.30 mcg	0.40 +
Hydroxyprogesterone Caproate	14.30 mg	0.35
Hydroxystilbamidine Isethionate	4.50 mg	1.10 +
Hydroxyzine HCl Injection	1.40 mg	3.60 +
Hyocyanine Sulfate	0.007 mg	714.30 +
Hyocyanine Sulfate and Scopolamine	0.007 mg	714.29

-I-

Idarubicin HCl Injection	0.31 mg	16.13 #
Ifosfamide	30.86 mg	0.16 #+
Imipenem	--- mg	0.23 +
Imipenem and Cilastatin	7.14 mg	0.70
Imipramine HCl Injection	1.00 mg	5.00 +

Immune Serum Globulin	5.50 mL	0.91
Indigotindisulfonate Sodium Injection	1.00 mL	5.00 +
Indium In111 Oxyquinoline	7.00 mL	25.00 +
*Indium Pentetate In111 Injection	0.50 mL	28.00 +
Indium Chlorides In113m Injection	2.00 mL	87.50 +
Indocyanine Green	0.70 mg	7.10 +
Indomethacin Sodium	0.20 mg	25.00
Insulin	2.00 units	2.50
Insulin Human	- - -	0.80 +
Interferon Alfa-n1	77142.00 units	0.65/10,000 #
Interferon Alfa-n3	3571.00 units	0.14/100 #
Interferon Alfa - 2a	428571.00 units	0.10/10,000 #
Interferon Alfa - 2b	514285.00 units	0.10/10,000 #
Inulin	50.00 mg	0.10 +
Invert Sugar	2.38 mL	2.10
Iodamide meglumine - 24%	4.30 mL	1.20
Iodide Sodium I123 Solution	7.00 mL	25.00 +
Iodinated I125 Albumin Injection	7.00 mL	25.00 +
Iodide Sodium I125 Solution	7.00 mL	25.00 +
Iodinated I131 Albumin Injection	7.00 mL	25.00 +
Iodinated I131 Albumin Aggregated Injection	7.00 mL	25.00 +
Iodohippurate Sodium I131 Injection	7.00 mL	25.00 +
Rose Bengal Sodium I131 Injection	7.00 mL	25.00 +
Iodide Sodium I131 Solution	7.00 mL	25.00 +
Iodipamide Meglumine Injection - 52%	0.60 mL	8.33
- 10.5%	1.40 mL	3.60 +
Iodipamide Meglumine - Diatrizoate meglumine	0.14 mL	35.71
*Iohexol	43.70 mg I	0.11
*Iopamidol	40.00 mg I	0.6 +
*Iophendylate Injection	0.22 mL	0.90 +
Iothalamate Meglumine Injection	80%- 1.40 mL	3.57
	60%- 2.00 mL	2.50
	43%- 5.70 mL	0.90 +
	30%- 4.30 mL	1.16
	17.2% -5.70 mL	0.90
Iothalamate Meglumine - Iothalamate Sodium 52%-26%	1.50 mL	3.35 +
Iothalamate Sodium	66.8% - 1.50 mL	3.35 +
	54.3% - 0.90 mL	5.56
Ioxaglate Meglumine	3.00 mL	1.67

Ioxaglate Sodium	3.00 mL	1.67
Iron Dextran Injection	0.90 mg	5.60
Iron Sorbitex	0.50 mL	10.00 +
Isobucaine HCl and Epinephrine	0.14 mL	35.70
Isoniazid	20.00 mg	0.30 +
Isoproterenol HCl Injection	0.014 mg	350.00
Isosulfan Sulfate	0.71 mg	7.00
Isoxsupine HCl Injection	0.14 mg	35.70 +

-K-

Kanamycin Sulfate Injection	7.50 mg	0.67 +
Ketamine HCl	13.00 mg	0.40 +

-L-

Labetalol HCl	4.30 mg	1.20 +
Leucovorin Calcium Injection	2.57 mg	1.95
Leuprolide Acetate	0.03 mg	16.67
Levallorphan Tartrate Injection	0.04 mg	125.00
Levarterenol	0.06 mg	83.33
Levorphanol Tartrate Injection	0.04 mg	125.00 +
Levothyroxine Sodium for Injection	0.007 mg	714.00
Lidocaine HCl Injection (with D5W)	4.50 mg	1.10 +
Lidocaine HCl with Epinephrine	7.00 mg	0.70 +
Lincomycin HCl	10.00 mg	0.50 +
Liver Derivative Complex	0.03 mg	166.67
Lorazepam	0.05 mg	100.00 +
Loxapine	0.71 mg	7.00

-M-

Magnesium Sulfate	57.10 mg	0.09 +
Manganese Chloride Injection	11.00 ug	0.45 +
Manganese Sulfate	11.00 ug	0.45 +
Mannitol <= 10%	120.00 mg	0.04
Mannitol > 10%	2.00 g	2.50
Mannitol and Sodium Chloride	120.00 mg	0.04 +
Mechlorethamine HCl for Injection	0.40 mg	12.50 #+
Medroxyprogesterone Acetate	14.30 mg	0.35
Menadiol Sodium Diphosphate (K-4)	0.20 mg	25.00 +

Menadione	0.09 mg	58.30 +
N. Meningococcal Polysaccharide Pur. Bulk, Group A	0.25 ug	20.00
N.Meningococcal Polysaccharide Pur. Bulk, Group C	0.25 ug	20.00
Meningococcal Polysaccharide Vaccine Group A	0.025 ug	200.00
Meningococcal Polysaccharide Vaccine Group C	0.025 ug	200.00
Meningococcal Polysaccharide Vaccine Group A and C	0.05 ug	100.00
Menotropin	2.00 units	2.50 +
Meperidine HCl Injection	2.14 mg	2.40 +
Mephentermine Sulfate	0.64 mg	7.80 +
Mepivacaine HCl	6.60 mg	0.80 +
Mepivacaine HCl and Levonordefrin	6.60 mg	0.80 +
Meprobamate Injection	1.00 mg	5.00
Meprylcaine HCl and Epinephrine	6.60 mg	0.80 +
Mercaptomerin Sodium	3.57 mg	1.40
Mersalyl with theophylline	2.90 mg	1.72
Merethoxylline Procaine	2.90 mg	1.72
Mesoridazine Besylate Injection	0.71 mg	7.00 +
Metaraminol Bitartrate	1.43 mg	3.50 +
Methadone HCl	0.57 mg	8.80 +
Methandroil	1.43 mg	3.50
Methapyrilene HCl	0.60 mg	8.33
Methicillin Sodium	50.00 mg	0.10 +
Methiodal Sodium Injection	1300.00 mg	0.004
Methocarbamol Injection	28.60 mg	0.20 +
Methohexital Sodium	10.00 mg	0.50 +
Methotrexate Sodium Injection	--- mg	2.00 +
Methotrimerprazine	0.28 mg	17.90 +
Methoxamine HCl	0.25 mg	20.00 +
Methyldopate HCl	10.00 mg	0.50 +
Methylene Blue Injection	2.00 mL	2.50 +
Methylergonovine Maleate	2.90 mcg	1.70 +
Methylprednisolone Acetate Sus	0.80 mg	6.25
Methylprednisolone Sodium Succinate for Injection	30.00 mg	0.17 +
Metoclopramide	2.00 mg	2.50 +
Metocurine Iodide	0.40 mg	12.50 +

Metoprolol Tartrate	0.20 mg	25.00 +
*Metrizamide	4.29 mg I	1.17
Metrizamide	634.00 mg I	0.008
Metrizoic	73% - 2.90 mL	1.72
	46.18% - 1.00 mL	5.00
Metronidazole HCl	15.00 mg	0.35 +
Metyrapone Tartrate Injection	15.00 mg	0.35
Mezlocillin Sodium	87.50 mg	0.06 +
Miconazole Injection	40.00 mg	0.10 +
*Miconazole Injection	0.29 mg	0.69
Midazolam HCl	0.35 mg	14.30
Minocycline HCl	4.00 mg	1.25 +
Mithramycin for Injection	0.05 mg	100.00
Mitomycin for Injection	0.51 mg	9.80 # +
Mitoxantrone HCl	0.36 mg	13.90 #
Molybdenum	2.30 ug	2.17
Morphine Sulfate	0.29 mg	17.00
*Morphine Sulfate	0.014 mg	14.29
Morrhuate Sodium	3.60 mg	1.40 +
Moxalactam	100.00 mg	0.05 +
Muromonab-CD3	0.10 mg	50.00

-N-

Nafcillin	40.00 mg	0.13 +
Nalbuphine HCl	3.00 mg	1.67
Nalorphine HCl	0.43 mg	11.60
Naloxone HCl Injection	0.01 mg	500.00
Neomycin Sulfate	3.80 mg	1.30 +
Neostigmine Methylsulfate	0.04 mg	125.00
Netilmicin	4.00 mg	1.25 +
Niacin	1.43 mg	3.50 +
Niacinamide Injection	1.43 mg	3.50 +
Nicotinamide	0.70 mg	7.14
Nikethamide	0.90 ml:25% sol	5.56
Nine Vitamin Injection	1.00 mL	5.00
Nitrofurantoin	2.50 mg	2.00
Nitroglycerin	50.00 ug	0.10 +
Nitroprusside Sodium	1.40 mcg	3.67
Norepinephrine bitartrate	0.06 mg	83.40 +
Novobiocin for Injection	7.10 mg	0.70

-O-

Opium Alkaloids HCl	0.28 mg	17.86
Orphenadrine Citrate Injection	0.86 mg	5.80 +
Ouabain	0.007 mg	714.29
Oxacillin Sodium	25.00 mg	0.20 +
Oxymorphone HCl	0.021 mg	238.10 +
Oxytetracycline	12.50 mg	0.40 +
Oxytocin	0.14 units	35.70

-P-

Pancuronium Bromide	0.10 mg	50.00
Papaverine HCl	1.70 mg	2.90 +
Paraldehyde	0.15 mL	33.33
Parathyroid Hormone	0.57 units	8.80
Penicillin G Benzathine Suspension	50,000.00 units	0.01/100 +
Penicillin G Potassium	50,000.00 units	0.01/100 +
Penicillin G Procaine and Suspension	50,000.00 units	0.01/100 +
Penicillin G Procaine, Dihydrostreptomycin Sulfate, Chlorpheniramine Maleate and Dexamethasone Suspension	--- units	0.01/100 +
Penicillin G Procaine, Dihydrostreptomycin Sulfate, Prednisolone Suspension	--- units	0.01/100 +
Penicillin G Sodium	50,000.00 units	0.01/100 +
Pentagastrin	0.006 mg	833.00
Pentamidine Isethionate	4.00 mg	1.25
Pentobarbital Sodium Injection	6.00 mg	0.83 +
Pentazocine Lactate Injection	0.86 mg	5.80 +
Perphenazine	0.14 mg	35.70 +
Phenobarbital Sodium Injection	20.00 mg	0.30 +
Phenolsulfonphthalein	0.09 mg	55.60
Phentolamine Mesylate	0.86 mg	5.80 +
Pentylene-tetrazol	7.14 mg	0.70
Phenylephrine HCl	0.20 mg	25.00 +
Phenytoin Sodium Injection	20.00 mg	0.30 +
Physostigmine Salicylate	0.06 mg	83.40 +
Phytonadione	0.36 mg	14.00 +
Piperacillin Sodium	75.00 mg	0.07 +
Piperocaine HCl	4.30 mg	1.16

Plasma Protein Fraction (5%)	10.00 mL	0.50
Plicamycin for Injection	0.05 mg	100.00 #
Polyestradiol Phosphate	1.10 mg	4.55
Polymyxin B Sulfate	1666.70 units	0.003
*Polymyxin B Sulfate	714.00 units	0.03/100
		units
Posterior Pituitary Injection	0.29 units	17.00 +
Potassium Acetate Injection	0.57 mEq	8.80 +
Potassium Chloride	0.57 mEq	8.80 +
Potassium Chloride, Lactated Ringers and Dextrose Injection	--- mL	0.50 +
Potassium Phosphate Injection	4.43 mg	1.10 +
Potassium Phosphate in Dextrose	10.00 mL	0.50
Potassium Phos., Lactated Ringers	10.00 mL	0.50
Pralidoxine Chloride	40.00 mg	0.10 +
Prednisolone Acetate Suspension	0.16 mg	31.25
Prednisolone Acetate and Prednisolone Sodium Phosphate Suspension	1.14 mg	4.39
	Pred. Acet.	
Prednisolone Sodium Phosphate	1.00 mg	5.00 +
Prednisolone Sodium Succinate	0.86 mg	5.80 +
Prednisolone Tebutate Suspension	0.57 mg	8.80 +
Prilocaine HCl	5.70 mg	0.90 +
Prilocaine HCl and Epinephrine	5.70 mg	0.90 +
Procaine HCl	8.60 mg	0.60 +
Procaine HCl & Epinephrine	--- mg	0.60 +
Procaine & Phenylephrine HCl	--- mg	0.60 +
Procaine, Tetracaine & Levonordefrin Injection	--- mg	0.60 +
Propofol	12.00 mg	0.42
Procainamide HCl Injection	14.30 mg	0.35 +
Prochlorperazine Edisylate & Mesylate	0.28 mg	17.90 +
Progesterone Aqueous & Suspension	1.43 mg	3.50
Promazine HCl Injection	2.80 mg	1.80 +
Promethazine HCl	1.00 mg	5.00 +
Propantheline Bromide	0.43 mg	11.60 +
Propiomazine HCl Injection	1.10 mg	4.60 +
Propoxycaine, Procaine HCl & Levonordefrin	6.60 mg	0.80 +
Propoxycaine, Procaine HCl & Norepinephrine Bitartrate	6.60 mg	0.80 +

Propranolol HCl Injection	0.09 mg	55.60 +
Protamine Sulfate Injection	0.71 mg	7.04 +
Protein Hydrolysate Injection	10.00 mL	0.50 +
Prothrombin Complex	50.00 units	0.10
Protirelin	7.00 mcg	0.70
Pyridostigmine Bromide	0.29 mg	17.00 +
Pyridoxine HCl	14.29 mg	0.40 +

-Q-

Quinidine Sulfate	8.60 mg	0.60 +
Quinidine Gluconate	8.60 mg	0.60

-R-

Ranitidine HCl	-.--	7.00
Reserpine	0.07 mg	71.50 +
Riboflavin	0.70 mg	7.10 +
Rifampin for Injection	-.-- mg	0.20 +
Ringer's Injection	10.00 mL	0.50 +
Ringer's Irrigation	10.00 mL	0.50 +
Ringer's in Dextrose	10.00 mL	0.50
Ringer's - Lactated Injection	10.00 mL	0.50 +
Ringer's - Lactated in Dextrose	10.00 mL	0.50
Ritodrine HCl	-.--	0.50 +
Rolitetraacycline for Injection	5.00 mg	1.00 +
Rolitetraacycline Nitrate	5.00 mg	1.00

-S-

Saralasin Acetate	0.26 mg	19.20
Secretin	1.00 unit	5.00
Scopolamine Butylbromide	0.29 mg	17.24
Scopolamine HBr	0.009 mg	555.60 +
Secobarbital Sodium Injection	5.50 mg	0.90 +
Selenious Acid (Selenium)	1.43 ug	3.50 +
Selenomethionine Se75 Injection	7.00 mL	25.00 +
Sincalide	0.02 mcg	250.00
Sisomicin Sulfate	10.00 mg	0.50 +
Sodium Acetate	1.29 mEq	3.90 +
Sodium Ascorbate	3.57 mg	1.40

Sodium Bicarbonate	1.00 mEq	5.00 +
Sodium Chloride 0.45-0.9%	10.00 mL	0.50 +
Sodium Chloride 3- 24.3%	1.40 mL	3.57 +
Sodium Chloride - Bacteriostatic	5.00 mL	1.00 +
Sodium Chloride 4.5%- Lactose 3%	10.00 mL	0.50
Sodium Chloride Irrigation	10.00 mL	0.50 +
Sodium Citrate	2.50 mEq	2.00
Sodium Iodide	14.30 mg	0.35
Sodium Lactate	2.40 mEq	2.00 +
Sodium Phosphate Injection	4.00 mg	1.10 +
Sodium Phosphate P32 Solution	7.00 mL	25.00 +
Sodium Salicylate	9.30 mg	0.54
Sodium Tetradecyl Sulfate	0.14 mL	35.71
Sodium Thiosalicylate	2.10 mg	2.38
Sodium Thiosulfate	167.00 mg	0.03 +
Somatrem for Injection	0.25 IU	20.00
Somatropin - Pituitary & Recombinant	0.20 IU	25.00
Soybean Oil Emulsion	3.13 mL	1.60
Spectinomycin HCl	57.00 mg	0.09 +
Streptokinase	21428.00 IU	0.02/100
Streptokinase-Streptodornase (Local)	3000.00 units	0.002
Streptokinase-Streptodornase (IM)	1000.00 units	0.005
Streptomycin Sulfate	20.00 mg	0.25 +
Streptozocin	38.60 mg	0.13 #
Succinylcholine Chloride	2.50 mg	2.00 +
Sufentanil citrate	0.05 mg	100.00
Invert Sugar	10.00 mL	0.50 +
Invert Sugar in Sodium Chloride	10.00 mL	0.50
Sulbactam Sodium	14.30 mg	0.35
Sulfadiazine Sodium	50.00 mg	0.10 +
Sulfamethoxazole & Trimethoprim	25.00 mg(sulf)	0.20
Sulfisoxazole Diolamine Injection	50.00 mg	0.10
Sulfobromophthalein	5.00 mg	1.00 +

-T-

Technetium Tc99m Albumin Aggregated	7.00 mL	25.00 +
Technetium Tc99m Antimony Trisulfate	7.00 mL	25.00
Technetium Tc99m Dsofenin	7.00 mL	25.00 +
Technetium Tc99m Etidronate	7.00 mL	25.00 +
Technetium Tc99m Ferpentetate	7.00 mL	25.00 +

Technetium Tc99m Gluceptate	7.00 mL	25.00 +
Technetium Tc99m Human Serum Albumin	7.00 mL	25.00 +
Technetium Tc99m Lidofenin	7.00 mL	25.00
Technetium Tc99m Mebrofenin	7.00 mL	25.00
Technetium Tc99m Medronate	7.00 mL	25.00 +
Technetium Tc99m Oxidronate	7.00 mL	25.00 +
Technetium Tc99m Pentetate	7.00 mL	25.00 +
Technetium Tc99m Sodium Pertechnetate	7.00 mL	25.00 +
Technetium Tc99m Pyrophosphate	7.00 mL	25.00 +
Technetium Tc99m (Pyro- and trimeta-) Phosphates	7.00 mL	25.00 +
Technetium Tc99m Succimer	7.00 mL	25.00 +
Technetium Tc99m Sulfur Colloid	7.00 mL	25.00 +
Terbutaline Sulfate	0.004 mg	1250.00 +
Teriparatide Acetate	5.00 units	1.00
Testolactone Suspension	1.43 mg	3.50
Testosterone (aqueous suspension)	1.43 mg	3.50 +
*Tetracaine Hydrochloride	0.29 mg	0.70 +
*Tetracaine HCl and Dextrose	0.20 mg	1.00 +
Tetracycline HCl	10.00 mg	0.50 +
Tetracycline Phosphate Complex	5.00 mg	1.00 +
Thallus Chloride Tl201 Injection	7.00 mL	25.00 +
Theophylline and Dextrose	5.00 mg	1.00
Thiamine HCl	1.43 mg	3.50
Thiamylal Sodium	5.00 mg	1.00 +
Thiethylperazine Maleate	0.14 mg	35.80 +
Thiopental Sodium	5.00 mg	1.00 +
Thiotepa for Injection	0.80 mg	6.20 #
Thiothixene HCl Injection	0.057 mg	88.00 +
Thyrotropin for Injection	0.14 IU	36.00
Ticarcillin Disodium	100.00 mg	0.05 +
Ticarcillin Disodium and Clavulanate	75.00 mg	0.07 +
Tobramycin Sulfate	2.50 mg	2.00 +
Tolazoline HCl	6.00 mg	0.80 +
Tolbutamide Sodium	14.20 mg	0.35 +
Tranexamic Acid	10.00 mg	0.50
Triamcinolone Acetate Suspension	1.14 mg	4.40 +
Triamcinolone Acetonide	1.14 mg	4.39
Triamcinolone Diacetate Suspension	0.70 mg	7.10 +
Triamcinolone Hexacetonide Suspension	0.29 mg	17.20 +
Tridihexethyl Chloride	3.00 mg	1.70 +

Triethylenethiophosphoramide	0.80 mg	6.25
Triethylperazine Maleate	0.43 mg	11.63
Trifluoperazine HCl Injection	0.029 mg	172.00 +
Triflupromazine HCl Injection	0.86 mg	5.80 +
Trimethaphan Camsylate	5.00 mg	1.00 +
Trimethobenzamide HCl	2.80 mg	1.80 +
Tromethamine	154.00 mg	0.03 +
Tubocurarine Chloride	0.50 mg	10.00 +

-U-

Urea	1500.00 mg	0.003 +
Urofollitropine	1.06 units	4.70
Urokinase	4,400.00 IU	0.002

-V-

Vancomycin HCl	15.00 mg	0.33 +
Vasopressin	0.14 units	35.70
Vecuronium Bromide	0.10 mg	50.00
Verapamil Hydrochloride	0.30 mg	16.70 +
Vidarabine for Injection	10.00 mg	0.50 +
Vinblastine Sulfate for Injection	0.50 mg	10.00 #+
Vincristine Sulfate for Injection	0.05 mg	100.00 #
Viomycin Sulfate	14.30 mg	0.35
Vitamin A	714.30 IU	0.007

-W-

Warfarin Sodium for Injection	0.21 mg	24.00 +
Water for Injection and Sterile WFI	---	0.25 +
Bacteriostatic WFI	---	0.50 +
Sterile Water for Inhalation	---	0.50 +
Sterile Water for Irrigation	---	0.25 +

-XYZ-

Xenon Xe133 Injection	2.00 mL	87.50 +
*Ytterbium Yb169 Pentetate Injection	2.50 mL	5.60 +
Zinc Chloride Injection	0.20 mg Zn	25.00 +
Zinc Sulfate Injection	0.20 mg Zn	25.00 +

(*) - Intrathecal Injections

(+) - USP Limit

NOTE: The limit formula for radiopharmaceuticals is $175/V$ except for intrathecally administered products $14/V$ for intrathecal products. V equals the maximum recommended dose (listed in the dose column), in mL, at the expiration date or time.

(#) - Drug Administered on a per Square Meter of Body Surface

Limit calculated according to the following formula:

$$5 \text{ EU/Kg} / ((\text{dose} * 1.80 \text{ sq. m.})/70 \text{ Kg})$$

References:

**Facts and Comparisons, Editors E. Kastrup and J. Boyd,
Facts and Comparisons, Inc.**

**United States Pharmacopeia Dispensing Information
1990, United States Pharmacopeia Convention, Inc.**

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Appendix C

ICCVAM Recommended Test Method Protocols

C1	The Human Whole Blood (WB)/Interleukin (IL)-1β <i>In Vitro</i> Pyrogen Test.....	C-3
C2	The Human WB/IL-1β <i>In Vitro</i> Pyrogen Test: Application of Cryopreserved (Cryo) Human WB.....	C-25
C3	The Human WB/IL-6 <i>In Vitro</i> Pyrogen Test	C-47
C4	The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 <i>In Vitro</i> Pyrogen Test.....	C-71
C5	The Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6 <i>In Vitro</i> Pyrogen Test.....	C-95

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Appendix C1

The Human Whole Blood (WB)/Interleukin (IL)-1 β *In Vitro* Pyrogen Test

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ICCVAM Final Recommended Protocol for Future Studies Using the Human Whole Blood (WB)/Interleukin (IL)-1 β *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-1 β from monocytoïd cells in human whole blood (WB). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)¹ WB/IL-1 β Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), and 2) information provided to the National Toxicology Program (NTP) Interagency Center for the Validation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ICCVAM BRD includes the ECVAM Standard Operating Procedures (SOPs) for the WB/IL-1 β test (could be referred to as Monocyte Activation Test), which is first described by Hartung and Wendel (1996). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOPs is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the WB/IL-1 β pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (<http://iccvam.niehs.nih.gov>) to ensure that the most current protocol is used.

¹ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOPs for the WB/IL-1 β Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM SOP ¹	ECVAM Validation SOP ¹
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test neat or at minimal dilution that produces no interference	Test at MVD
Number of Blood Donors	Minimum of 3 (independent or pooled)	Minimum of 1	Minimum of 1
Decision Criteria for Interference	Mean OD ² of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC
Incubation Plate (The number of samples or controls measured in quadruplicate)	NSC (1)	NSC (1) in triplicate	NSC (1)
	EC (5)	EC (5) in triplicate	EC (2)
	TS (14)	TS (25) in triplicate	TS (3) x EC (5) spikes = 15 TS
	PPC ³ (0)	PPC (0)	PPC (3) = 3 TS
	NPC ³ (0)	NPC (0)	NPC (3) = 3 TS
	LTAC ⁴ (0)	LTAC (1) in triplicate	LTAC (0)
ELISA Plate	Includes seven point IL-1 β SC and blank in duplicate	Not included	Not included
Assay Acceptability Criteria	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test ⁶	Outliers rejected using Dixon's test ⁶
	Mean OD of NSC \leq 0.15	Not included	Not included
	Quadratic function of IL-1 β SC $r \geq$ 0.95 ⁵	Not included	Not included
	EC SC produces OD values that ascend in a sigmoidal concentration response	Not included	Not included
	Not included	Mean OD of 0.5 EU/mL EC \geq 1.6x Mean OD of NSC	Mean OD of 0.5 EU/mL EC \geq 1.6x Mean OD of NSC
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC ⁷ TS	OD TS > OD 0.5 EU/mL EC	OD TS > OD 0.5 EU/mL EC

Abbreviations: EC = Endotoxin control; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-1 β = Interleukin-1 β ; LTAC = Lipoteichoic acid (LTA) control; MVD = Maximum valid dilution; NPC = Negative product control; NSC = Negative saline control; OD = Optical density; PPC = Positive product control; SC = Standard curve; SOP = Standard operating procedure; TS = Test substance; WB = Whole blood

¹ECVAM WB/IL-1 β SOPs are presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).

²Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

³In the ICCVAM WB/IL-1 β protocol, PPC and NPC are assessed in the interference test described in **Section 4.2**, which is performed prior to the ELISA. In the ECVAM SOP, PPC and NPC were only included in the ECVAM validation study.

⁴LTAC was only included in the ECVAM SOP.

⁵Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

⁶Included in the ECVAM Trial data report presented in Appendix D of the ICCVAM BRD.

⁷Where unknown, the ELC is calculated (see **Section 12.2**).

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of interleukin (IL)-1 β from monocytoid cells in whole blood (WB). The concentration of IL-1 β released by incubation of WB cells with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-1 β . The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by WB cells exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)¹ or an equivalent standard expressed in Endotoxin Units (EU)/mL. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures that use human blood-derived materials should follow national /international procedures for handling blood potentially contaminated with pathogens. An example of such guidelines is the Universal Precautions available at <http://www.niehs.nih.gov/odhsb/biosafe/univers.htm>. For non-human blood procedures (e.g., ELISAs), standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes (min). If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

¹RSEs are internationally harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Blood Donor Eligibility

Monocytoid cells from WB are the source of cytokine production in the WB/IL-1 β test method as described by Hartung and Wendel (1996) and Schindler et al. (2006). In the United States (U.S.), the collection of blood and blood components for transfusion and further manufacture (including the use of resulting monocytes in a licensed test) is currently regulated under Section 351 of the Public Health Service (PHS) Act (U.S. Code [U.S.C.], Title 42, Chapter 6A) and/or the Federal Food Drug and Cosmetic Act (U.S.C., Title 21, Chapter 9), both of which require compliance with Current Good Manufacturing Practice (cGMP) regulations (21 CFR Parts 210, 211 and 600-640²).

These regulations and the associated FDA guidance's provide an important resource for information regarding the currently accepted practice for blood manufacture and collection (including donor screening) (<http://www.fda.gov/cber/blood.htm>). Specifically, guidance regarding donor screening questionnaires and links to currently acceptable questionnaires can be found at <http://www.fda.gov/cber/gdlns/donorhistques.htm#iv>. If you have questions regarding the applicability of blood regulations to your specific situation³, it is recommended that you e-mail the Manufacturers Assistance and Technical Training (MATT) Branch established by FDA at matt@cber.fda.gov for advice.

Any participating blood establishment should address how unused components of blood donations will be accounted for and ultimately destroyed, and if the establishment will store the blood preparation, describe the storage procedures to be followed.

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA procedure, the materials that will be in close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be sterile and pyrogen-free.

3.2.1 Blood Incubation

3.2.1.1 *Equipment*

- Centrifuge
- Hood; Bio-safety, laminar flow (recommended)
- Incubator; cell culture (37 \pm 1 $^{\circ}$ C + 5% CO₂)

²The requirements for WB can be found at 21 CFR 640.1 *et seq.* In addition, there are specific regulations applicable to red blood cells, platelets, and other blood components. See, for example, 21 CFR 640.10-640.27. Other regulations applicable to the manufacture of blood and blood components include 21 CFR Part 606, the cGMP requirements for blood and blood components, 21 CFR 610.40, the requirements for testing of WB donations, and 21 CFR 640.3, the requirements for determining the suitability of the donor. Blood that enters into U.S. interstate commerce should be tested for antibodies to HIV 1/2, HCV, HTLV I and II, HBc, HBsAg and RPR, WNV and Chagas.

³The collection of blood for research and development purposes or as a component of an *in vitro* test (that is not subject to licensure) may potentially not be required to adhere to the FDA regulations outlined above.

- Pipetter; multichannel (8- or 12-channel)
- Pipetters; single-channel adjustable (20 and 200 μ L)
- Repeating pipetter
- Vortex mixer

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (1.0 and 2.5 mL)
- Needle set; multily, pyrogen-free, 19 mm, 21 gauge
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- Reservoirs; for blood collection
- Tips; pipetter, sterile, pyrogen-free (20 and 200 μ L)

3.2.2 ELISA

3.2.2.1 *Equipment*

- Microplate mixer
- Microplate reader (450 nm with an optional reference filter in the range of 600-690 nm⁴)
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, 96-well, polystyrene
- Pyrogen-free water (PFW)
- Reservoirs; fluid

⁴The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

- Tips; pipetter, nonsterile
- Tubes; polystyrene (12 mL)

3.2.2.3 ELISA Kit

An ELISA that measures IL-1 β release is used. A variety of IL-1 β ELISA kits are commercially available and the IL-1 β ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. The IL-1 β ELISA should be calibrated using an IL-1 β international reference standard (e.g., World Health Organization [WHO] 86/680) prior to use. The IL-1 β cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-1 β ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-1 β capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-1 β reference standard
- PFS
- Stop solution
- TMB⁵/substrate solution

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

3.4 Solutions

ELISA solutions are listed in **Section 3.2**.

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The collection of WB is outlined in **Section 6.1**.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the WB/IL-1 β pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control

⁵The use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable.

(NSC) and five RSE concentrations (0.25, 0.50, 1.0, 2.5, and 5.0 EU/mL) are included in the incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of the stock vial by following the instructions provided by the manufacturer (e.g., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL ¹	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000 ^{2,3}	50	1950	50 ⁴
50	100	900	5.0
5.0	500	500	2.5
2.5	500	500	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

Each stock tube should be vortexed prior to its use to make the subsequent dilution.

¹To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.

²A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

³The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.

⁴This concentration is not used in the assay.

4.2 Interference Test

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.2.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethyl sulfoxide (DMSO) and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be diluted beyond its Maximum Valid Dilution (MVD) (refer to **Section**

12.3). The calculation of the MVD is dependent on the ELC for a test substance. The ELC can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to blood cells.

4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.2.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 1.0 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin-spiking solutions is shown in **Table 4-2**. For non-spiked solutions, 200 µL of PFS is added to a well followed by 20 µL of the test substance (i.e., equivalent to the negative product control [NPC]) and 20 µL of WB. Endotoxin-spiked solutions are prepared by adding 180 µL of PFS to each well followed by 20 µL of the test substance, and 20 µL of WB. Then, 20 µL of an endotoxin-spike solution (1.0 EU/mL) (i.e., equivalent to the positive product control [PPC]) is added to each well. The contents of the wells are mixed and incubated as outlined in **Section 6.1.3, Steps 6-8**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-1β standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

Sample Addition	Spiked	Non-spiked
	µL/well ¹	
PFS	180	200
Endotoxin-spike solution ²	20	0
Test substance (neat and each serial dilution)	20	20
WB	20	20
Total ³	240	240

Abbreviations: PFS = Pyrogen-free saline; WB = Whole blood

¹ n=4 replicates each

² Endotoxin concentration is 1.0 EU/mL in PFS.

³ A total volume of 240 µL per well is used for the incubation.

The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The spike recovery for each sample dilution is calculated as a percentage by setting the theoretical value (i.e., endotoxin-spike concentration of 1.0 EU/mL) at 100%. For example, consider the following interference test results in **Table 4-3**:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-1 β relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not to exceed the MVD, for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1.0 EU/mL EC).

4.2.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., WB incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., WB incubated with PFS instead of the test substance) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.4 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-1 β Release

6.1.1 Collection of Human Blood

Human volunteers that have met the donor eligibility criteria described in **Section 3.1** are used as the source of WB. All components of the blood collection system (e.g., syringes, tubes, connecting lines) must be sterile and pyrogen-free. WB is drawn by venipuncture⁶ from the medial cubital or cephalic vein of either the right or left arm and collected in a sterile container that contains anticoagulant solution (e.g., heparin). The total volume of blood collected per donor (i.e., up to 500 mL) will be dictated by experimental design and determined by the test method user. WB should be stored at room temperature (RT) and must be used within 4 hr. All subsequent handling of WB should be performed in a laminar flow hood using sterile technique to prevent contamination.

Prior to use in the assay, an equal volume of WB from multiple individual donors should be pooled⁷.

6.1.2 Incubation Plate

Test substances are prepared at a level of dilution that did not show interference with the test system, provided that this dilution does not exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test samples (see **Table 6-1**).

⁶WB is obtained using Universal Precautions (e.g., latex gloves, labcoats, safety glasses) and sterile equipment (e.g., syringes, needles, collection tubes) within a hospital or clinical setting by qualified and adequately trained personnel (i.e., registered nurse, licensed phlebotomist, or medical doctor).

⁷Multiple donors (i.e., a minimum of three) should meet the acceptability criteria as outlined in **Section 8.0** either as a pool of multiple individual donors or as multiple individual donors tested independently.

Table 6-1 Overview of Incubation Plate Preparation in the WB/IL-1 β Pyrogen Test

Number of Wells	Sample	PFS	EC	Test Sample	WB	Mix the samples; incubate for 10 to 24 hr at 37 \pm 1 $^{\circ}$ C in a humidified atmosphere with 5% CO ₂ .	Mix the samples; immediately transfer to an ELISA plate ³ and run ELISA or store plate in a -20 $^{\circ}$ C or -80 $^{\circ}$ C freezer.
20 ¹	EC	200	20	0	20		
4	NSC	220	0	0	20		
56 ²	Test samples (1-14)	200	0	20	20		

Abbreviations: EC = Endotoxin control; IL-1 β = Interleukin-1 β ; NSC = Negative saline control; PFS = Pyrogen-free saline; WB = Whole blood

¹Five EC concentrations (0.25, 0.50, 1.0, 2.5, and 5.0 EU/mL) in quadruplicate

²14 test samples (n=4) per plate

³An IL-1 β standard curve is prepared in Columns 11 and 12 on the ELISA plate (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.3 Incubation Assay for IL-1 β Release

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. Blood samples are prepared in a microtiter plate using a laminar flow hood. All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

Step 1. Refer to the incubation plate template presented in **Table 6-2**.

Step 2. Using a pipetter, transfer 200 μ L of PFS into each well.

Step 3. Transfer 20 μ L of test sample or 20 μ L of PFS for the NSC into the appropriate wells as indicated in the template.

Step 4. Transfer 20 μ L of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.

Step 5. Transfer 20 μ L of WB into each well and mix by gently swirling the plate.

Step 6. Mix the contents of the wells thoroughly by gently pipetting up and down five times using a multichannel pipetter, changing the tips between each row to avoid cross-contamination.

Step 7. Place the covered plate in a tissue culture incubator for 10 to 24 hr at 37 \pm 1 $^{\circ}$ C in a humidified atmosphere containing 5% CO₂.

Step 8. Prior to transferring the test samples onto the ELISA plate, mix the contents of the wells by pipetting up and down three times using a multichannel pipetter, changing the tips between each row to avoid cross-contamination.

Note: The aliquots may be tested immediately in the ELISA or stored in a -20°C or -80°C freezer for testing at a later time. After transfer to the ELISA plate, freeze the remaining aliquots in a -20°C or -80°C freezer for subsequent experiments, if necessary.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	Void ³	Void
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

¹EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.

²TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

³Columns 11 and 12 are reserved for the IL-1 β standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-1 β Release

6.2.1 IL-1 β Standard Curve

An IL-1 β standard, supplied with the ELISA kit, is used. IL-1 β standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in PFS to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL. Each well on the ELISA plate will receive 100 μ L of an IL-1 β blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed by heating them in a water bath. A sample ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, an eight-point IL-1 β standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-1 β standard curve is prepared as

described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4**.

Step 1. Add 100 μL of enzyme-labeled detection antibody to each well.

Step 2. After pipetting up and down three times to mix the supernatant, transfer 100 μL from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 3. Add 100 μL of each IL-1 β standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 90 min on a microplate mixer at 350-400 rpm at RT.

Step 5. Decant and wash each well five to six times with 300 μL Buffered Wash Solution per well and then rinse three times with deionized water. Place the plates upside down and tap to remove the wash solution.

Step 6. Add 200 μL of TMB/Substrate Solution to each well and incubate at RT in the dark for 10 to 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 μL of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD₄₅₀ within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 600-690 nm is recommended.⁸

⁸The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-1 β ³ 0	IL-1 β 0
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	IL-1 β 62.5	IL-1 β 62.5
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	IL-1 β 125	IL-1 β 125
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	IL-1 β 250	IL-1 β 250
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	IL-1 β 500	IL-1 β 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-1 β 1000	IL-1 β 1000
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-1 β 2000	IL-1 β 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-1 β 4000	IL-1 β 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

¹EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.

²TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

³IL-1 β values in columns 11 and 12 are in pg/mL.

Table 6-4 Overview of ELISA Procedure

Enzyme-labeled Antibody (μ L)	Material transfer from Incubation Plate (μ L)	IL-1 β standard (0 to 4000 pg/mL) (μ L)	Incubate 90 min on a plate mixer at 350 to 400 rpm at RT.	Decant and wash each well three times with 300 μ L Buffered Wash Solution and three times with deionized water.	TMB/Substrate Solution (μ L)	Incubate for less than 15 min at RT in dark.	Stop Solution (μ L)	Read each well at OD ₄₅₀ with a 600 to 690 nm reference filter.
100	100	100			200		50	

Abbreviations: OD₄₅₀ = Optical density at 450 nm; RT = Room temperature

7.0 EVALUATION OF TEST RESULTS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD₄₅₀) with a 600 to 690 nm reference filter (recommended)⁹. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-1 β standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-1 β standard curve produces an $r \geq 0.95^{10}$ and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

An outlying observation that represents either a pool of multiple independent donors or a single individual donor may be excluded if there is confirmation that the accuracy of the medical information provided by an individual donor is suspect, or if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett et al. 1994], Grubbs' test [Barnett et al. 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance
- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)

⁹The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

¹⁰Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, resuspension solvent)

Justification of the In Vitro Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time
- If the test method employs proprietary components, documentation on the procedure used to ensure their integrity from “lot-to-lot” and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)¹

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)^{1,2}

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M , where:

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product were used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)^{1,2}

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is¹:

$$\text{MVD} = (\text{ELC} \times \text{Product Potency [PP]})/\lambda$$

As an example, for Cyclophosphamide Injection, the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is¹:

$$\text{MVD} = \text{PP}/\text{Minimum Valid Concentration (MVC)}$$

$$\text{where, MVC} = (\lambda \times M)/K$$

where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

¹From FDA (1987)

²From USP (2007)

12.5 Parenteral Threshold Pyrogen Dose (K)^{1,2}

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, the PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces $\frac{1}{2}$ the maximal increase in optical density (OD) from the endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.7 Product Potency (PP)^{1,2}

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)^{1,2}

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.

Appendix C2

The Human WB/IL-1 β *In Vitro* Pyrogen Test: Application of Cryopreserved (Cryo) Human WB

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ICCVAM Final Recommended Protocol for Future Studies Using the Cryopreserved (Cryo) Whole Blood (WB)/Interleukin (IL)-1 β *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-1 β from monocytoïd cells in human whole blood (WB) that have been cryopreserved (Cryo). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)¹ Cryo WB/IL-1 β Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), and 2) information provided to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ICCVAM BRD includes the ECVAM Standard Operating Procedure (SOP) for the Cryo WB/IL-1 β test (could be referred to as Monocyte Activation Test), which is based on the Cryo WB/IL-1 β method first described by Schindler et al. (2004). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOP is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the Cryo WB/IL-1 β pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (<http://iccvam.niehs.nih.gov>) to ensure that the most current protocol is used.

¹ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOP for the Cryo WB/IL-1 β Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM Catch-Up Validation SOP ¹
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test at MVD
Number of Blood Donors	Minimum of 3 (independent or pooled)	5 (pooled) ²
Decision Criteria for Interference	Mean OD ³ of PPC is 50% to 200% of 0.5 EU/mL EC	Mean OD of PPC is 50% to 200% of 0.5 EU/mL EC
	Not included	Mean OD of PPC \geq 1.6x Mean OD of NPC
Incubation Plate (The number of samples or controls measured in quadruplicate)	NSC (1)	NSC (1)
	EC (5)	EC (2)
	TS (14)	TS (3) x EC (5) spikes = 15 TS
	PPC ⁴ (0)	PPC (3) = 3 TS
	NPC ⁴ (0)	NPC (3) = 3 TS
ELISA Plate	Includes seven point IL-1 β SC and blank in duplicate	Not included
Assay Acceptability Criteria	Mean OD of NSC \leq 0.15	Mean OD of NSC \leq 100 m OD
	Quadratic function of IL-1 β SC $r \geq$ 0.95 ⁵	Not included
	EC SC produces OD values that ascend in a sigmoidal concentration response	Not included
	Not included	Mean OD of 0.5 EU/mL EC \geq 1.6x Mean OD of NSC
	Not included	If one OD of 1.0 EU/mL EC > Max, ELISA may be repeated using reduced incubation time
	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test ⁶
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC ⁷ TS	OD TS > OD 0.5 EU/mL EC ⁶

Abbreviations: Cryo = Cryopreserved; EC = Endotoxin control; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-1 β = Interleukin-1 β ; MVD = Maximum valid dilution; NPC = Negative product control; NSC = Negative saline control; OD = Optical density; PPC = Positive product control; SC = Standard curve; SOP = Standard operating procedure; TS = Test substance; WB = Whole blood

¹ECVAM Cryo WB/IL-1 β catch-up validation SOP is presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).

²Samples are collected from five donors and pooled prior to cryopreservation.

³Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

⁴In the ICCVAM Cryo WB/IL-1 β protocol, PPC and NPC are assessed in the interference test described in **Section 4.2**, which is performed prior to the ELISA.

⁵Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

⁶Included in the ECVAM Trial data report presented in Appendix D of the ICCVAM BRD.

⁷Where unknown, the ELC is calculated (see **Section 12.2**).

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of interleukin (IL)-1 β from monocytoid cells in whole blood (WB) that have been cryopreserved (Cryo). The concentration of IL-1 β released by incubation of Cryo WB cells with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-1 β . The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by Cryo WB cells exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)¹ or an equivalent standard expressed in Endotoxin Units (EU)/mL. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures that use human blood-derived materials should follow national/international procedures for handling blood potentially contaminated with pathogens. An example of such guidelines is the Universal Precautions available at <http://www.niehs.nih.gov/odhsb/biosafe/univers.htm>. For non-human blood procedures (e.g., ELISAs), standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes (min). If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as

¹RSEs are internationally harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Blood Donor Eligibility

Monocytoid cells from WB are the primary source of cytokine production in the Cryo WB/IL-1 β test method as described by Hartung and Wendel (1999) and Schindler et al. (2004, 2006). In the United States (U.S.), the collection of blood and blood components for transfusion and further manufacture (including the use of resulting monocytes in a licensed test) is currently regulated under Section 351 of the Public Health Service (PHS) Act (U.S. Code [U.S.C.], Title 42, Chapter 6A) and/or the Federal Food Drug and Cosmetic Act (U.S.C., Title 21, Chapter 9), both of which require compliance with Current Good Manufacturing Practice (cGMP) regulations (21 CFR Parts 210, 211 and 600-640²).

These regulations and the associated FDA guidance's provide an important resource for information regarding the currently accepted practice for blood manufacture and collection (including donor screening) (<http://www.fda.gov/cber/blood.htm>). Specifically, guidance regarding donor screening questionnaires and links to currently acceptable questionnaires can be found at <http://www.fda.gov/cber/gdlns/donorhistques.htm#iv>. If you have questions regarding the applicability of blood regulations to your specific situation³, it is recommended that you e-mail the Manufacturers Assistance and Technical Training (MATT) Branch established by FDA at matt@cber.fda.gov for advice.

Any participating blood establishment should address how unused components of blood donations will be accounted for and ultimately destroyed, and if the establishment will store the blood preparation, describe the storage procedures to be followed.

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA, the materials that will be in close contact with samples and/or blood cells (e.g., pipet tips, containers, and solutions) should be sterile and pyrogen-free.

3.2.1 Blood Incubation

3.2.1.1 *Equipment*

- Centrifuge

²The requirements for WB can be found at 21 CFR 640.1 *et seq.* In addition, there are specific regulations applicable to red blood cells, platelets, and other blood components. See, for example, 21 CFR 640.10-640.27. Other regulations applicable to the manufacture of blood and blood components include 21 CFR Part 606, the cGMP requirements for blood and blood components, 21 CFR 610.40, the requirements for testing of WB donations, and 21 CFR 640.3, the requirements for determining the suitability of the donor. Blood that enters into U.S. interstate commerce should be tested for antibodies to HIV 1/2, HCV, HTLV I and II, HBc, HBsAg and RPR, WNV and Chagas.

³The collection of blood for research and development purposes or as a component of an *in vitro* test (that is not subject to licensure) may potentially not be required to adhere to the FDA regulations outlined above.

- Hood; Bio-safety, laminar flow (recommended)
- Incubator; cell culture ($37\pm 1^{\circ}\text{C} + 5\% \text{CO}_2$)
- Pipetter; multichannel (8- or 12-channel)
- Pipettors; single-channel adjustable (20 and 200 μL)
- Repeating pipetter
- Vortex mixer

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (1.0 and 2.5 mL)
- Needle set; multify, pyrogen-free, 19 mm, 21 gauge
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- Reservoirs; for fluid collection
- RPMI-1640 cell culture medium
- Tips; pipetter, sterile, pyrogen-free (20 and 200 μL)

3.2.2 ELISA

3.2.2.1 *Equipment*

- Microplate mixer
- Microplate reader (450 nm with an optional reference filter in the range of 600-690 nm⁴)
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, 96-well, polystyrene
- Pyrogen-free water (PFW)
- Reservoirs; fluid
- Tips; pipetter, nonsterile

⁴The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

- Tubes; polystyrene (12mL)

3.2.2.3 ELISA Kit

An ELISA that measures IL-1 β release is used. A variety of IL-1 β ELISA kits are commercially available and the IL-1 β ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. The IL-1 β ELISA should be calibrated using an international reference standard (e.g., World Health Organization [WHO] 86/680) prior to use. The IL-1 β cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-1 β ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-1 β capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-1 β reference standard
- PFS
- Stop solution
- TMB⁵/substrate solution

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

3.4 Solutions

- RPMI-1640 cell culture medium

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The collection of WB and the procedure for cryopreservation of WB is outlined in **Section 6.1**.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the Cryo WB/IL-1 β pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control (NSC) and five RSE concentrations (0.25, 0.50, 1.0, 2.5, and 5.0 EU/mL) are included in the

⁵The use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable.

incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of the stock vial by following the instructions provided by the manufacturer (i.e., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL ¹	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000 ^{2,3}	50	1950	50 ⁴
50	100	900	5.0
5.0	500	500	2.5
2.5	400	600	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

¹To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.

²A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

³The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.

⁴This concentration is not used in the assay.

4.2 Interference Test

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.2.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethyl sulfoxide (DMSO) and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be diluted beyond its Maximum Valid Dilution (MVD) (refer to **Section 12.3**). The calculation of the MVD is dependent on the ELC for a test substance. The ELC

can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to blood cells.

4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.2.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 1.0 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin-spiking solutions is shown in **Table 4-2**. For non-spiked solutions, 200 µL of RPMI is added to a well followed by 20 µL of the test substance (i.e., equivalent to the negative product control [NPC]) and 20 µL of Cryo WB (**Section 6.1**). Endotoxin-spiked solutions are prepared by adding 180 µL of RPMI to each well followed by 20 µL of the test substance and 20 µL of Cryo WB. Then, 20 µL of an endotoxin-spike solution (1.0 EU/mL) (i.e., equivalent to the positive product control [PPC]) is added to each well. The contents of the wells are mixed and incubated as outlined in **Section 6.1.5, Steps 6-9**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-1β standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

Sample Addition	Spiked	Non-spiked
	µL/well ¹	
RPMI	180	200
Endotoxin-spike solution ²	20	0
Test substance (neat and each serial dilution)	20	20
Cryo WB	20	20
Total ³	240	240

Abbreviations: Cryo = Cryopreserved; WB = Whole blood

¹n=4 replicates each

²Endotoxin concentration is 1.0 EU/mL in RPMI.

³A total volume of 240 µL per well is used for the incubation.

The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The spike recovery for each sample dilution is calculated as a percentage by setting the theoretical value (i.e., endotoxin-spike concentration of 1.0 EU/mL) at 100%. For example, consider the following interference test results in **Table 4-3**:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-1 β relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not to exceed the MVD, for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1.0 EU/mL EC).

4.2.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., WB incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., Cryo WB incubated with PFS instead of the test substance) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.4 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-1 β Release

6.1.1 Collection of Human Blood

Human volunteers that have met the donor eligibility criteria described in **Section 3.1** are used as the source of WB. All components of the blood collection system (e.g., syringes, tubes, connecting lines) must be sterile and pyrogen-free. WB is drawn by venipuncture⁶ from the medial cubital or cephalic vein of either the right or left arm and collected in a sterile container that contains anticoagulant solution (e.g., heparin). The total volume of blood collected per donor (i.e., up to 500 mL) will be dictated by experimental design and determined by the test method user. All subsequent handling of WB should be performed in a laminar flow hood using sterile technique to prevent contamination.

6.1.2 Cryopreservation Procedure

The two methods available for cryopreservation of blood are 1) the PEI method developed at the Paul Ehrlich Institute (Langen, Germany) and 2) the Konstanz method developed at the University of Konstanz (Konstanz, Germany).

6.1.3 PEI Method of Cryopreservation

In the PEI method (Schindler et al. 2006), an equal volume of WB from multiple independent donors is pooled⁷ and frozen in a cryoprotective phosphate buffer (Sorensen's) containing 20% (v/v) pyrogen-free, clinical-grade DMSO. The tubes can be stored in a -80°C freezer or in the vapor phase of liquid nitrogen until used.

6.1.3.1 *Konstanz Method of Cryopreservation*

In the Konstanz method (Schindler et al. 2004), pyrogen-free, clinical grade DMSO is added to WB of individual donors at a final concentration of 10% (v/v). An equal volume of WB from multiple independent donors is pooled⁷ and frozen in a computer-controlled freezer using several cycles of programmed freezing down to -120°C. Tubes of WB are then removed from the instrument and stored in the vapor phase of liquid nitrogen until used.

⁶WB is obtained using Universal Precautions (e.g., latex gloves, labcoats, safety glasses) and sterile equipment (e.g., syringes, needles, collection tubes) within a hospital or clinical setting by qualified and adequately trained personnel (i.e., registered nurse, licensed phlebotomist, or medical doctor).

⁷Multiple donors (i.e., a minimum of three) should meet the acceptability criteria as outlined in **Section 8.0** either as a pool of multiple individual donors or as multiple individual donors tested independently.

6.1.3.2 *Thawing Procedure*

The tubes are thawed in an incubator at 37±1°C for 15 min. Prior to use in the assay, the pooled⁷ WB cells should be examined under a microscope to determine that the morphology of the cells is consistent with the appearance of cells that previously yielded acceptable results. The results of this examination should be included in the study report.

6.1.4 Incubation Plate

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test samples (see **Table 6-1**).

Table 6-1 Overview of Incubation Plate Preparation in the Cryo WB/IL-1β Pyrogen Test (PEI Method)

Number of Wells	Sample	RPMI	EC	Test Sample	Cryo WB ¹	Mix the samples; incubate for 10 to 24 hr at 37±1°C in a humidified atmosphere with 5% CO ₂ .	Mix the samples; immediately transfer to an ELISA plate ⁵ and run ELISA or store plate in a -20°C or -80°C freezer.
		μL					
20 ²	EC	180	20	0	40		
4	NSC	180	0	0 ³	40		
56 ⁴	Test samples (1-14)	180	0	20	40		

Abbreviations: Cryo = Cryopreserved; EC = Endotoxin control; IL-1β = Interleukin-1β; NSC = Negative saline control; PEI = Paul Ehrlich Institute; PFS = Pyrogen-free saline; WB = Whole blood

¹For the Konstanz method of cryopreservation, 20 μL of Cryo WB is used and the volume of RPMI is adjusted to 200 μL.

²Five EC concentrations (0.25, 0.50, 1.0, 2.5, 5.0 EU/mL) in quadruplicate

³20 μL of PFS is added instead of the test sample.

⁴14 test samples (n=4) per plate

⁵An IL-1β standard curve is prepared in Columns 11 and 12 on the ELISA plate (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.5 Incubation Assay for IL-1β Release

Cryo WB is prepared in a microtiter plate using a laminar flow hood (refer to **Section 6.1.1**). All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

Step 1. Refer to the incubation plate template presented in **Table 6-2**.

Step 2. Using a pipetter, transfer either 180 or 200 μL of RPMI into each well (for the PEI or Konstanz method of cryopreservation, respectively – refer to **Step 5** below).

Step 3. Transfer 20 µL of test sample or 20 µL of PFS for the NSC into the appropriate wells as indicated in the template.

Step 4. Transfer 20 µL of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.

Step 5. Transfer either 40 or 20 µL of Cryo WB (for the PEI or Konstanz method of cryopreservation, respectively) into each well and mix by gently swirling the plate.

Step 6. Mix the contents of the wells thoroughly by gently pipetting up and down five times using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Step 7. Place the covered plate in a tissue culture incubator for 10 to 24 hr at 37±1°C in a humidified atmosphere containing 5% CO₂.

Step 8. If using the Konstanz method, freeze the plate in a -20°C or -80°C freezer until the contents of the well are completely frozen and then, thaw the plate at RT or in a water bath not exceeding 37±1°C.

Step 9. Prior to transferring the test samples onto the ELISA plate, mix the contents of the wells by pipetting up and down three times using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Note: The aliquots may be tested immediately in the ELISA or stored in a -20°C or -80°C freezer for testing at a later time. After transfer to the ELISA plate, freeze the remaining aliquots in a -20°C or -80°C freezer for subsequent experiments, if necessary.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	Void ³	Void
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

¹EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.

²TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

³Columns 11 and 12 are reserved for the IL-1β standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-1 β Release

6.2.1 IL-1 β Standard Curve

An IL-1 β standard, supplied with the ELISA kit, is used. IL-1 β standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in RPMI to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL. Each well on the ELISA plate will receive 100 μ L of an IL-1 β blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed by heating them in a water bath. A suggested ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, an eight-point IL-1 β standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-1 β standard curve is prepared as described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4**.

Step 1. Add 100 μ L of enzyme-labeled detection antibody to each well.

Step 2. After pipetting up and down three times to mix the supernatant, transfer 100 μ L from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 3. Add 100 μ L of each IL-1 β standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 90 min on a microplate mixer at 350-400 rpm at RT.

Step 5. Decant and wash each well three times with 300 μ L Buffered Wash Solution and then rinse three times with deionized water. Place the plates upside down and tap to remove water.

Step 6. Add 200 μ L of TMB/Substrate Solution to each well and incubate at RT in the dark for 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 μ L of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD₄₅₀ within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 600-690 nm is recommended.⁸

⁸The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-1 β ³ 0	IL-1 β 0
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	IL-1 β 62.5	IL-1 β 62.5
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	IL-1 β 125	IL-1 β 125
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	IL-1 β 250	IL-1 β 250
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	IL-1 β 500	IL-1 β 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-1 β 1000	IL-1 β 1000
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-1 β 2000	IL-1 β 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-1 β 4000	IL-1 β 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

¹EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.

²TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

³IL-1 β values in columns 11 and 12 are in pg/mL.

Table 6-4 Overview of ELISA Procedure

Enzyme-labeled Antibody (μ L)	Material transfer from Incubation Plate (μ L)	IL-1 β standard (0 to 4000 pg/mL) (μ L)	Incubate 90 min on a plate mixer at 350 to 400 rpm at RT.	Decant and wash each well three times with 300 μ L Buffered Wash Solution and three times with deionized water.	TMB/Substrate Solution (μ L)	Incubate for less than 15 min at RT in dark.	Stop Solution (μ L)	Read each well at OD ₄₅₀ with a 600 to 690 nm reference filter.
100	100	100			200		50	

Abbreviations: OD₄₅₀ = Optical density at 450 nm; RT = Room temperature

7.0 EVALUATION OF TEST RESULTS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD₄₅₀) with

a 600 to 690 nm reference filter (recommended)⁹. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-1 β standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-1 β standard curve produces an $r \geq 0.95$ ¹⁰ and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

An outlying observation that represents either a pool of multiple independent donors or a single individual donor may be excluded if there is confirmation that the accuracy of the medical information provided by an individual donor is suspect, or if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett et al. 1984], Grubbs' test [Barnett et al. 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance
- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)
- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, and resuspension solvent)

Justification of the In Vitro Test Method and Protocol Used

Test Method Integrity

⁹The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

¹⁰Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time
- If the test method employs proprietary components, documentation on the procedure used to ensure their integrity from “lot-to-lot” and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)¹¹

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)^{11,12}

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M, where:

¹¹From FDA (1987)

¹²From USP (2007)

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product were used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)^{11,12}

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is¹¹:

$$\text{MVD} = (\text{ELC} \times \text{Product Potency [PP]})/\lambda$$

As an example, for Cyclophosphamide Injection, the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is¹¹:

$$\text{MVD} = \text{PP}/\text{Minimum Valid Concentration (MVC)}$$

$$\text{where, MVC} = (\lambda \times \text{M})/\text{K}$$

where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

12.5 Parenteral Threshold Pyrogen Dose (K)^{11,12}

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, the PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces ½ the maximal increase in optical density (OD) from the endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.7 Product Potency (PP)^{11,12}

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)^{11,12}

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.

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Appendix C3
The Human WB/IL-6 *In Vitro* Pyrogen Test

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ICCVAM Final Recommended Protocol for Future Studies Using the Human Whole Blood (WB)/Interleukin (IL)-6 *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-6 from monocytoïd cells in human whole blood (WB). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)¹ WB/IL-6 Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), and 2) information provided to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ICCVAM BRD includes the ECVAM Standard Operating Procedure (SOP) for the WB/IL-6 test (could be referred to as Monocyte Activation Test), which is based on the WB/IL-6 method first described by Pool et al. (1998). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOP is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the WB/IL-6 pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (<http://iccvam.niehs.nih.gov>) to ensure that the most current protocol is used.

¹ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

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Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOP for the WB/IL-6 Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM SOP ¹
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test neat or at minimal dilution that produces no interference
Number of Blood Donors	Minimum of 3 (independent or pooled)	Minimum of 3 (independent)
Decision Criteria for Interference	Mean OD ² of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC
Incubation Plate for ELISA (The number of samples or controls measured in quadruplicate)	NSC (1)	NSC (1)
	EC (5)	EC (5)
	TS (14)	TS (14)
	PPC ³ (0)	PPC (0)
	NPC ³ (0)	NPC (0)
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Includes seven point IL-6 SC and blank in duplicate
Assay Acceptability Criteria	Mean OD of NSC ≤ 0.15	NSC < 200 pg/mL IL-6
	Quadratic function of IL-6 SC $r \geq 0.95^4$	EC SC satisfies ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures Methodology; ICH Q2B, Nov 1996
	EC SC produces OD values that ascend in a sigmoidal concentration response	Wilcoxon rank-sum test used to show that at least 3 of 4 replicates at each increasing EC concentration are higher relative to the next lowest concentration
	High responder blood donors (i.e., >200 pg/mL IL-6) may be excluded	High responder blood donors (i.e., >200 pg/mL IL-6) may be excluded
	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test ⁵
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC ⁶ TS	Endotoxin concentration TS > ELC TS OR Limit test is run to determine whether or not a TS after correction and dilution contains < 0.5 EU/mL of endotoxin

Abbreviations: EC = Endotoxin control; ELC = Endotoxin limit concentration; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-6 = Interleukin-6; MVD = Maximum valid dilution; NSC = Negative saline control; OD = Optical density; PPC = Positive product control; SC = Standard curve; TS = SOP = Standard operating procedure; Test substance; WB = Whole blood

¹ECVAM WB/IL-6 SOP is presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).

²Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

³In the ICCVAM WB/IL-6 protocol, PPC and NPC are assessed in the interference test described in **Section 4.2**, which is performed prior to the ELISA. In the ECVAM SOP, PPC and NPC were only included in the ECVAM validation study.

⁴Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

⁵Included in the ECVAM Trial data report presented in Appendix D of the ICCVAM BRD.

⁶Where unknown, the ELC is calculated (see **Section 12.2**)

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of interleukin (IL)-6 from monocytoïd cells in whole blood (WB). The concentration of IL-6 released by incubation of WB with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-6. The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by WB cells exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)¹ or an equivalent standard expressed in Endotoxin Units (EU)/mL. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures that use human blood-derived materials should follow national/international procedures for handling blood potentially contaminated with pathogens. An example of such guidelines is the Universal Precautions available at <http://www.niehs.nih.gov/odhsb/biosafe/univers.htm>. For non-human blood procedures (e.g., ELISAs), standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes (min). If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

¹RSEs are internationally-harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Blood Donor Eligibility

Monocytoid cells from fresh WB are the source of cytokine production in the WB/IL-6 test method as described by Hartung and Wendel (1996), Pool et al. (1998), and Schindler et al. (2006). In the United States (U.S.), the collection of blood and blood components for transfusion and further manufacture (including the use of resulting monocytes in a licensed test) is currently regulated under Section 351 of the Public Health Service (PHS) Act (U.S. Code [U.S.C.], Title 42, Chapter 6A) and/or the Federal Food Drug and Cosmetic Act (U.S.C., Title 21, Chapter 9), both of which require compliance with Current Good Manufacturing Practice (cGMP) regulations (21 CFR Parts 210, 211 and 600-640²).

These regulations and the associated FDA guidance's provide an important resource for information regarding the currently accepted practice for blood manufacture and collection (including donor screening) (<http://www.fda.gov/cber/blood.htm>). Specifically, guidance regarding donor screening questionnaires and links to currently acceptable questionnaires can be found at <http://fda.gov/cber/gdlns/donorshitques.htm#iv>. If you have any questions regarding the application of blood regulations to your specific situation³, it is recommended that you e-mail the Manufacturers Assistance and Technical Training (MATT) Branch established by FDA at matt@cber.fda.gov for advice.

Any participating blood establishment should address how the unused components of blood donations will be accounted for and ultimately destroyed, and if the establishment will store the blood preparation, describe the storage procedures to be followed.

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA procedure, the materials that will be in close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be sterile and pyrogen-free.

3.2.1 Blood Incubation

3.2.1.1 *Equipment*

- Centrifuge
- Hood; Bio-safety, laminar flow (recommended)
- Incubator; cell culture (37±1°C + 5% CO₂)

²The requirements for WB can be found at 21 CFR 640.1 *et seq.* In addition, there are specific regulations applicable to red blood cells, platelets, and other blood components. See, for example, 21 CFR 640.10-640.27. Other regulations applicable to the manufacture of blood and blood components include 21 CFR Part 606, the cGMP requirements for blood and blood components, 21 CFR 610.40, the requirements for testing of WB donations, and 21 CFR 640.3, the requirements for determining the suitability of the donor. Blood that enters into U.S. interstate commerce should be tested for antibodies to HIV 1/2, HCV, HTLV I and II, HBc, HBsAg and RPR, WNV and Chagas.

³The collection of blood for research and development purposes or as a component of an *in vitro* test (that is not subject to licensure) may potentially not be required to adhere to the FDA regulations outlined above.

- Pipetter, multichannel (8- or 12-channel)
- Pipetters, single-channel adjustable (20 and 200 μ L)
- Repeating pipetter
- Vortex mixer

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (1.0 and 2.5 mL)
- Needle set; multily, pyrogen-free, 19 mm, 21 gauge
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- Reservoirs; for blood collection
- Tips; pipetter, sterile, pyrogen-free (20 and 200 μ L)

3.2.2 ELISA

3.2.2.1 *Equipment*

- Microplate mixer
- Microplate reader (450 nm with an optional reference filter in the range of 540-590 nm)⁴
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, 96-well, polystyrene
- Pyrogen-free water (PFW)
- Reservoirs; fluid
- Tips; pipetter, nonsterile
- Tubes; polystyrene (12 mL)

⁴The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

3.2.2.3 ELISA Kit

An ELISA that measures IL-6 release is used. A variety of IL-6 ELISA kits are commercially available and the IL-6 ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. The IL-6 ELISA should be calibrated using an IL-6 international reference standard (e.g., World Health Organization [WHO] 89/548) prior to use. The IL-6 cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-6 reference standard
- PFS
- Stop solution
- TMB⁵/substrate solution

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

3.4 Solutions

ELISA solutions are listed in **Section 3.2**.

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The collection of WB is outlined in **Section 6.1**.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the WB/IL-6 pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control (NSC) and five RSE concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) are included in the incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of

⁵The use of an IL-6 ELISA kit with a chromagen other than TMB is acceptable.

the stock vial by following the instructions provided by the manufacturer (e.g., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL ¹	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration in Tube EU/mL
2000 ^{2,3}	20	1980	20 ⁴
20	100	900	2.0
2.0	500	500	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

Each stock tube should be vortexed prior to its use to make the subsequent dilution.

¹To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.

²A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

³ The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.

⁴This concentration is not used in the assay.

4.2 Interference Test

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.2.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethyl sulfoxide (DMSO) and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be diluted beyond its Maximum Valid Dilution (MVD) (refer to **Section 12.3**). The calculation of the MVD

is dependent on the ELC for a test substance. The ELC can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to blood cells.

4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.2.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 1.0 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin-spiking solutions is shown in **Table 4-2**. For non-spiked solutions, 50 µL of PFS is added to a well followed by 50 µL of WB and mixed by inversion. Then, 50 µL of the test substance (i.e., equivalent to the negative product control [NPC]) is added followed by 100 µL of PFS and the well contents are mixed. Endotoxin-spiked solutions are prepared by adding 50 µL of PFS to each well followed by 50 µL of WB and mixed by inversion. Then, 50 µL of the test substance, 50 µL of an endotoxin-spike solution (1.0 EU/mL), and 50 µL of PFS (i.e., equivalent to the positive product control [PPC]) are added to each well. The contents of the wells are mixed and incubated as outlined in **Section 6.1.3, Steps 6-8**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-6 standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

Sample Addition	Spiked	Non-spiked
	µL/well ¹	
PFS (total volume added)	100 ²	150 ²
Endotoxin-spike solution ³	50	0
Test substance (neat and each serial dilution)	50	50
WB	50	50
Total ⁴	250	250

Abbreviations: PFS = Pyrogen-free saline; WB = Whole blood

¹n=4 replicates each

²50 µL of WB and 50 µL of PFS are added to each well and mixed by inversion prior to the addition of the remaining components and volume of PFS.

³Endotoxin concentration is 1.0 EU/mL in PFS.

⁴A total volume of 250 µL per well is used for the incubation.

The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The spike recovery for each sample dilution is calculated as a percentage by

setting the theoretical value (i.e., endotoxin-spike concentration of 1.0 EU/mL) at 100%. For example, consider the following interference test results in **Table 4-3**:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-6 relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not exceed the MVD for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1.0 EU/mL EC).

4.2.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., WB incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., WB incubated with PFS instead of the test substance) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.4 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-6 Release

6.1.1 Collection of Human Blood

Human volunteers that have met the donor eligibility criteria described in **Section 3.1** are used as the source of WB. All components of the blood collection system (e.g., syringes, tubes, connecting lines) must be sterile and pyrogen-free. WB is drawn by venipuncture⁶ from the medial cubital or cephalic vein of either the right or left arm and collected in a sterile container that contains anticoagulant solution (e.g., heparin). The total volume of blood collected per donor (i.e., up to 500 mL) will be dictated by experimental design and determined by the test method user. WB should be stored at room temperature (RT) and must be used within 4 hr⁷. All subsequent handling of WB should be performed in a laminar flow hood using sterile technique to prevent contamination.

Prior to use in the assay, an equal volume of WB from multiple individual donors should be pooled⁸.

6.1.2 Incubation Plate

Test substances are prepared at a level of dilution that did not show interference with the test system, provided that this dilution does not exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test substances (see Table **6-1**).

⁶WB is obtained using Universal Precautions (e.g., latex gloves, labcoats, safety glasses) and sterile equipment (e.g., syringes, needles, collection tubes) within a hospital or clinical setting by qualified and adequately trained personnel (i.e., registered nurse, licensed phlebotomist, or medical doctor).

⁷Although the ECVAM SOP did not describe the use of cryopreserved WB for the WB/IL-6 test method, the use of cryopreserved WB with the WB/IL-1 test method was outlined and this methodology may also be appropriate for the WB/IL-6 test method, but this has yet to be demonstrated.

⁸Multiple donors (i.e., a minimum of three) should meet the acceptability criteria as outlined in **Section 8.0** either as a pool of multiple individual donors or as multiple individual donors tested independently.

Table 6-1 Overview of Incubation Plate Preparation in the WB/IL-6 Pyrogen Test

Number of Wells	Sample	PFS	EC	Test Sample	WB	Mix the samples; incubate for 16 to 24 hr at 37±1°C in a humidified atmosphere with 5% CO ₂ .	Mix the samples; immediately transfer to an ELISA plate ³ and run ELISA.
20 ¹	EC	100	50	0	50		
4	NSC	150	0	0	50		
56 ²	Test samples (1-14)	100	0	50	50		

Abbreviations: EC = Endotoxin control; IL-6 = Interleukin-6; NSC = Negative saline control; PFS = Pyrogen-free saline WB = Whole blood

¹Five EC concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) in quadruplicate

²14 test samples (n=4 each) per plate

³An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.3 Incubation Assay for IL-6 Release

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. Blood samples are prepared in a microtiter plate using a laminar flow hood. All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

- **Step 1.** Refer to the incubation plate template presented in **Table 6-2**.
- **Step 2.** Using a pipetter, transfer 100 µL of PFS into each well.
- **Step 3.** Transfer 50 µL of test sample or 50 µL of PFS for the NSC into the appropriate wells as indicated in the template.
- **Step 4.** Transfer 50 µL of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.
- **Step 5.** Transfer 50 µL of WB into each well and mix by gently swirling the plate.
- **Step 6.** Mix the contents of the wells thoroughly by gently pipetting up and down several times using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.
- **Step 7.** Place the covered plate in a tissue culture incubator for 16 to 24 hr at 37±1°C in a humidified atmosphere containing 5% CO₂.

- **Step 8.** Prior to transferring the test samples to the ELISA plate, mix the contents of the wells by pipetting up and down using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	Void ³	Void
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

¹ EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.

² TS number (e.g., TS 1) represents an arbitrary sequence for individual test substances.

³ Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-6 Release

6.2.1 IL-6 Standard Curve

An IL-6 standard, supplied with the ELISA kit, is used. IL-6 standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in PFS to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 µL. Each well on the ELISA plate will receive 50 µL of an IL-6 blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed by heating them in a water bath. A suggested ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, an eight-point IL-6 standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-6 standard curve is prepared as described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4**.

Step 1. After pipetting up and down three times to mix the supernatant, transfer 50 µL from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 2. Add 50 µL of each IL-6 standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 3. Add 200 µL of the enzyme-labeled detection antibody (neat as supplied, or diluted, if necessary) to each of the wells.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 2 to 3 hr at RT.

Step 5. Decant and wash each well three times with 300 µL Buffered Wash Solution and then rinse three times with deionized water. Place the plates upside down and tap to remove water.

Step 6. Add 200 µL of TMB/Substrate Solution to each well and incubate at RT in the dark for 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 µL of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD₄₅₀ within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 540 to 590 nm is recommended.⁹

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6 ³ 0	IL-6 0
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	IL-6 500	IL-6 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-6 1000	IL-6 1000
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-6 2000	IL-6 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-6 4000	IL-6 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

¹EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.

²TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

³IL-6 values in columns 11 and 12 are in pg/mL.

⁹The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-4 Overview of ELISA Procedure

Material transfer from Incubation Plate (µL)	IL-6 standard (0 to 4000 pg/mL) (µL)	Enzyme-labeled Antibody (µL)		Decant and wash each well three times with 300 µL Buffered Wash Solution and three times with deionized water.	TMB/Substrate Solution (µL)	Incubate for less than 15 min at RT in the dark.	Stop Solution (µL)	Read each well at OD ₄₅₀ with a 540 to 590 nm reference filter.
50	50	200	Cover the Incubation Plate and incubate for 2 to 3 hr at RT.		200		50	

Abbreviations: OD₄₅₀ = Optical density at 450 nm; RT = Room temperature

7.0 EVALUATION OF TEST METHODS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD₄₅₀) with a 540 to 590 nm reference filter (recommended)¹⁰. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6 standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-6 standard curve produces an $r \geq 0.95$ ¹¹ and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

Blood donors (or a pool of blood donors) are considered to be high responders if their concentration of IL-6 is greater than 200 pg/mL. High responders should be excluded from analysis. The preparation being examined is required to pass the test with blood donations from at least three different donors (i.e., either as a pool of three individual donors or as three individual donors tested independently).

¹⁰The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

¹¹Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

An outlying observation that represents either a pool of multiple independent donors or a single individual donor may be excluded if there is confirmation that the accuracy of the medical information provided by an individual donor is suspect, or if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett and Lewis 1994], Grubbs' test [Barnett and Lewis 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance
- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)
- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, resuspension solvent)

Justification of the Test Method and the Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time
- If the test method employs proprietary components, documentation on the procedure used to ensure their integrity from “lot-to-lot” and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure used

- Description of any modification to the test procedure
- Reference to historical data of the model
- Description of the evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)¹

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)^{1,2}

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M , where:

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product were used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)^{1,2}

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is¹:

$$\text{MVD} = (\text{ELC} \times \text{Product Potency [PP]})/\lambda$$

As an example, for Cyclophosphamide Injection, the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is¹:

$$\text{MVD} = \text{PP}/\text{Minimum Valid Concentration (MVC)}$$

$$\text{where, MVC} = (\lambda \times M)/K$$

where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

¹From FDA (1987)

²From USP (2007)

12.5 Parenteral Threshold Pyrogen Dose (K)^{1,2}

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, the PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces $\frac{1}{2}$ the maximal increase in optical density (OD) from the endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.7 Product Potency (PP)^{1,2}

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)^{1,2}

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.

Appendix C4

The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test

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ICCVAM Final Recommended Protocol for Future Studies Using the Human Peripheral Blood Mononuclear Cell (PBMC)/Interleukin (IL)-6 *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-6 from human peripheral blood mononuclear cells (PBMCs). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)¹ PBMC/IL-6 Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), and 2) information provided to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ICCVAM BRD includes the ECVAM Standard Operating Procedures (SOPs) for the PBMC/IL-6 test (could be referred to as Monocyte Activation Test), which is based on various methods that use human PBMCs to detect cytokine production as a measure of pyrogen presence (Bleeker et al. 1994; Dinarello et al. 1984; Poole et al. 2003). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOPs is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the PBMC/IL-6 pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (<http://iccvam.niehs.nih.gov>) to ensure that the most current protocol is used.

¹ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

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Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOPs for the PBMC/IL-6 Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM Catch-Up Validation SOP ¹	ECVAM Validation SOP ¹
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test at MVD	Test at MVD
Number of Blood Donors	Minimum of 3 (independent or pooled)	Minimum of 3 ² (independent)	Minimum of 4 (independent)
Decision Criteria for Interference	Mean OD ³ of PPC is 50% to 200% of 0.25 EU/mL EC	Mean OD of PPC is 50% to 200% of 0.25 EU/mL EC	Mean OD of PPC is 50% to 200% of 0.25 EU/mL EC
Incubation Plate for ELISA (The number of samples or controls measured in quadruplicate)	NSC (1)	NSC (1)	NSC (1)
	EC (5)	EC (5)	EC (5)
	TS (14)	TS (2) x EC (5) spikes = 10 TS	TS (2) x EC (5) spikes = 10 TS
	PPC ⁴ (0)	PPC (2) = 2 TS	PPC (2) = 2 TS
	NPC ⁴ (0)	NPC (2) = 2TS	NPC (2) = 2TS
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Includes seven point IL-6 SC and blank in duplicate	Includes seven point IL-6 SC and blank in duplicate
Assay Acceptability Criteria	Mean OD of NSC ≤ 0.15	Mean OD of NSC ≤ 0.15	Mean OD of NSC ≤ 0.15
	Quadratic function of IL-6 SC $r \geq 0.95$ ⁵	Quadratic function of IL-6 SC $r \geq 0.95$	Quadratic function of IL-6 SC $r \geq 0.95$
	EC SC produces OD values that ascend in a sigmoidal concentration response	EC SC produces OD values that ascend in a sigmoidal concentration response	EC SC produces OD values that ascend in a sigmoidal concentration response
	High responder blood donors (i.e., > 200 pg/mL IL-6) or low responder blood donors (i.e., Mean OD of 1EU/mL EC is significantly less than that of 1000 pg/mL IL-6) may be excluded	High responder blood donors (i.e., > 200 pg/mL IL-6) or low responder blood donors (i.e., Mean OD of 1EU/mL EC is significantly less than that of 1000 pg/mL IL-6) may be excluded	High responder blood donors (i.e., > 200 pg/mL IL-6) or low responder blood donors (i.e., Mean OD of 1EU/mL EC is significantly less than that of 1000 pg/mL IL-6) may be excluded
	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC ⁶ TS	Endotoxin concentration TS > ELC TS	Endotoxin concentration TS > ELC TS ⁷

Abbreviations: EC = Endotoxin control; ELC = Endotoxin limit concentration; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-6 = Interleukin-6; MVD = Maximum valid dilution; NSC = Negative saline control; OD = Optical density; PBMC = Peripheral blood mononuclear cell; PPC = Positive product control; SC = Standard curve; SOP = Standard operating procedure; TS = Test substance

¹ECVAM PBMC/IL-6 SOPs are presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).

²Sample are cryopreserved prior to use in the assay.

³Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

⁴In the ICCVAM PBMC/IL-6 protocol, PPC and NPC are assessed in the interference test described in **Section 4.2**, which is performed prior to the ELISA.

⁵Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

⁶Where unknown, the ELC is calculated (see **Section 12.2**).

⁷Decision criteria for individual donors were defined in the ECVAM Validation SOP for the PBMC/IL-6 test method. Test method users should refer to these criteria if multiple donors are tested independently.

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of interleukin (IL)-6 from human peripheral blood mononuclear cells (PBMCs). The concentration of IL-6 released by incubation of PBMCs with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-6. The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by PBMCs exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)¹ or an equivalent standard expressed in Endotoxin Units (EU)/mL. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures that use human blood-derived materials should follow national/international procedures for handling blood potentially contaminated with pathogens. An example of such guidelines is the Universal Precautions available at <http://www.niehs.nih.gov/odhsb/biosafe/univers.htm>. For non-human blood procedures (e.g., ELISAs), standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'- TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes (min). If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

¹RSEs are internationally-harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Blood Donor Eligibility

PBMCs from fresh whole blood (WB) are the source of cells for cytokine production in the PBMC/IL-6 test method as reported by Poole et al. (2003)². In the United States (U.S.), the collection of blood and blood components for transfusion and further manufacture (including the use of resulting PBMCs in a licensed test) is currently regulated under Section 351 of the Public Health Service (PHS) Act (U.S. Code [U.S.C.], Title 42, Chapter 6A) and/or the Federal Food Drug and Cosmetic Act (U.S.C., Title 21, Chapter 9), both of which require compliance with Current Good Manufacturing Practice (cGMP) regulations (21 CFR Parts 210, 211 and 600-640³).

These regulations and the associated FDA guidance's provide an important resource for information regarding the currently accepted practice for blood manufacture and collection (including donor screening) (<http://www.fda.gov/cber/blood.htm>). Specifically, guidance regarding donor screening questionnaires and links to currently acceptable questionnaires can be found at <http://www.fda.gov/cber/gdlns/donorhistques.htm#iv>. If you have questions regarding the applicability of blood regulations to your specific situation⁴, it is recommended that you e-mail the Manufacturers Assistance and Technical Training (MATT) Branch established by FDA at matt@cber.fda.gov for advice.

Any participating blood establishment should address how unused components of blood donations will be accounted for and ultimately destroyed, and if the establishment will store the blood preparation, describe the storage procedures to be followed.

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA procedure, the materials that will be in close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be sterile and pyrogen-free.

3.2.1 Preparation of PBMCs

3.2.1.1 *Equipment*

- Centrifuge
- Hood; Bio-safety, laminar flow (recommended)

²As indicated by the ECVAM Catch-Up Validation SOP for the PBMC/IL-6 test method, PBMCs that have been cryopreserved can also be used as the source of cells in the PBMC/IL-6 test method.

³The requirements for WB can be found at 21 CFR 640.1 *et seq.* In addition, there are specific regulations applicable to red blood cells, platelets, and other blood components. See, for example, 21 CFR 640.10-640.27. Other regulations applicable to the manufacture of blood and blood components include 21 CFR Part 606, the cGMP requirements for blood and blood components, 21 CFR 610.40, the requirements for testing of WB donations, and 21 CFR 640.3, the requirements for determining the suitability of the donor. Blood that enters into U.S. interstate commerce should be tested for antibodies to HIV 1/2, HCV, HTLV I and II, HBc, HBsAg and RPR, WNV and Chagas.

⁴The collection of blood for research and development purposes or as a component of an *in vitro* test (that is not subject to licensure) may potentially not be required to adhere to the FDA regulations outlined above.

- Incubator; cell culture ($37\pm 1^{\circ}\text{C} + 5\% \text{CO}_2$)
- Lymphoprep™
- Pipetter; multichannel (8- or 12-channel)
- Pipettors; single-channel adjustable (20, 200, and 1000 μL)
- Repeating pipetter
- Vortex mixer

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (2.5 and 5.0 mL)
- Cryotubes; screw-cap, 2 mL
- Filters; sterile, 0.22 μm
- Needle set; multify, pyrogen-free, 19 mm, 21 gauge
- Phosphate buffered saline (PBS); sterile
- Pipettes; serological, sterile (5, 10, and 25 mL)
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- Reservoirs; for blood collection
- RPMI-1640 cell culture medium (500 mL); supplemented with the following reagents to yield RPMI-C
 - Human serum albumin (HSA); 5 mL or a 1% final concentration)
 - L-Glutamine; 200 mM
 - Penicillin/streptomycin (10,000 IU/mL penicillin, 10 mg/mL streptomycin)
- Syringes; sterile (100 μL and 30 mL)
- Tips; pipetter, sterile, pyrogen-free (20, 200, and 1000 μL)

3.2.2 ELISA

3.2.2.1 *Equipment*

- Microplate mixer

- Microplate reader (450 nm with an optional reference filter in the range of 540-590 nm)⁵
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, 96-well, polystyrene
- Pyrogen-free water (PFW)
- Reservoirs; fluid
- Tips; pipetter, nonsterile
- Tubes; polystyrene (12 mL)

3.2.2.3 *ELISA Kit*

An ELISA that measures IL-6 release is used. A variety of IL-6 ELISA kits are commercially available and the IL-6 ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. The IL-6 ELISA should be calibrated using an IL-6 international reference standard (e.g., World Health Organization [WHO] 89/548) prior to use. The IL-6 cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-6 reference standard
- PFS
- Stop solution
- TMB⁶/substrate solution

⁵The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

⁶The use of an IL-6 ELISA kit with a chromagen other than TMB is acceptable.

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

3.4 Solutions

- RPMI-C cell culture medium; supplemented as described in **Section 3.2.1.2**

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The collection of WB, the isolation of PBMCs from WB, and the procedure for cryopreservation of PBMCs is outlined in **Section 6.1**.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the PBMC/IL-6 pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control (NSC) and five RSE concentrations (0.063, 0.125, 0.25, 0.50, and 1.0 EU/mL) are included in the incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL ¹	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000 ^{2,3}	40	3960	20 ⁴
20	100	1900	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0.125	500	500	0.063
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

Each stock tube should be vortexed vigorously prior to its use to make the subsequent dilution.

¹To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.

²A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

³The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.

⁴This concentration is not used in the assay.

To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of the stock vial by following the instructions provided by the manufacturer (e.g., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when

stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

4.2 Interference Test

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.2.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethyl sulfoxide (DMSO) and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be diluted beyond its Maximum Valid Dilution (MVD) (refer to **Section 12.3**). The calculation of the MVD is dependent on the ELC for a test substance. The ELC can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to blood cells.

4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.2.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 0.25 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin-spiking solutions is shown in **Table 4-2**. For non-spiked solutions, 150 µl of RPMI-C is added to a well followed by 50 µl of the test substance (i.e., equivalent to the negative product control [NPC]) and 50 µL of PBMCs and the well contents are mixed. Endotoxin-spiked solutions are prepared by adding 100 µL of RPMI-C to each well followed by 50 µL of the test substance, and 50 µL of an endotoxin-spike solution (0.25 EU/mL) (i.e., equivalent to the positive product control [PPC]). Finally, 50 µL of PBMCs are added to each well and the wells are mixed and incubated as outlined in **Section 6.1.3, Steps 6-8**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-6 standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

Sample Addition	Spiked	Non-spiked
	µL/well ¹	
RPMI-C	100	150
Endotoxin-spike solution ²	50	0
Test substance (neat and each serial dilution)	50	50
PBMCs ³	50	50
Total ⁴	250	250

Abbreviations: PBMC = Peripheral blood mononuclear cells

¹n=4 replicates each

²Endotoxin concentration is 0.25 EU/mL in RPMI-C.

³PBMCs are resuspended in RPMI-C (1 x 10⁶ cells/mL).

⁴A total volume of 250 µL per well is used for the incubation.

The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The spike recovery for each sample dilution is calculated as a percentage by setting the theoretical value (i.e., endotoxin-spike concentration of 0.25 EU/mL) at 100%. For example, consider the following interference test results in **Table 4-3**:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-6 relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not to exceed the MVD, for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance to be used in the *in vitro* pyrogen test would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 0.25 EU/mL EC).

4.2.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., PBMCs incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., PBMCs incubated with PFS instead of the test substance) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.4 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-6 Release

6.1.1 Collection of Human Blood

Human volunteers that have met the donor eligibility criteria described in **Section 3.1** are used as the source of WB. All components of the blood collection system (e.g., syringes, tubes, connecting lines) must be sterile and pyrogen-free. WB is drawn by venipuncture⁷ from the medial cubital or cephalic vein of either the right or left arm and collected in a sterile container that contains anticoagulant solution (e.g., heparin). The total volume of

⁷WB is obtained using Universal Precautions (e.g., latex gloves, labcoats, safety glasses) and sterile equipment (e.g., syringes, needles, collection tubes) within a hospital or clinical setting by qualified and adequately trained personnel (i.e., registered nurse, licensed phlebotomist, or medical doctor).

blood collected per donor (i.e., up to 500 mL) will be dictated by experimental design and determined by the test method user. All subsequent handling of WB should be performed in a laminar flow hood using sterile technique to prevent contamination.

6.1.1.1 *Isolation of PBMCs from WB*

PBMCs are isolated from WB using density gradient centrifugation. The PBMC suspension must be isolated within 2 hr of WB collection using Lymphoprep™. The isolated PBMC suspension may be used immediately (**Section 6.1.2**) or frozen for later use (**Section 6.1.1.3**). The isolation procedure described below is a modification of the manufacturer's instructions as outlined in the ECVAM SOP for the PBMC/IL-6 pyrogen test.

To form a lower, denser layer, 15 mL of PBS and 20 mL of Lymphoprep™ should be added to each tube containing 15 mL of WB. The tubes are then centrifuged at 340 x g for 45 min at RT. After centrifugation, a white band of PBMCs should be visible at approximately the 25 mL graduation mark on the tube. If cryopreservation of PBMCs is to be performed (see **Section 6.1.1.3**), carefully remove 18 mL of supernatant above the PBMC band and transfer it to a new tube for preparing a cryoprotective solution. The remaining supernatant above the PBMC band should be aspirated and discarded. Using a 10 mL pipet, transfer the PBMC layer to a new centrifuge tube.

6.1.1.2 *Washing PBMCs*

The PBMCs are resuspended in a total volume of 50 mL of PBS and centrifuged at 340 x g for 15 min. The supernatant is poured off and the cellular sediment resuspended in 10 mL of PBS by pipetting up and down several times with a serological pipet. The total volume in each tube is adjusted to 50 mL with PBS and centrifuged at 340 x g for 10 min. After centrifugation, the PBMCs should be resuspended in RPMI-C and an equal volume of cell suspension from multiple individual donors should be pooled⁸.

Prior to use in the assay, the pooled PBMCs should be examined under a microscope to determine that the morphology of the cells is consistent with the appearance of cells that previously yielded acceptable results. It is advisable that cell number and cell viability be determined using appropriate methods (e.g., hemocytometer and vital dye or flow cytometer and fluorescent marker). The cell count of the PBMC suspension should be adjusted to 1 x 10⁶ cells/mL in RPMI-C. The percentage of viable PBMCs should exceed 80% for their inclusion in the test. The results of these examinations should be included in the study report. If PBMCs are prepared from fresh WB, then the cell suspension must be used in the assay within 4 hr from the time of WB collection.

6.1.1.3 *Procedure for Cryopreservation and Thawing of PBMCs*

To freeze the PBMCs, prepare a cryoprotective solution by adding 2 mL of pyrogen-free DMSO to the supernatant (18 mL) collected in the centrifugation procedure outlined in **Section 6.1.1.1**. Cool the cryoprotective solution to between 2 and 8°C. Centrifuge the isolated PBMCs as instructed in **Section 6.1.1.2** and then add 6 mL of the chilled cryoprotective solution to the cell sediment and prepare aliquots in cryotubes. The cryotubes

⁸Multiple donors (i.e., a minimum of three) should meet the acceptability criteria as outlined in **Section 8.0** either as a pool of multiple individual donors or as multiple individual donors tested independently.

are placed in a Styrofoam box for thermal insulation and slowly frozen in a -80°C freezer. After 72 hr, the tubes can be transferred to liquid nitrogen for prolonged storage.

To thaw the cryopreserved PBMCs, submerge the tubes in a water bath at 37±1°C. After thawing, the cell suspensions are pooled in a single 50 mL centrifuge tube and RPMI-C is added to give a total volume of 40 mL. The PBMCs are centrifuged at 340 x g for 10 min, the supernatant removed, and the cells resuspended in 10 mL of RPMI-C.

Prior to use in the assay, it is advisable that cell number and cell viability be examined as described in **Section 6.1.1.2**. The cell count of the PBMC suspension should be adjusted to 1 x 10⁶ cells/mL in RPMI-C. The percentage of viable PBMCs should exceed 80% for their inclusion in the test. The results of this examination should be included in the study report.

6.1.2 Incubation Plate

Test substances are prepared at a level of dilution that did not show interference with the test system, provided that this dilution does not exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test samples (see **Table 6-1**).

Table 6-1 Overview of Incubation Plate Preparation in the PBMC/IL-6 Pyrogen Test

Number of Wells	Sample	RPMI-C	EC	Test Sample	PBMCs	Mix the samples; incubate for 16 to 24 hr at 37±1°C in a humidified atmosphere with 5% CO ₂ .	Mix the samples; immediately transfer to an ELISA plate ⁴ and run ELISA.
		µL					
20 ¹	EC	100	50	0	100		
4	NSC	100	0	0 ²	100		
56 ³	Test samples (1-14)	100	0	50	100		

Abbreviations: EC = Endotoxin control; IL-6 = Interleukin-6; NSC = Negative saline control; PBMC = Peripheral blood mononuclear cell

¹ Five EC concentrations (0.063, 0.125, 0.25, 0.50, and 1.0 EU/mL) in quadruplicate

² 50 µL of PFS is added instead of the test sample.

³ 14 test samples (n=4 each) per plate

⁴ An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.3 Incubation Assay for IL-6 Release

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. PBMC samples are prepared in a microtiter plate using a laminar flow hood (refer to **Section 6.1.1**). All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled

appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

Step 1. Refer to the suggested incubation plate template presented in **Table 6-2**.

Step 2. Using a pipetter, transfer 100 µL of RPMI-C into each well.

Step 3. Transfer 50 µL of test sample or 50 µL of PFS for the NSC into the appropriate wells as indicated in the template.

Step 4. Transfer 50 µL of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.

Step 5. Transfer 100 µL of a well-mixed PBMC suspension into each well and mix by gently swirling the plate.

Step 6. Mix the contents of the wells thoroughly by pipetting up and down several times using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Step 7. Place the covered plate in a tissue culture incubator for 16 to 24 hr at 37±1°C in a humidified atmosphere containing 5% CO₂.

Step 8. Prior to transferring the test samples to the ELISA plate, mix the contents of the wells by pipetting up and down using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 1.0	EC 1.0	EC 1.0	EC 1.0	TS3	TS3	TS3	TS3	TS11	TS11	Void ³	Void
B	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.063	EC 0.063	EC 0.063	EC 0.063	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

¹EC value (e.g., EC 1.0) represents the endotoxin concentration in EU/mL.

²TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

³Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-6 Release

6.2.1 IL-6 Standard Curve

An IL-6 standard, supplied with the ELISA kit, is used. IL-6 standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in RPMI-C to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 μ L. Each well on the ELISA plate will receive 50 μ L of an IL-6 blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed by heating them in a water bath. A suggested ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, an eight-point IL-6 standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-6 standard curve is prepared as described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4**.

Step 1. After pipetting up and down very carefully three times (avoid detachment of the adherent PBMCs) to mix the supernatant, transfer 50 μ L from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 2. Add 50 μ L of each IL-6 standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 3. Add 200 μ L of the enzyme-labeled detection antibody (neat as supplied, or diluted, if necessary) to each of the wells.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 2 to 3 hr at RT.

Step 5. Decant and wash each well three times with 300 μ L Buffered Wash Solution and then rinse three times with deionized water. Place the plates upside down and tap to remove water.

Step 6. Add 200 μ L of TMB/Substrate Solution to each well and incubate at RT in the dark for 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 μ L of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD₄₅₀ within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 540 to 590 nm is recommended.⁹

⁹The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 1.0	EC 1.0	EC 1.0	EC 1.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6 ³ 0	IL-6 0
B	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.063	EC 0.063	EC 0.063	EC 0.063	TS7	TS7	TS7	TS7	TS13	TS13	IL-6 500	IL-6 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-6 1000	IL-6 1000
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-6 2000	IL-6 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-6 4000	IL-6 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

¹EC value (e.g., EC 1.0) represents the endotoxin concentration in EU/mL.

²TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

³IL-6 values in columns 11 and 12 are in pg/mL.

Table 6-4 Overview of ELISA Procedure

Material transfer from Incubation Plate (µL)	IL-6 standard (0 to 4000 pg/mL) (µL)	Enzyme-labeled Antibody (µL)	Cover the Incubation Plate and incubate for 2 to 3 hr at RT.	Decant and wash each well three times with 300 µL Buffered Wash Solution and three times with deionized water.	TMB/Substrate Solution (µL)	Incubate for less than 15 min at RT in dark.	Stop Solution (µL)	Read each well at OD ₄₅₀ with a 540 to 590 nm reference filter.
50	50	200			200		50	

Abbreviations: OD₄₅₀ = Optical density at 450 nm; RT = Room temperature

7.0 EVALUATION OF TEST METHODS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD₄₅₀) with

a 540 to 590 nm reference filter (recommended)¹⁰. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6 standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-6 standard curve produces an $r \geq 0.95$ ¹¹ and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

Blood donors (or a pool of blood donors) are considered to be low responders if their OD₄₅₀ value obtained for 1.0 EU/mL EC is below the OD₄₅₀ value obtained for 1000 pg/mL IL-6. Blood donors (or a pool of blood donors) who produce an OD₄₅₀ value for the NSC that is above the OD₄₅₀ value at 500 pg/mL IL-6 are considered to be high responders. Low and high responders should be excluded from analysis. The preparation being examined is required to pass the test with blood donations from at least three different donors (i.e., either as a pool of three individual donors or as three individual donors tested independently).

An outlying observation that represents either a pool of multiple independent donors or a single individual donor may be excluded if there is confirmation that the accuracy of the medical information provided by an individual donor is suspect, or if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett and Lewis 1994], Grubbs' test [Barnett and Lewis 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection¹²

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance

¹⁰The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

¹¹Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

¹²Decision criteria for individual donors were defined in the ECVAM SOP for the PBMC/IL-6 test method. Test method users should refer to these criteria if multiple donors are tested independently.

- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)
- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, resuspension solvent)

Justification of the In Vitro Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time
- If the test method employs proprietary components, documentation on the procedure used to ensure their integrity from “lot-to-lot” and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure used
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

11.0 REFERENCES

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)¹

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)^{1,2}

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M , where:

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product is used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)^{1,2}

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is¹:

$$\text{MVD} = (\text{ELC} \times \text{Product Potency [PP]})/\lambda$$

As an example, for Cyclophosphamide Injection, the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is¹:

$$\text{MVD} = \text{PP}/\text{Minimum Valid Concentration (MVC)}$$

$$\text{where, MVC} = (\lambda \times M)/K$$

where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

¹From FDA (1987)

²From USP (2007)

12.5 Parenteral Threshold Pyrogen Dose (K)^{1,2}

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, the PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces $\frac{1}{2}$ the maximal increase in optical density (OD) from the endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.7 Product Potency (PP)^{1,2}

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)^{1,2}

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.

Appendix C5

The Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6 *In Vitro* Pyrogen Test

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ICCVAM Final Recommended Protocol for Future Studies Using the Monocytoid Cell Line Mono Mac 6 (MM6)/Interleukin (IL)-6 *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-6 from the monocytoid cell line Mono Mac 6 (MM6). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)¹, MM6/IL-6 Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), and 2) information provided to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ICCVAM BRD includes the ECVAM Standard Operating Procedures (SOPs) for the MM6/IL-6 test (could be referred to as Monocyte Activation Test), which are based on the methodology published by Taktak et al. (1991). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOPs is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the MM6/IL-6 pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (<http://iccvam.niehs.nih.gov>) to ensure that the most current protocol is used.

¹ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

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Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOPs for the MM6/IL-6 Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM SOP ¹	ECVAM Validation SOP ¹
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test neat or at minimal dilution that produces no interference	Test at MVD
Decision Criteria for Interference	Mean OD ² of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC
Incubation Plate for ELISA (The number of samples or controls in quadruplicate)	NSC (1)	NSC (1)	NSC (1)
	EC (5)	EC (5)	EC (5)
	TS (14)	TS (14)	TS (2) x EC (5) spikes = 10 TS
	PPC ³ (0)	PPC (0)	PPC (2) = 2 TS
	NPC ³ (0)	NPC (0)	NPC (2) = 2 TS
	PC ⁴ (0)	PC (0)	PC (1) = 1 TS
	NC ⁴ (0)	NC (0)	NC (1) = 1 TS
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Includes seven point IL-6 SC and blank in duplicate	Not included
Assay Acceptability Criteria	Quadratic function of IL-6 SC $r \geq 0.95^5$	Not included	Not included
	Mean OD of NSC ≤ 0.15	Not included	Not included
	EC SC produces OD values that ascend in a sigmoidal concentration response	Endotoxin concentration (0.5 IU/mL) > background (defined as the mean +2SD (n-1))	Mean OD of each EC > Mean OD of next lower EC concentration (minimum of 4 data points needed for valid SC)
	Not included	Not included	PC = $\pm 20\%$ of the theoretical value
	Not included	Not included	OD NC < 0.200
	Not included	Not included	OD PC > LOQ ⁶
	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC ⁷ TS	Endotoxin concentration TS > ELC TS	OD TS > OD 0.5 EU/mL EC

Abbreviations: EC = Endotoxin control; ELC = Endotoxin limit concentration; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-6 = Interleukin-6; IU = International units; LOQ = Limit of quantification; MM6 = Mono Mac 6; MVD = Maximum valid dilution; NC = Negative control; NPC = Negative product control; NSC = Negative saline control; OD = Optical density; PC = Positive control;

PPC = Positive product control; SC = Standard curve; SD = Standard deviation; SOP = Standard operating procedure; TS = Test substance

¹ECVAM MM6/IL-6 SOP and ECVAM MM6/IL-6 Validation SOP are presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).

²Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

³In the ICCVAM MM6/IL-6 protocol, PPC and NPC are assessed in the interference test described in **Section 4.3**, which is performed prior to the ELISA. In the ECVAM SOPs, PPC and NPC were only included in the ECVAM validation study.

⁴PC and NC were only included in the ECVAM validation study. PC is 50 pg/mL endotoxin in saline. NC is 0.9% saline.

⁵Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

⁶LOQ is the mean OD of the NSC + 10x the SD of the mean OD for the NSC.

⁷Where unknown, the ELC is calculated (See **Section 12.2**).

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of IL-6 from Mono Mac 6 (MM6) cells, a human cell line derived from a patient with acute monocytic leukemia (Zeigler-Heitbrock et al. 1988). The concentration of IL-6 released by incubation of MM6 cells with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-6. The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by MM6 cells exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)¹ or an equivalent standard expressed in Endotoxin Units (EU)/mL. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PROCEDURES

All procedures should be performed following standard laboratory precautions, including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes (min). If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

¹RSEs are internationally harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

3.0 MATERIALS, EQUIPMENT AND SUPPLIES

3.1 Source of Cells

The MM6 cell line is a human monocytic cell line originally described by Professor H.W.L. Ziegler-Heitbrock at the Institute for Immunology, University of Munich, Germany (Ziegler-Heitbrock et al. 1988). The MM6 cell line may be purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, <http://www.dsmz.de>) by individuals working at non-profit organizations. Prior to transaction, a legal agreement must be reached with Professor H.W.L. Ziegler-Heitbrock stating that the cells will be used for research purposes only. Any contract research organization or pharmaceutical company wanting to obtain the MM6 cell line must contact Professor H.W.L. Ziegler-Heitbrock to negotiate a fee for provision and a royalty payment per batch of product tested. Contact information for Professor H.W.L. Ziegler-Heitbrock is as follows: Professor Dr. H.W.L. Ziegler-Heitbrock, University of Leicester, Dept. of Microbiology, University Road, Leicester LE1 9HN, United Kingdom, e-mail: ziehei@gmx.de.

MM6 cells should be maintained according to the instructions provided by the DSMZ and Professor Dr. H.W.L. Ziegler-Heitbrock, which should stipulate the permissible limit to the passage number for these cells.

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA procedure, the materials that will be in close contact with samples (e.g., pipet tips, containers, solutions) should be sterile and pyrogen-free.

3.2.1 Utilization of MM6 cells

3.2.1.1 Equipment

- Centrifuge
- Hood; Bio-safety, laminar flow (recommended)
- Incubator; cell culture ($37\pm 1^{\circ}\text{C} + 5\% \text{CO}_2$)
- Inverted Microscope
- pH meter
- Pipetter; multichannel (8- or 12-channel)
- Pipettors; single-channel adjustable (20, 200, and 1000 μL)
- Repeating pipetter
- Vortex mixer
- Water bath

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (1.0 and 2.5 mL)

- Cryotubes; screw-cap (2 mL)
- Filters; sterile, 0.22 µm
- Flasks; tissue culture
- Phosphate buffered saline (PBS); sterile
- Pipettes; sterile
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- RPMI-1640 cell culture medium supplemented as described in **Section 4.3** to yield either RPMI-C or RPMI-M
- Tips; pipetter, sterile, pyrogen-free (20 and 200 µL)
- Tubes; polystyrene

3.2.2 ELISA

3.2.2.1 *Equipment*

- Microplate mixer
- Microplate reader (450 nm with an optional reference filter in the range of 540-590 nm)²
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, 96-well, polystyrene
- Pyrogen-free water (PFW)
- Reservoirs; fluid
- Tips; pipetter, sterile and nonsterile
- Tubes; polystyrene (12 mL)

3.2.2.3 *ELISA Kit*

An ELISA that measures IL-6 release is used. A variety of IL-6 ELISA kits are commercially available and the IL-6 ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. The IL-6 ELISA should be calibrated using an IL-6

² The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

international reference standard (e.g., World Health Organization [WHO] 89/548) prior to use. The IL-6 cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-6 reference standard
- PFS
- Stop solution
- TMB³/substrate solution

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; U.S. Food and Drug Administration [FDA] *E. coli* Lot EC6)

3.4 Solutions

- RPMI-1640 cell culture medium; supplemented as described in **Section 4.3**

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The preparation of MM6 cells for use in the assay is outlined in **Section 6.1**.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the MM6/IL-6 pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control (NSC) and five RSE concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) are included in the incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of the stock vial by following the instructions provided by the manufacturer (e.g., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in

³The use of an IL-6 ELISA kit with a chromagen other than TMB is acceptable.

a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL ¹	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000 ^{2,3}	40	3960	20 ⁴
20	100	900	2.0
2.0	500	500	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

Each stock tube should be resonicated and vortexed vigorously before the subsequent dilution.

¹To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.

²A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

³The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.

⁴This concentration is not used in the assay.

4.2 Cell Culture Medium

MM6 cells are maintained in RPMI containing 10% FBS, denoted as RPMI-M. For use in the ELISA procedure, the concentration of FBS is reduced to 2% and referred to as RPMI-C. Each medium is prepared and stored as described by the manufacturer.

4.2.1 RPMI-M

- Bovine insulin; 0.23 IU/mL
- FBS; heat-inactivated at 55±1°C (50 mL or a 10% final concentration)
- HEPES buffer; 20 mM
- L-Glutamine; 2 mM
- MEM non-essential amino acids; 0.1 mM
- Oxaloacetic acid; 1 mM
- Penicillin/streptomycin (10,000 IU/mL penicillin, 10 mg/mL streptomycin)
- RPMI-1640 medium (500 mL)
- Sodium pyruvate; 1 mM

4.2.2 Starting a Culture of MM6 Cells

To initiate a culture of MM6 cells, remove a vial of the primary stock from liquid nitrogen. Thaw the vial on ice. Gently mix and transfer the cells to a 50 mL centrifuge tube and add 10 mL of RPMI-M. Centrifuge at 100 x g for 5 min at room temperature (RT). Remove the supernatant and resuspend the cells in ice-cold RPMI-M. Centrifuge at 100 x g for 5 min at RT. Remove the supernatant and resuspend the MM6 cells in 2 mL of RPMI-M. Add 8 mL of RPMI-M to a tissue culture flask and transfer the cell suspension to the flask. Cells should be examined microscopically to ensure that the cells are not clumped together. Place the flasks in a cell culture incubator and maintain the cells at $37\pm 1^{\circ}\text{C} + 5\% \text{CO}_2$.

4.2.3 Propagation of MM6 Cells

Remove the cell culture flask from the incubator and examine the cells under a microscope to determine that the morphology of the cells is consistent with the appearance of MM6 cells that previously yielded acceptable results. Centrifuge at 100 x g for 8 min at RT. Remove the supernatant, resuspend the cell pellet in 4 mL of RPMI-M, and gently pipet up and down to mix. It is advisable that cell number and cell viability be determined using appropriate methods (e.g., hemocytometer and vital dye or flow cytometer and fluorescent marker). The percentage of cell viability should exceed 80% for further propagation. The results of these examinations should be included in the study report. Transfer the cells (2×10^5 cells/mL) to new tissue culture flasks and add RPMI-M. Place the flasks in a cell culture incubator and maintain the cells at $37\pm 1^{\circ}\text{C} + 5\% \text{CO}_2$.

4.2.4 Preparation of a MM6 Cell Bank

To initiate a bank of MM6 cells, centrifuge the cell culture(s) at 100 x g for 8 min at 2 to 8°C . Remove the supernatant and resuspend the cells in FBS at 2 to 8°C . It is advisable to determine cell number and cell viability as outlined in **Section 4.2.3** and adjust the cell concentration to 4×10^6 cells/mL and store on ice for 10 min. Add an equal volume of ice-cold FBS containing 10% dimethyl sulfoxide (DMSO) drop-wise to the cell suspension (final concentration is 2×10^6 cells/mL with 5% DMSO). Transfer the cell suspension to sterile, pyrogen-free cryotubes (1 mL/tube). Place the tubes in a well-insulated polystyrene box and store in a -80°C freezer for greater than 48 hours (hr) and then transfer to a liquid nitrogen container.

4.3 **Interference Test**

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.3.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in DMSO and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be

diluted beyond its Maximum Valid Dilution (MVD) (refer to **Section 12.3**). The calculation of the MVD is dependent on the ELC for a test substance. The ELC can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to MM6 cells.

4.3.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.3.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 1.0 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin spiking solutions is shown in **Table 4-2**. For non-spiked solutions, 150 µL of RPMI-C and 50 µL of the test substance (i.e., equivalent to the negative product control [NPC]) are added to a well. Endotoxin-spiked solutions are prepared by adding 100 µL of RPMI-C, 50 µL of the test substance, and 50 µL of an endotoxin-spike solution (1.0 EU/mL) (i.e., equivalent to the positive product control [PPC]). Finally, MM6 cells (50 µL) are added to each well and the wells are mixed and incubated as outlined in **Section 6.1.3, Steps 6-7**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-6 standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

Sample Addition	Spiked	Non-spiked
	µL/well ¹	
RPMI-C	100	150
Endotoxin-spike solution ²	50	0
Test substance (neat and each serial dilution)	50	50
MM6 cells ³	50	50
Total ⁴	250	250

Abbreviations: MM6 cells = Mono Mac 6

¹n=4 replicates each

²Endotoxin concentration is 1.0 EU/mL in RPMI-C.

³MM6 cells are resuspended in RPMI-C (2.5 x 10⁶ cells/mL).

⁴A total volume of 250 µL per well is used for the incubation.

The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The spike recovery for each sample dilution is calculated as a percentage by setting the theoretical value (i.e., endotoxin-spike concentration of 1.0 EU/mL) at 100%. For example, consider the following interference test results in Table 4-3:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-6 relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not to exceed the MVD, for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance to be used in the *in vitro* pyrogen test would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1.0 EU/mL EC).

4.3.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., MM6 cells incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., MM6 cells incubated with PFS instead of the test substance) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.4 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-6 Release

6.1.1 Preincubation of MM6 Cells

To perform an ELISA on the following day, obtain an MM6 cell suspension (30 to 50 mL) from propagation flasks and centrifuge at 100 x g for 8 min at RT. Remove the supernatant, resuspend the cell pellet in 2 mL of RPMI-C and gently pipet up and down to mix. It is advisable to determine cell number and cell viability as outlined in **Section 4.2.3**. The percentage of viable MM6 cells should exceed 80% to be suitable for use in the test. The results of these examinations should be included in the study report. Transfer the cells (4×10^5 cells/mL) to new tissue culture flasks and add RPMI-C. Place the flasks in a cell culture incubator and maintain the cells at $37 \pm 1^\circ\text{C} + 5\% \text{CO}_2$ for 16 to 24 hr. In general, the preincubation of 2.0×10^7 cells in 50 mL RPMI-C will provide enough cells for one 96-well assay plate

6.1.2 Preparation of MM6 Cells for the Incubation Assay

Prepare the MM6 cells just prior to addition to the incubation plate (**Section 6.1.3, Step 5**). Centrifuge 30 to 50 ml of cell suspension at 100 x g for 8 min at RT. Pour off the supernatant and resuspend the cells in approximately 2 ml of RPMI-C. It is advisable that cell number and cell viability be determined as outlined in **Section 4.2.3**. The percentage of viable MM6 cells should exceed 80% to be suitable for use in the test. The results of these examinations should be included in the study report. Dilute the cells with RPMI-C to a volume that gives a concentration of 2.5×10^6 cells/ml.

6.1.3 Incubation Plate

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test substances (see **Table 6-1**).

Table 6-1 Overview of Incubation Plate Preparation in the MM6/IL-6 Pyrogen Test

Number of Wells	Sample	RPMI-C	EC	Test Sample	MM6 ¹	Mix the samples; incubate for 16 to 24 hr at 37±1°C in a humidified atmosphere with 5% CO ₂ .	Mix the samples; immediately transfer to an ELISA plate ⁵ and run ELISA.
		µL					
20 ²	EC	100	50	0	100		
4	NSC	100	0	0 ³	100		
56 ⁴	Test samples (1-14)	100	0	50	100		

Abbreviations: EC = Endotoxin control; IL-6 = Interleukin-6; NSC = Negative saline control; MM6 = Mono Mac 6

¹MM6 cell concentration is 2.5 x 10⁶ cells/mL.

²Five EC concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) in quadruplicate

³50 µl of PFS is added instead of the test sample.

⁴14 test samples (n=4 each) per plate

⁵An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.4 Incubation Assay for IL-6 Release

MM6 cells are prepared in a microtiter plate using a laminar flow hood (refer to **Section 6.1.2**). All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

Step 1. Refer to the suggested incubation plate template presented in **Table 6-2**.

Step 2. Using a pipetter, transfer 100 µL of RPMI-C into each well.

Step 3. Transfer 50 µL of test sample or 50 µL of PFS for the NSC into the appropriate wells as indicated in the template.

Step 4. Transfer 50 µL of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.

Step 5. Transfer 100 µL of a well-mixed MM6 cell suspension into each well.

Step 6. Place the covered plate in a tissue culture incubator for 16 to 24 hr at 37±1°C in a humidified atmosphere containing 5% CO₂.

Step 7. Remove 150 µL of the supernatant from each well, without disrupting the cells, and transfer to the IL-6 ELISA plate.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	Void ³	Void
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

¹EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.

²TS number (e.g., TS 1) represents an arbitrary sequence for individual test substances.

³Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-6 Release

6.2.1 IL-6 Standard Curve

An IL-6 standard supplied with the ELISA kit is used. IL-6 standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in RPMI-C to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 µL. Each well on the ELISA plate will receive 50 µL of an IL-6 blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed by heating them in a water bath. A suggested ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, an eight-point IL-6 standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-6 standard curve is prepared as described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4**.

Step 1. After pipetting up and down very carefully three times (avoid detachment of the adherent MM6 cells) to mix the supernatant, transfer 50 μ L from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 2. Add 50 μ L of each IL-6 standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 3. Add 200 μ L of the enzyme-labeled detection antibody (neat as supplied, or diluted, if necessary) to each of the wells.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 2 to 3 hr at RT.

Step 5. Decant and wash each well three times with 300 μ L Buffered Wash Solution and then rinse three times with deionized water. Place the plates upside down and tap to remove water.

Step 6. Add 200 μ L of TMB/Substrate Solution to each well and incubate at RT in the dark for 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 μ L of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD₄₅₀ within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 540 to 590 nm is recommended⁴.

⁴The TMB chromagen is measured at OD₄₅₀. However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6 ³ 0	IL-6 0
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	IL-6 500	IL-6 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-6 1000	IL-6 1000
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-6 2000	IL-6 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-6 4000	IL-6 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

¹EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.

²TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

³IL-6 values in columns 11 and 12 are in pg/mL.

Table 6-4 Overview of ELISA Procedure

Material transfer from Incubation Plate (µL)	IL-6 standard (0 to 4000 pg/mL) (µL)	Enzyme-labeled Antibody (µL)	Cover the Incubation Plate and incubate for 2 to 3 hr at RT.	Decant and wash each well three times with 300 µL Buffered Wash Solution and three times with deionized water.	TMB/Substrate Solution (µL)	Incubate for less than 15 min at RT in dark.	Stop Solution (µL)	Read each well at OD ₄₅₀ with a 540 to 590 nm reference filter.
50	50	200			200		50	

Abbreviations: OD₄₅₀ = Optical density at 450 nm; RT = Room temperature

7.0 EVALUATION OF TEST RESULTS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD_{450}) with a reference filter of 540 to 590 nm (recommended)⁵. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6 standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-6 standard curve produces an $r \geq 0.95$ ⁶ and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

An outlying observation may be excluded if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett and Lewis 1994] or Grubbs' test [Barnett and Lewis 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance
- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)
- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, resuspension solvent)

⁵The TMB chromagen is measured at OD_{450} . However, the use of an IL-1 β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

⁶Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

*Justification of the In Vitro Test Method and Protocol Used**Test Method Integrity*

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time
- If the test method employs proprietary components, documentation on the procedure used to ensure their integrity from “lot-to-lot” and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure used
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples

*Description of Other Effects Observed**Discussion of the Results**Conclusion**A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies*

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

11.0 REFERENCES

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)¹

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)^{1,2}

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M , where:

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product were used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)^{1,2}

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is¹:

$$\text{MVD} = (\text{ELC} \times \text{Product Potency [PP]})/\lambda$$

As an example, for Cyclophosphamide Injection, the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is¹:

$$\text{MVD} = \text{PP}/\text{Minimum Valid Concentration (MVC)}$$

$$\text{where, MVC} = (\lambda \times M)/K$$

where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

¹From FDA (1987)

²From USP (2007)

12.5 Parenteral Threshold Pyrogen Dose (K)^{1,2}

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, the PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces $\frac{1}{2}$ the maximal increase in optical density (OD) from the endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.7 Product Potency (PP)^{1,2}

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)^{1,2}

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.

Appendix D

Federal Register Notices, Public Comments, and Relevant SACATM Meeting Minutes

D1	<i>Federal Register Notices</i>.....	D-3
	D1-1 Vol. 70, No. 241, pp. 74833-74834, December 16, 2005: Peer Panel Evaluation of <i>In Vitro</i> Pyrogenicity Testing Methods: Request for Comments, Nominations of Experts, and Submission of <i>In Vivo</i> and <i>In Vitro</i> Data.....	D-5
	D1-2 Vol. 71, No. 238, pp. 74533-74534, December 12, 2006: Announcement of an Independent Scientific Peer Review Meeting on the Use of <i>In Vitro</i> Pyrogenicity Testing Methods; Request for Comments.....	D-7
	D1-3 Vol. 72, No. 89, pp. 26395-26396, May 9, 2007: Peer Review Panel Report on Five <i>In Vitro</i> Pyrogen Test Methods: Availability and Request for Public Comments.....	D-9
D2	Public Comments Received in Response to <i>Federal Register</i> Notices.....	D-11
D3	ICCVAM Evaluation of <i>In Vitro</i> Pyrogen Test Methods: Excerpt from SACATM Draft Meeting Minutes	D-43

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Appendix D1

***Federal Register* Notices**

- D1-1 *Federal Register*, Vol. 70, No. 241, pp. 74833-74834, December 16, 2005: Peer Panel Evaluation of *In Vitro* Pyrogenicity Testing Methods: Request for Comments, Nominations of Experts, and Submission of *In Vivo* and *In Vitro* Data.....D-5**
- D1-2 *Federal Register*, Vol. 71, No. 238, pp. 74533-74534, December 12, 2006: Announcement of an Independent Scientific Peer Review Meeting on the Use of *In Vitro* Pyrogenicity Testing Methods; Request for CommentsD-7**
- D1-3 *Federal Register*, Vol. 72, No. 89, pp. 26395-26396, May 9, 2007: Peer Review Panel Report on Five *In Vitro* Pyrogen Test Methods: Availability and Request for Public CommentsD-9**

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DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Toxicology Program (NTP), NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Peer Panel Evaluation of In Vitro Pyrogenicity Testing Methods: Request for Comments, Nominations of Experts, and Submission of In Vivo and In Vitro Data

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes Of Health (NIH).

ACTION: Request for comments, nominations of scientific experts, and submission of data.

SUMMARY: NICEATM, in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), is considering convening an independent peer review panel (hereafter, "Panel") to evaluate the validation status of five *in vitro* pyrogenicity test methods: (1) Human PBMC/IL-6 *in vitro* pyrogen test (PBMC/IL-6), (2) human whole blood/IL-1 *in vitro* pyrogen test (WB/IL-1), (3) human whole blood/IL-1 *in vitro* pyrogen test: application of cryopreserved human whole blood cryo (WB/IL-1), (4) the human whole blood/IL-6 *in vitro* pyrogen test (WB/IL-6), and (5) an alternative *in vitro* pyrogen test using the human monocytoid cell line MONO MAC-6 (MM6/IL6). NICEATM requests public comments as to the appropriateness and relative priority of this activity. In addition, NICEAM requests the nomination of expert scientists for consideration as potential Panel members in the event a Panel meeting occurs. Finally, NICEATM requests the submission of data from the rabbit pyrogenicity test, the bacterial endotoxin test (BET), and *in vitro* pyrogenicity testing with the methods listed above.

DATES: Comments, nominations of expert scientist, and data submissions should be received by January 17, 2006.

ADDRESSES: Correspondence should be sent by mail, fax, or email to Dr. William S. Stokes, NICEATM, NIEHS, P. O. Box 12233, MD EC-17, Research Triangle Park, NC, 27709, (phone) 919-541-2384, (fax) 919-541-0947, (e-mail) niceatm@niehs.nih.gov.

SUPPLEMENTARY INFORMATION:

Background

The European Committee on the Validation of Alternative Methods (ECVAM) conducted a validation study to independently evaluate the

usefulness of five *in vitro* pyrogenicity assays (PBMC/IL-6, WB/IL-1, cryo WB/IL-1, WB/IL-6, and MM6/IL6). In June 2005, ECVAM submitted background review documents (BRDs) for these five methods to NICEATM for consideration as replacements for the currently required tests (i.e., rabbit pyrogen tests and the BET). ICCVAM and NICEATM reviewed the BRDs for completeness and concluded that these five *in vitro* test methods appear to have considerable potential for pyrogenicity testing, but the sponsors needed to provide additional information prior to a formal review by a Panel. Pending receipt and review of the requested information, ICCVAM and NICEATM will determine the priority of an evaluation of these test methods. If convened, the Panel would (1) peer review the BRDs for the test methods, and (2) determine whether the data cited in the BRDs support draft ICCVAM Test Method Recommendations regarding the proposed usefulness, limitations, and validation status of the test methods. If appropriate, the Panel might also formulate conclusions on the adequacy of any draft recommended performance standards, any proposed future validation studies, draft standardized test method protocols, and/or reference substances. In making their conclusions and recommendations, the Panel considers all available information including the scientific studies cited in the draft BRD, public comments, and any new information identified during the peer review.

Request for Public Comments and Nominations of Scientific Experts

NICEATM requests public comments on the appropriateness and relative priority of the proposed Panel review activity. In addition, NICEAM requests the nomination of scientists with relevant knowledge and experience to potentially serve on the Panel should it be convened. Areas of relevant expertise include, but are not limited to: physiology, pharmacology, immunology, pyrogenicity testing in animals, development and use of *in vitro* methodologies, biostatistical data analysis, knowledge of chemical data sets useful for validation of toxicity studies, and hazard classification of chemicals and products. Each nomination should include the person's name, affiliation, contact information (i.e., mailing address, e-mail address, telephone and fax numbers), and a brief summary of relevant experience and qualifications.

Request for Data

NICEATM invites the submission of data from standard *in vivo* rabbit pyrogen testing, the BET, and *in vitro* pyrogenicity testing using the methods detailed above. Although data can be accepted at any time, data submitted by the deadline listed in this notice would be considered during an evaluation of the validation status of the five pyrogenicity testing methods should this activity occur. Submitted data will be used to further evaluate the usefulness and limitations of *in vitro* pyrogenicity test methods and may be included in future NICEATM and ICCVAM reports and publications as appropriate. The data will also be included in a NICEATM database to support the investigation of other test methods for assessing pyrogenicity.

When submitting chemical and protocol information/test data, please reference this **Federal Register** notice and provide appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, as applicable).

NICEATM prefers data to be submitted as copies of pages from study notebooks and/or study reports, if available. Raw data and analyses available in electronic format may also be submitted. Each submission for a chemical should preferably include the following information, as appropriate:

- Common and trade name
- Chemical Abstracts Service Registry Number (CASRN)
- Chemical class
- Product class
- Commercial source
- *In vitro* pyrogenicity test protocol used
- *In vitro* pyrogenicity test results
- BET test protocol used
- BET test results
- *In vivo* rabbit pyrogen test protocol used
- Individual animal responses
- The extent to which the study complied with national or international Good Laboratory Practice (GLP) guidelines
- Date and testing organization

Background Information on ICCVAM and NICEATM

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use or generate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more

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accurately assess the safety and hazards of chemicals and products and that refine, reduce, or replace animal use. The ICCVAM Authorization Act of 2000 (Pub. L. 106-545, available at <http://iccvam.niehs.nih.gov/about/PL106545.htm>) establishes ICCVAM as a permanent interagency committee of the NIEHS under the NICEATM. NICEATM administers the ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of Federal agencies. Additional information about ICCVAM and NICEATM can be found at the following Web site: <http://www.iccvam.niehs.nih.gov>.

Dated: December 5, 2005.

Samuel H. Wilson,

Deputy Director, National Institute of Environmental Health Sciences.

[FR Doc. E5-7410 Filed 12-15-05; 8:45 am]

BILLING CODE 4140-01-P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Toxicology Program (NTP), NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Announcement of an Independent Scientific Peer Review Meeting on the Use of In Vitro Pyrogenicity Testing Methods; Request for Comments

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: Meeting announcement and request for comments.

SUMMARY: NICEATM in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) announces an independent scientific peer review meeting to evaluate the validation status of five *in vitro* pyrogenicity test methods: (1) Human PBMC/IL-6 *in vitro* pyrogen test (PBMC/IL-6), (2) human whole blood/IL-1 *in vitro* pyrogen test (WB/IL-1), (3) human whole blood/IL-1 *in vitro* pyrogen test: application of cryopreserved human whole blood (cryo WB/IL-1), (4) the human whole blood/IL-6 *in vitro* pyrogen test (WB/IL-6), and (5) an alternative *in vitro* pyrogen test using the human monocytoid cell line MONO MAC-6 (MM6/IL6). These five *in vitro* test methods are proposed as replacements for the *in vivo* rabbit pyrogen test (RPT). At this meeting, a scientific panel will peer review the draft background review document (BRD) on each test method, evaluate the extent that the BRD addresses established validation and acceptance criteria for each test method, and provide comment on draft ICCVAM recommendations on the proposed use of these test methods, draft test method protocols, and draft performance standards. NICEATM invites public comments on the draft BRDs, draft ICCVAM test method recommendations,

draft test method protocols, and draft performance standards.

DATES: The meeting will be held on February 6, 2007, from 8:30 a.m. to 5 p.m. The meeting is open to the public with attendance limited only by the space available. In order to facilitate planning for this meeting, persons wishing to attend are asked to register by January 23, 2007, via the ICCVAM/NICEATM Web site (<http://iccvam.niehs.nih.gov>). Comments should be sent by mail, fax, or email to the address given below by January 26, 2007.

ADDRESSES: The meeting will be held at the National Institutes of Health (NIH), Natcher Conference Center, 45 Center Drive, Bethesda, MD 20892.

FOR FURTHER INFORMATION CONTACT: Dr. William S. Stokes, Director of NICEATM, NIEHS, P.O. Box 12233, MD EC-17, Research Triangle Park, NC, 27709, (phone) 919-541-2384, (fax) 919-541-0947, (e-mail) niceatm@niehs.nih.gov. Courier address: NICEATM, 79 T.W. Alexander Drive, Building 4401, Room 3128, Research Triangle Park, NC 27709.

SUPPLEMENTARY INFORMATION:

Background

The European Centre for the Validation of Alternative Methods (ECVAM) conducted a validation study to independently evaluate the usefulness and limitations of five *in vitro* pyrogenicity test methods (PBMC/IL-6, WB/IL-1, cryo WB/IL-1, WB/IL-6, and MM6/IL6). In June 2005, ECVAM submitted BRDs for these five methods to NICEATM for consideration as replacements for the currently required test, the RPT. ICCVAM and NICEATM reviewed the BRDs for completeness and concluded that these five *in vitro* test methods appear to have considerable potential for pyrogenicity testing, but that the sponsor needed to provide additional information prior to a formal scientific review by an expert panel. In anticipation of proceeding with an evaluation of these test methods, ICCVAM and NICEATM requested public comments as to the appropriateness and relative priority of a panel review activity and the nomination of scientists with relevant knowledge and experience to potentially serve on the panel (**Federal Register** Vol. 70, No. 241, pp. 74833-4, December 16, 2005). NICEATM also requested submission of data using the standard *in vivo* rabbit pyrogen test, the bacterial endotoxin test (BET), and *in vitro* pyrogenicity tests. These requests were sent directly to over 100 interested

stakeholders; no additional data were received.

In March 2006, ECVAM responded to the ICCVAM/NICEATM request for information by providing a revised BRD for each test method. ICCVAM and NICEATM drafted a BRD that combines all of the available information on the five *in vitro* pyrogenicity test methods into a single document and includes each of the ECVAM BRDs as an appendix. Based on this information, ICCVAM developed draft test method recommendations regarding the proposed usefulness, limitations, and validation status of these test methods. ICCVAM subsequently recommended that an independent scientific panel be convened to (1) peer review the draft BRD for the test methods and (2) determine whether the data and analyses in the draft BRDs support the draft ICCVAM test method recommendations. The panel will also be asked to comment on the adequacy of the draft recommended performance standards, proposed future validation studies, draft standardized test method protocols, and recommended reference substances. In making their conclusions and recommendations, NICEATM will ask the panel to consider all available information including the scientific studies cited in the draft BRD, public comments, and any new information identified during the peer review.

Peer Review Panel Meeting

The purpose of this meeting is the scientific peer review evaluation of the validation status of five *in vitro* pyrogenicity test methods as replacements for the RPT. First, the panel will review the draft BRD on the current status of five *in vitro* test methods for the detection of pyrogenicity and evaluate the extent that established validation and acceptance criteria are addressed for each test method (Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods, NIH Publication No. 97-981, <http://iccvam.niehs.nih.gov>). Next, the panel will comment on the extent to which the ICCVAM recommendations are supported by the information provided in the BRD and on the proposed use of these test methods, draft test method protocols, draft performance standards, and any proposed validation studies.

Information about the panel meeting, including a roster of the panel members and the draft agenda, will be made available two weeks prior to the meeting on the ICCVAM/NICEATM Web site (<http://iccvam.niehs.nih.gov>) or can be

obtained after that date by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** above).

Attendance and Registration

This public meeting will take place February 6, 2007, at the NIH Campus, Natcher Conference Center, Bethesda, MD (a map of the NIH campus and other visitor information are available at <http://www.nih.gov/about/visitor/index.htm>). The meeting begins at 8:30 a.m. and will conclude at approximately 5 p.m. Persons needing special assistance, such as sign language interpretation or other reasonable accommodation in order to attend, should contact 919-541-2475 (voice), 919-541-4644 TTY (text telephone), through the Federal TTY Relay System at 800-877-8339, or by e-mail to niehsoeeo@niehs.nih.gov. Requests should be made at least seven business days in advance of the event.

Availability of the BRD and Draft ICCVAM Recommendations

NICEATM prepared a BRD on five *in vitro* pyrogenicity test methods that describes the current validation status of the *in vitro* test methods and contains all of the data and analyses supporting this validation status. The draft BRDs, draft ICCVAM test method recommendations, draft test method protocols, and draft test method performance standards are available from the ICCVAM/NICEATM Web site (<http://iccvam.niehs.nih.gov>) or by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** above).

Request for Comments

NICEATM invites the submission of written comments on the BRDs, draft ICCVAM test method recommendations, draft test method protocols, and draft test method performance standards. When submitting written comments, it is important to refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). Written comments should be sent by mail, fax, or e-mail to Dr. William Stokes, Director of NICEATM, at the address listed above, not later than January 26, 2007. All comments received will be placed on the ICCVAM/NICEATM Web site (<http://iccvam.niehs.nih.gov>), sent to the panel and ICCVAM agency representatives, and made available at the meeting.

This meeting is open to the public and time will be provided for the presentation of public oral comments at designated times during the peer review. Members of the public who

wish to present oral statements at the meeting (one speaker per organization) should contact NICEATM (see **FOR FURTHER INFORMATION CONTACT** above) no later than January 26, 2007. Speakers will be assigned on a consecutive basis and up to seven minutes will be allotted per speaker. Persons registering to make comments are asked to provide NICEATM a written copy of their statement by January 26, 2007, so that copies can be distributed to the panel prior to the meeting or if this is not possible to bring 40 copies to the meeting. Written statements can supplement and expand the oral presentation. Each speaker is asked to provide contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable) when registering to make oral comments.

Summary minutes and the panel's final report will be available following the meeting on the ICCVAM/NICEATM Web site (<http://iccvam.niehs.nih.gov>). ICCVAM will consider the panel's conclusions and recommendations and any public comments received in finalizing their test method recommendations and performance standards for these methods.

Background Information on ICCVAM and NICEATM

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use or generate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 2851-3, available at <http://iccvam.niehs.nih.gov/about/PL106545.htm>) establishes ICCVAM as a permanent interagency committee of the NIEHS under the NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of federal agencies. Additional information about ICCVAM and NICEATM can be found at the following Web site: <http://iccvam.niehs.nih.gov>.

Dated: November 27, 2006.

Samuel H. Wilson,

Deputy Director, National Institute of Environmental Health Sciences and National Toxicology Program.

[FR Doc. E6-21038 Filed 12-11-06; 8:45 am]

BILLING CODE 4140-01-P

DEPARTMENT OF HEALTH AND HUMAN SERVICES**National Toxicology Program (NTP), NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Peer Review Panel Report on Five In Vitro Pyrogen Test Methods: Availability and Request for Public Comments**

AGENCY: National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

ACTION: Request for comments.

SUMMARY: NICEATM in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) convened an independent scientific peer review panel meeting on February 6, 2007, to evaluate the validation status of five in vitro pyrogen test methods proposed as replacements for the Rabbit Pyrogen Test (RPT). The peer review panel ("the Panel") report from this meeting is now available. The report contains (1) the Panel's evaluation of the validation status of the methods and (2) the Panel's comments and conclusions on draft ICCVAM test method recommendations. NICEATM invites public comment on the Panel's report. The report is available on the NICEATM/ICCVAM Web site at (<http://iccvam.niehs.nih.gov/methods/pyrogen/pyrogen.htm>) or by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** below).

DATES: Written comments on the Panel report should be received by June 25, 2007.

ADDRESSES: Comments should preferably be submitted electronically via the NICEATM/ICCVAM Web site: http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm. Comments can also be submitted by e-mail to niceatm@niehs.nih.gov. Written comments can be sent by mail or fax to Dr. William S. Stokes, NICEATM Director, NIH/NIEHS, P.O. Box 12233, MD EC-17, Research Triangle Park, NC 27709, (fax) 919-541-0947, (e-mail) niceatm@niehs.nih.gov. Courier address: NICEATM, 79 T.W. Alexander Drive, Building 4401, Room 3128, Research Triangle Park, NC 27709.

FOR FURTHER INFORMATION CONTACT: Other correspondence should be directed to Dr. William S. Stokes, NICEATM Director (919-541-2384 or niceatm@niehs.nih.gov).

SUPPLEMENTARY INFORMATION**Background**

The European Centre for the Validation of Alternative Methods (ECVAM) submitted five *in vitro* pyrogen test methods to ICCVAM for evaluation in 2006. The proposed test methods include:

1. The Human Whole Blood (WB)/IL-1 *In Vitro* Pyrogen Test: Application of Cryopreserved Human WB
2. The Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6 *In Vitro* Pyrogen Test
3. The Human PBMC/IL-6 *In Vitro* Pyrogen Test
4. The Human WB/IL-1 *In Vitro* Pyrogen Test
5. The Human WB/IL-6 *In Vitro* Pyrogen Test

These test methods are based on the measurement of proinflammatory cytokines released from either fresh or cryopreserved human blood cells or a human monocytoid line in response to the presence of Gram-negative endotoxin in parenteral pharmaceuticals. NICEATM and ICCVAM prepared a comprehensive background review document (BRD) that included the available data for the five test methods and a separate document containing ICCVAM test method recommendations. At the peer review meeting, the Panel reviewed the BRD and evaluated the extent to which the ICCVAM criteria for validation and acceptance had been adequately addressed for the intended purpose of these test methods. The Panel also provided comments on the ICCVAM draft test method recommendations regarding the proposed usefulness and limitations, standardized protocols, performance standards, and future studies. The Panel's conclusions and recommendations on the five in vitro pyrogen test methods are described in the *Peer Review Panel Final Report: Five In Vitro Pyrogen Test Methods* (available at: <http://iccvam.niehs.nih.gov/methods/pyrogen/pyrogen.htm>). The draft BRD and the draft test method recommendations are available at <http://iccvam.niehs.nih.gov/methods/pyrogen/pyrogen.htm>.

Request for Comments

NICEATM invites the submission of written comments on the Panel's report. When submitting written comments please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, email, and sponsoring organization, if applicable). All comments received by the deadline listed above will be placed on the

NICEATM/ICCVAM Web site (<http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm>) and made available to ICCVAM. In addition, there will be an opportunity for oral public comments on the Panel's report during a meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) scheduled for June 12, 2007. Information concerning the SACATM meeting will be published in a separate **Federal Register** notice and available on the SACATM website: (<http://ntp.niehs.nih.gov/go/7441>). Any written comments on the Panel report received prior to June 7, 2007, will be distributed to SACATM.

ICCVAM will consider the Panel report along with the SACATM and public comments as it finalizes recommendations for the five *in vitro* pyrogen test methods. An ICCVAM test method evaluation report, which includes the ICCVAM final recommendations, will be forwarded to appropriate federal agencies for their consideration. This report will also be available to the public on the NICEATM/ICCVAM Web site and by request from NICEATM.

Background Information on ICCVAM, NICEATM, and SACATM

ICCVAM is an interagency committee composed of representatives from 15 federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes scientific validation and regulatory acceptance of toxicological test methods that more accurately assess safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 2851-3, available at http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf) establishes ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of Federal agencies. Additional information about ICCVAM and NICEATM can be found at the ICCVAM/NICEATM Web site (<http://iccvam.niehs.nih.gov>).

Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at <http://ntp.niehs.nih.gov/go/167>.

26396

Federal Register / Vol. 72, No. 89 / Wednesday, May 9, 2007 / Notices

Dated: April 30, 2007.

Samuel H. Wilson,

*Deputy Director, National Institute of
Environmental Health Sciences and National
Toxicology Program.*

[FR Doc. E7-8896 Filed 5-8-07; 8:45 am]

BILLING CODE 4140-01-P

Appendix D2

Public Comments Received in Response to *Federal Register* Notices

Comments in Response to *Federal Register*, Vol. 70, No. 241, pp. 74833-74834, December 16, 2005: Peer Panel Evaluation of *In Vitro* Pyrogenicity Testing Methods: Request for Comments, Nominations of Experts, and Submission of *In Vivo* and *In Vitro* Data

- 1. Dr. Pilar Vindarell (Facultat de Farmacia, Barcelona, Spain)D-13
- 2. Sadhana Dhruvakumar (People for the Ethical Treatment of Animals [PETA]), Dr. Martin Stephens (Humane Society of the United States [HSUS]), Dr. Chad Sandusky (Physician’s Committee for Responsible Medicine [PCRM]), Sue Leary (Alternatives Research and Development Foundation [ARDF]).....D-14

Comments in Response to *Federal Register*, Vol. 71, No. 238, pp. 74533-74534, December 12, 2006: Announcement of an Independent Scientific Peer Review Meeting on the Use of *In Vitro* Pyrogenicity Testing Methods; Request for Comments

- 1. Kristie Stoick and Dr. Chad Sandusky (PCRM), Dr. Martin Stephens (HSUS), Dr. Catherine Willett (PETA), Sue Leary (ARDF), Tracie Letterman (American Anti-Vivisection Society), Sara Amundson (Doris Day Animal League).....D-19
- 2. Dr. Mary Lou Chapek (MVP Laboratories Inc.)D-24
- 3. Steven Myers and Anita Sawyer (Becton, Dickinson and Co.)D-27
- 4. Dr. Erik Wind Hansen and Michael Timm (University of Copenhagen)D-29

Comments in Response to *Federal Register*, Vol. 72, No. 89, pp. 26395-26396, May 9, 2007: Peer Review Panel Report on Five *In Vitro* Pyrogen Test Methods: Availability and Request for Public Comments

- 1. Kristie Stoick (PCRM)D-34
- 2. Dr. Thomas Montag-Lessing and Dr. Ingo Spreitzer (Paul Ehrlich Institute)D-37

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From: MA. PILAR VINARDELL MARTINEZ-HIDALGO
Sent: Tuesday, December 20, 2005 8:08 AM
To: NIEHS NICEATM
Subject: peer panel pyrogens

Dear Dr Stockes

I am pleased to send you our paper* related to studies of pyrogens in vitro. I hope it will be of interest for your work. I have collaborated before with the het-cam test.

Sincerely yours

Dr. Pilar Vinardell
Dept Fisiologia-Divisio IV
Facultat de Farmacia
Av. Joan XXIII s/n
08028 Barcelona (Spain)

*Martinez V, Mitjans M, Vinardell MP. 2004. TNF α Measurement in Rat and Human Whole Blood as an *In Vitro* Method to Assay Pyrogens and its Inhibition by Dexamethasone and Erythromycin. J. Pharm. Sci. 93:2718-2723.

January 17, 2006

Dr. William Stokes
Director, NICEATM
National Institute of Environmental Health Sciences
P.O. Box 12233, MD EC-17
Research Triangle Park, NC 27709

Via electronic transmission to: niceatm@niehs.nih.gov

Dear Dr. Stokes:

These comments are submitted on behalf of People for the Ethical Treatment of Animals, Humane Society of the United States, Physicians Committee for Responsible Medicine, and the Alternatives Research & Development Foundation, a coalition of animal protection, alternatives development, and health advocacy organizations representing more than 10 million Americans in response to a December 16, 2005 notice in the *Federal Register* inviting public comment on a proposed peer review panel evaluation of five human biology-based *in vitro* pyrogenicity test methods. We consider these methods to have great potential to replace the existing animal-based methods and we appreciate the work that has gone into the development of the background review documents (BRDs) by the European Center for the Validation of Alternative Methods (ECVAM) and into their preliminary review by Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM).

We believe that an international peer review of these novel pyrogenicity test methods is appropriate, necessary, and should be given extremely high priority. A thorough yet expeditious review of these tests by an expert panel resulting in the endorsement of at least one proposed test method should be viewed as a potential quick win in the efforts of ICCVAM to meet its statutory mandate to promote the replacement, reduction, or refinement of animal-based testing (42 *U.S.C.* Sec. 2851-3(b)).

Need for Speedy Review of Novel Pyrogenicity Tests

Pyrogenicity testing is most commonly used to ensure that medical treatments (particularly injectable medicines or implanted devices) are free of fever-inducing contaminants. Currently used methods of animal-based pyrogenicity testing have significant scientific and practical limitations (described below). Human-biology based pyrogenicity tests are more sensitive, more consistent, and more versatile, but most importantly, more accurate. Since they are based on human immune system responses, they represent the most relevant and best possible means of predicting human pyrogenic potential. Swift validation of the proposed *in vitro* tests and replacement of animal-based pyrogenicity tests is necessary to best safeguard consumer safety.

Use of the five novel test methods will also better protect the public because they enable testing that was not previously possible due to the limitations of the animal tests. For example, they

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enable direct testing of air filters in buildings so that airborne pyrogens can be detected and eliminated; they enable direct testing for pyrogens bound to the surfaces of medical devices, previously not possible; and they enable the testing of cell culture media in order to guarantee its pyrogen-free status. The existing animal-based testing methods are inadequate for testing many important upcoming areas of therapeutics (especially cellular products) which can be tested using the novel human biology-based methods (Hartung et al. *ATLA* 29, 99-123; 2001).

The proposed methods are already in use by over 200 laboratories around the world (EU press release 12/5/03 Reference: IP/03/662) and interest from industry is quite high, thus it is imperative that US federal agencies issue a stance on the validity of these methods. Of the methods under consideration, those utilizing human whole blood (fresh or cryopreserved) and measuring the production of Interleukin-1 are particularly advanced (Methods #2 & 3 in Federal Register notice). These methods have been commercialized as test kits that produce results within a day by the European company Milenia as “PyroCheck” and in the US by Charles River Labs as “Endosafe-IPT.” Customers are already using these test kits but cannot stop using the animal-based tests until they know that Agencies will accept their results.

The EU, primarily through ECVAM, has invested considerable resources into the development and international validation of the five submitted *in vitro* test methods with the involvement of over 60 groups from academia, industry, and regulatory bodies. Descriptions of this work have been published in numerous scientific journals. The European Pharmacopeia has installed an international expert group to draft a General Method for these tests and we understand that the ECVAM Scientific Advisory Committee (ESAC) will shortly review the methods and make a statement on their validity. Whereas the vast majority of novel non-animal test methods are validated in the EU years prior to consideration in the US, this submission represents an exciting first opportunity for the US to concurrently evaluate a test method in parallel with the EU. This adds to the imperative that a panel is convened and a review is conducted in a timely manner.

Limitations of Currently Used Pyrogenicity Tests

The considerable limitations of the existing animal-based pyrogenicity tests create another important imperative. The rabbit pyrogenicity test, developed in the 1940s, still consumes an estimated 400,000 rabbits per year (Hartung et al. *ATLA* 30, 49-51; 2002). Animals are locked in full-body restraints while their temperature is monitored through rectal probes and suffer effects which can include fever, breathing problems, organ failure, and fatal shock. Like all animal-based tests, the rabbit pyrogenicity test is time-consuming, costly, and gives results that are species-specific: The potency of pyrogens varies by up to 10,000 in different mammals ((Hartung et al. *ALTEX* 15, 17-18; 1998). However, the rabbit test is scientifically problematic in many additional ways. Even at the highest injected volumes, the detection limit of the rabbit test is above the human fever threshold: humans show a fever response at concentrations as low as 30pg LPS/ml while rabbits’ sensitivity varies between 50 and 350 pg LPS/ml. In contrast, the human whole blood IL-1 test has a sensitivity of 10pg LPS/ml (Hartung et al. *ATLA* 29, 99-123; 2001). In addition, the sensitivity of the rabbit test varies depending on the strain, age and gender of rabbit used. Other important problems include the fact that the rabbit test often only gives a

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pass/fail, rather than a quantitative, answer; that results are influenced by animal distress as well as seasonal variation; and that inconclusive results necessitating test repetition are common. Lastly, the rabbit pyrogenicity test does not work for many classes of substances including important new therapies such as cellular products or species-specific agents, as well as chemotherapeutics, radiopharmaceuticals, certain biologicals and antibiotics, drugs that cause immune reactions, drugs that influence body temperature such as sedatives/analgesics/anesthetics, and vitamins.

The *in vitro* Limulus Amoebocyte Lysate (LAL) assay, also known as the bacterial endotoxin test or BET, was developed in the 1970s and has largely replaced the rabbit test where possible, but it has severe limitations as well. The most important limitation of the LAL assay is that it only detects endotoxins (components of gram-negative bacteria) but not other pyrogens including gram-positive bacteria, fungi, and viruses. Thus, the LAL assay is used extensively for pharmaceutical testing and for in-process monitoring in biological production but is not suitable as a final release test for complex biologically-derived products that may contain non-endotoxin pyrogens, for material-mediated pyrogenicity, or for substances that chemically or physically interfere with the clotting reaction in the LAL test such as proteins or lipids. It cannot be used for the testing of biological products such as vaccines, immunoglobulins, and clotting factors. In addition, the accuracy of the LAL test for predicting human pyrogens and their potencies is questionable since it is based on the defense system of an arthropod (the coagulation of horseshoe crabs' blood) which is not mechanistically relevant to the human response (Hartung et al. *ALTEX* 15, 9-10; 1998). It is also important to note that the blood used in the LAL assay is obtained by harvesting crabs from the ocean floor and draining ~30% of their blood, which can cause them injury, disrupts their natural life cycles, and depletes their populations, which may make availability of their blood more limited in the future. For ethical and welfare reasons, this test should be replaced as soon as possible.

The rabbit assay is a poor and inadequate test in numerous ways, but the limitations of the LAL assay have led to its continued use. For decades, these tests have been used complementary, but in fact, they are simply limited in different ways and their combined use leaves many gaps in consumer protection and much to be desired. In addition, the two animal-based tests are difficult to correlate with each other. Since the proposed human biology-based tests can detect non-endotoxin pyrogens, they should at the very least completely replace the outdated rabbit pyrogenicity test in final release testing. However, the human biology-based test should also replace the LAL test which should not be conducted if a more humane and relevant human biology-based *in vitro* test is available, which will clearly better safeguard human health.

Human Biology-Based Pyrogenicity Tests

Our understanding of human immunology has advanced rapidly over the last 20 years, and this represents the first opportunity to reflect this in our methods of testing for pyrogenicity. The first interleukins were cloned in 1984, leading to an understanding of the mechanism of pyrogenicity: When an "exogenous pyrogen" enters the bloodstream, cells of the immune system produce "endogenous pyrogens" (interleukins) that signal the brain to generate a fever. The first human

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blood-based *in vitro* pyrogenicity tests were developed over a decade ago (Hartung & Wendel *ALTEX* 12, 70-75; 1995), based on measuring the production of interleukins in response to the test substance. Such methods are physiologically and mechanistically relevant and thus are capable of detecting all classes of human pyrogens (though there are a few limitations, such as testing for contamination of drugs that interact with immune cells, however this limitation also applies to the rabbit test).

Human biology-based pyrogenicity tests have since undergone extensive development and evaluation. The five tests proposed for consideration vary in their use of human whole blood (fresh or cryopreserved), cells isolated from blood, or immune cell lines, and in the interleukin response they measure, but otherwise work on the same principle. It will be up to the panel to decide whether all of these test methods accurately model the pyrogenic response with the necessary accuracy and sensitivity, and whether it varies by application.

The proposed human biology-based tests have almost every advantage over the existing animal-based tests: They are more biologically relevant, more reproducible, and more broadly applicable than the animal-based alternatives. They are speedier, more cost-effective, less laborious, and more humane. They are very sensitive; as mentioned above, the whole blood IL-1 test has been shown to have a sensitivity of 10pg LPS/ml, far below the human fever threshold. (As previously discussed, the rabbit test is far less sensitive and consistent, and neither the rabbit or LAL tests have ever been formally validated to demonstrate either intra- and inter-laboratory reproducibility, much less their relevance to human beings. Thus, when the expert panel considers the proposed novel methods, it is especially important to avoid the common pitfall of using the animal data as the “gold standard” in assessing false positive and negative rates.)

In conclusion, the submitted BRDs represent an ideal opportunity to conduct an expeditious review of well-validated non-animal methods and fully replace outdated animal tests with modern, improved alternatives as per ICCVAM’s mandate. ICCVAM’s endorsement will be key in encouraging US government agencies and industry to develop the necessary confidence in these innovative methods. Led by the FDA, Agencies should require these tests as the new standards in place of the animal tests, for which there will be no adequate rationale for continued use. The rabbit test in particular should be deleted from pharmacopeias and regulatory guidance and not accepted by Agencies once the new tests are validated.

With all this in mind, we strongly urge ICCVAM to move ahead quickly to convene a panel of experts who can make the necessary scientific judgments regarding the proposed tests with a view towards a speedy affirmation of their respective values in assessing pyrogenic potential. Consumer safety, scientific rigor, and animal welfare concerns will all be best served by promoting the use of these accurate, sensitive, and humane tests.

Thank you for your attention and responsiveness to these comments.

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Sincerely,



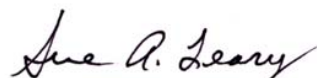
Sadhana Dhruvakumar
Director, Medical Testing Issues
People for the Ethical Treatment of Animals



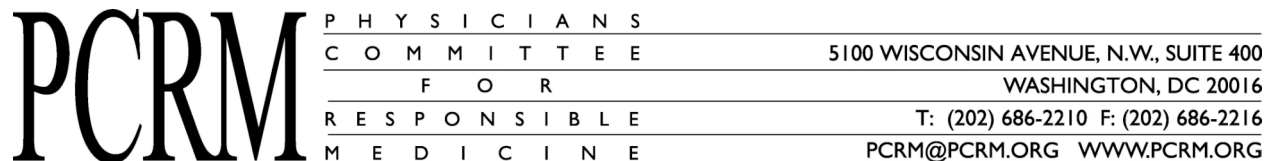
Martin L. Stephens, Ph.D.
Vice President, Animal Research Issues
Humane Society of the United States



Chad B. Sandusky, Ph.D.
Director of Toxicology and Research
Physicians Committee for Responsible Medicine



Sue Leary
President
Alternatives Research & Development Foundation



January 26, 2007

Via email to: niceatm@niehs.nih.gov

Dr. William Stokes
 Director, NICEATM
 National Institute of Environmental Health Sciences
 P.O. Box 12233, MD EC-17
 Research Triangle Park, NC 27709

Re: Federal Register Vol. 70, No. 238, pp 74533-4, December 12, 2006: NTP Interagency Center for the Evaluation of Alternative Toxicological Methods; Announcement of an Independent Scientific Peer Review Meeting on the Use of *In Vitro* Pyrogenicity Testing Methods; Request for Comments

Dear Dr. Stokes:

These comments are submitted on behalf of the more than 10 million U.S. members of the Physicians Committee for Responsible Medicine, People for the Ethical Treatment of Animals, the Humane Society of the United States, the Alternatives Research & Development Foundation, the American Anti-Vivisection Society, and the Doris Day Animal League. We appreciate the opportunity to review ICCVAM's recommendations for five *in vitro* pyrogenicity tests (IVPTs) conducted using either human whole blood or human monocytic cell lines, and to provide comments regarding ICCVAM's "Draft Test Method Recommendations" (Recommendations) and "Draft Background Review Document" (BRD) on these methods. These comments incorporate by reference an earlier submission dated January 17, 2006.

At the outset, it should be stated that the parties to this submission have always endeavored to regard ICCVAM and its member agencies as federal partners who share our commitment to reducing, refining, and ultimately replacing the use of animals in regulatory toxicology. However, the abbreviated number of methods reviewed by ICCVAM and accepted by federal agencies in recent years raises concern over the genuine commitment to progress in the 3Rs by some federal agencies and/or their representatives on ICCVAM. The pyrogenicity BRD and Recommendations currently under discussion represent a glaring case in point.

ICCVAM's Recommendations accept the use of IVPTs only for the detection of lipopolysaccharide-mediated (LPS) pyrogenicity induced by gram-negative bacterial endotoxins "in materials currently tested in the RPT" (rabbit pyrogen test). Thus, for practical purposes, ICCVAM's Recommendations do not support the use or regulatory acceptance of these methods for the detection of gram-positive bacterial, fungal, or viral pyrogens. Moreover, ICCVAM specifically states that it does not regard the IVPTs as full replacements for the Limulus amoebocyte lysate (LAL). Its Recommendations further state that in order to be considered as potential replacements for the RPT for the detection of non-LPS-mediated pyrogenicity, "additional studies that include a broader range of pyrogenic materials are recommended...such studies should include parallel RPT testing." More specifically, "when a positive non-endotoxin-mediated RPT result is encountered, this same sample should be subsequently tested *in vitro*."¹

Despite the extensive discussion of the 3Rs throughout the BRD and Recommendations, it is not clear if or how ICCVAM's Recommendations could contribute to a meaningful reduction in animal use in

pyrogenicity testing if in fact we are not looking to replace the BET and continued comparisons to—and confirmatory testing in—the RPT are required for these methods.

We therefore strongly urge ICCVAM to significantly revise its Recommendations and BRD to more accurately reflect the potential use of these methods as full replacements for both the LAL and RPT. The available evidence shows that the IVPTs are fully valid for the detection of all pyrogens. We also strongly encourage ICCVAM to delete the recommendation regarding the conduct of de novo RPTs to further demonstrate *in vivo/in vitro* concordance.

General Comments

There are a number of disadvantages to current pyrogen-detection methods. These have been discussed previously, but necessitate a brief mention. The RPT exposes live rabbits to painful or distressing experiences; requires trans-species extrapolation; is less sensitive than the human fever threshold;² and is ill equipped to handle substances such as cellular products, radiopharmaceuticals, certain biologicals, and medical devices. The LAL also requires species extrapolation, can only detect LPS, and cannot be used for substances that interfere with the clotting process, biologicals, or the direct assessment of medical devices.

Despite references to the 3Rs, the RPT is still used extensively, especially for complex biologically derived products and end-product release testing. Indeed, it is estimated that up to 400,000 rabbits per year are used,³ and the LAL, despite catch-and-release procedures, results in an approximate 15% mortality rate.⁴ It is therefore imperative, for both ethical and scientific reasons, that both of these tests are replaced by the alternatives presented here for endorsement.

In addition to the obvious ethical advantages of human whole and/or cellular blood pyrogenicity tests, the IVPTs have numerous scientific advantages. The first is the elimination of species extrapolation issues, since the proposed test methods are direct *in vitro* models of the human fever response. Additionally, because the pyrogenic response is a blood-mediated reaction, IVPTs are not limited by potential *in vivo/in vitro* extrapolation considerations, as some *in vitro* tests might be. The IVPTs are sensitive and can detect all potential pyrogens, not only LPS. They can be used to evaluate traditional pharmaceuticals as well as medical devices, species-specific cellular/biological therapies, cell culture media, air quality assessments, and human serum albumin, among other materials. The IVPTs could also be easily adapted into species-specific pyrogenicity tests for veterinary products.

The methods presented to the panel have undergone a full quantitative validation study. The validation studies were conducted in order to certify the IVPTs as appropriate for replacement of both the RPT and the LAL. The concordances and sensitivities for all five human blood-based methods are over 90%; specificities are above 80%; and all methods demonstrate low false-positive and -negative rates.⁵ In comparison, historical data from 171 rabbits were used to calculate a theoretical sensitivity of 57.9% and a theoretical specificity of 88.3% for the RPT.⁴

Clearly, the IVPT methods, after 20 years of research and refinement, are a wholly superior way to detect pyrogens in medicinal products. However, the animal protection community has serious concerns related to the duplication of review efforts, as evidenced by the time ICCVAM has taken to arrive at this point with the IVPTs. As discussed in another recent set of public comments, ICCVAM continues to invest substantial time and resources in what are regarded by many as redundant and unnecessarily duplicative evaluations of 3Rs methods that have already undergone successful validation, independent peer review, and/or international acceptance in other jurisdictions. We therefore question the value of subjecting the IVPTs to multiple peer reviews—particularly when the animal-based RPT and LAL have not been subject to a level of scrutiny even closely resembling that of an ECVAM or ICCVAM validation study.

Specific Recommendations

Accept IVPTs as full replacements for the LAL

It is unclear why ICCVAM has chosen not to consider the IVPTs as appropriate for replacement of both the RPT and the LAL. With the validation of the IVPTs using an endotoxin standard, the LAL has become redundant. If there are specific cases of which we are not aware that require the LAL, exceptions can be made, but surely for ethical and scientific reasons the IVPTs should in general replace the LAL.

Certify the IVPTs valid for the detection of all pyrogens; conduct a "retrospective validation," if needed.

The mechanism of action behind the detection of LPS in the LAL, and hence the reason for its pyrogen specificity, is unique to arthropods. The mechanism of action, if not the magnitude of response, behind the detection of pyrogens in the RPT and the IVPTs is the same. Since the RPT is currently used to detect all pyrogens, there is no biologically sound rationale to conclude that the IVPTs cannot also detect all pyrogens—at a level at least equivalent to the RPT. ICCVAM documents drafted for review today state as much.

Indeed, BRDs submitted by ECVAM, draft BRDs posted by ICCVAM, and other materials list between 15 and 30 published studies discussing the detection of pyrogens, including non-LPS pyrogens, in human serum albumin, pharmaceuticals, and other materials. Some studies used clinically positive materials, and some used comparisons to the traditional *in vivo* or an *in vitro* version of the RPT.⁶⁻⁸ One of these studies⁶ compared the WB/IL-1 IVPT and the RPT using 96 batches of parenteral pharmaceuticals. Of all test substances, only one tested positive in all three (RPT, LAL, and WB/IL-1) test systems. The remaining 95 were negative in all test systems. ECVAM has also provided detailed testing results of materials with the IVPT methods that were determined to be positive for pyrogenic activity during clinical experience. Results were favorable in all assessments.⁴

It is at best perplexing to see peer review reports and testing recommendations stop short of giving the IVPT methods full validated certification, and only recommend the use of these methods for the detection of LPS-mediated pyrogenicity. While most pyrogenicity is indeed related to LPS, the ICCVAM draft recommended test method uses and future studies virtually guarantee that the RPT will not be replaced in the foreseeable future, as it will be needed to certify regulated end products completely "pyrogen free."

Given the results of Jahnke⁶ above, it is further difficult to envision the concurrent *in vivo/in vitro* study recommended by ICCVAM. Hundreds of rabbits could be used in an unnecessary quest to get enough non-LPS-mediated pyrogenicity reactions in rabbits to subsequently confirm using the IVPT methods.

For ethical reasons, the ECVAM validation did not include such concurrent testing. Instead, the study chose LPS, a model pyrogen, to represent the pyrogen reaction and validate the *in vitro* test systems. There is no scientific reason to suspect that the IVPTs will not detect the full range of pyrogens. Published evidence supports this hypothesis,⁷⁻¹⁰ as does supporting evidence submitted by ECVAM in early 2006.⁴ If necessary, a coordinated assessment of such evidence—a retrospective validation of sorts—should more than allay any concerns about the applicability of the IVPTs to all varieties of pyrogen.

Articulate more clearly a path to full replacement

Investments in IVPTs by industry and the public sector are increasing. At least one American company, Charles River Laboratories, has for some time offered an IVPT assay for use in the detection of the range of pyrogens for research use. At least 200 laboratories worldwide have worked with or offer similar assays. Faith in the continued growth of these methods is clearly held by industry, academia, and government alike. With approval and continued use, we are confident that the IVPT methods will become

the “Gold Standard” for human pyrogen detection. The ICCVAM recommendations as currently written will limit the usefulness of these assays, and fail to achieve real reductions in animal use in a timely manner. We urge ICCVAM to revise its Recommendations as outlined above—and offer detailed guidance on how prospective end-users can adopt the IVPTs and put them into immediate practice.

Thank you for your attention to these comments.

Kristie Stoick, MPH
Chad Sandusky, PhD
Physicians Committee for Responsible Medicine

Martin Stephens, PhD
The Humane Society of the United States

Catherine Willett, PhD
People for the Ethical Treatment of Animals

Sue Leary
Alternatives Research & Development Foundation

Tracie Letterman, Esq
American Anti-Vivisection Society

Sara Amundson
Doris Day Animal League

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February 5, 2007

Via email to: niceatm@niehs.nih.gov

Dr. William Stokes
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National Institute of Environmental Health Sciences
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Research Triangle Park, NC 27709

Re: Federal Register Vol. 70, No. 238, pp 74533-4, December 12, 2006: NTP
Interagency Center for the Evaluation of Alternative Toxicological Methods;
Announcement of an Independent Scientific Peer Review Meeting on the Use of
In Vitro Pyrogenicity Testing Methods; Request for Comments

Dear Dr. Stokes:

I have taken the opportunity to review ICCVAM's recommendations for five *in vitro* pyrogenicity tests (IVPTs) and to provide comments regarding ICCVAM's "Draft Test Method Recommendations" (Recommendations) and "Draft Background Review Document" (BRD) on these methods.

I have always regarded ICCVAM and its member agencies as federal partners who share my commitment to the 3 R's, reducing, refining, and ultimately replacing the use of animals in regulatory testing. I have been greatly disappointed at the minimal number of methods reviewed by ICCVAM and accepted by federal agencies over the past 15 years and would like to see progress in this area, not just stagnation. The pyrogenicity BRD and Recommendations currently under discussion indicate to me that there is a lack of logical focus. I propose a two phase approach whereby ICCVAM can demonstrate success.

The summaries and data provided in the BRD indicate that the five proposed *in vitro* pyrogenicity tests are only being evaluated and validated for their ability to measure the

pyrogenic response produced by endotoxin. Even then, only a few pharmaceutical products were tested by spiking with known amounts of endotoxin. Replacing the RPT fully with the *in vitro* pyrogenicity tests is a noble and worthwhile project. I support it fully. However, the testing still to be conducted is extraordinary. Numerous types of products need to be evaluated (some of which have been reported by ECVAM) and non-endotoxin pyrogens must be tested. I would strongly suggest that the ICCVAM proceed with a phased project in order to demonstrate that something can be accomplished rather quickly and animals' lives can be saved.

I propose that Phase I would concentrate on replacing the BET with one or more of the *in vitro* pyrogenicity tests, a task that appears less daunting than replacement of the RPT. The Phase I testing is important because use of the *in vitro* pyrogenicity tests instead of the BET would eliminate the need for horseshoe crabs to die during or after the process of removing the hemolymph. Additionally, the *in vitro* pyrogenicity tests use human components instead of non-human horseshoe crab hemolymph that could be argued to be less relevant to the human fever response.

There is already a significant amount of work reported in the BRD indicating that the five *in vitro* pyrogenicity tests can detect endotoxin pyrogens with accuracy and sensitivity. Thus, Phase I would only require validation against the BET for those products that can currently be tested in the BET.

It appears from the ECVAM information that the *in vitro* pyrogenicity tests can actually test more varied products since there is no interference with these test systems. Such lack of interference could also be demonstrated during Phase I by spiking an array of test products with known endotoxin levels and demonstrating accuracy, specificity and lack of interference.

As for Phase II, I would strongly suggest that the ICCVAM select one or two of the *in vitro* tests based on the results obtained so far, and use them in validation studies against the RPT in order to replace that test completely. The reason for selecting only one or two of the *in vitro* tests is based on the fact that three of the five proposed *in vitro* pyrogenicity tests require fresh human blood that must be collected within 4 hours of running the test. In today's world, such a task is difficult to say the least. The cell culture assay appears much more adaptable to ease of use. That would certainly be one of my choices.

Phase II would still be complex, as now the focus would be on total replacement of the RPT with one or two of the *in vitro* pyrogenicity tests. However, evaluation and initial validation of one or two tests is less of a challenge than trying to evaluate and validate five tests.

Phase II evaluation would require evaluation and validation of all materials currently tested in the RTP as well as all of the types of pyrogens currently quantified in the RPT.

Because standards are not available for all of the types of pyrogens, such standards would have to be developed. Another possibility would be to find products that failed the RPT and use those for validation purposes (less difficult but less scientific). As you already know, this could require years. At least, if Phase I was complete, there could be a demonstration that ICCVAM had accomplished some of its goal of replacement of animal tests with *in vitro* tests.

I hope that ICCVAM will consider my recommendations.

Best Regards,

Mary Lou Chapek, President and CEO
MVP Laboratories, Inc.

May 2008
Rec'd 1/29/07

26 January 2007



Dr. William Stokes
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NIEHS
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The following comments are made in response to: FR Notice (Vol. 71, No. 238, pp. 74533-74534, 12/12/06), Scientific Peer Review Meeting on the Use of In Vitro Pyrogenicity Testing Methods; Request for Comments.

We would like to acknowledge the efforts that NICEATM and ICCVAM have made towards implementing *in vitro* testing as a replacement for that of the standard *in vivo* methods for pyrogenicity. Towards this common goal we are all in agreement. However, even though we share the goal of replacement of methods, which use animals wherever possible, we in the medical device industry have had to continue to use the rabbit pyrogen test to assure that new material components for our products do not contain substances known as “material-mediated” pyrogens. The known substances of this type, listed in ISP 10993-11 Annex F, are generally chemicals, which are mostly understood to directly stimulate the thermoregulatory center in the brain to produce a pyrogenic response. This type of non-endotoxin pyrogen is rare, I have been working in the medical device industry now since 2004 and in this capacity have never observed a pyrogen test conducted on a medical device that did induce a febrile reaction in an animal. This testing is performed by government mandate: Code of Federal Regulations, Title 21, (21CFR610.13) and as such, is not an option for the medical device industry. Further, ISO 10993-11:2006 Annex F states that medical devices containing new chemical entities or substances which have previously elicited a pyrogenic response, should be evaluated for material-mediated pyrogenicity.

The proposed *in vitro* methods for assessing pyrogenicity do not include any data that would support the validity of these methods for the indication of material-mediated pyrogenicity. *In vitro* pyrogen tests appear from studies cited and summarized to be a suitable substitute for the LAL test for endotoxin testing (which we use routinely for product lot release) with additional capability to detect pyrogenic substances from gram positive cell walls and fungi; but it is mechanistically unlikely these methods can detect the majority of material-mediated pyrogens (Annex F list), because there is no macrophage/ cytokine involvement. To accept any/all of these methods as replacements



for the rabbit pyrogen test in all cases without data to support their intended use/s for the acceptance of medical devices would at the very least be deemed to be an equivocal representation for safety considerations in human practices. A minimum consideration should be given to a further study to evaluate some of the non-endotoxin material-mediated pyrogens contained in Annex F of the ISO 10993-11 document by the *in-vitro* pyrogen methods. We strongly recommend that such a study be initiated.

The ICCVAM background document itself notes the following items of concern regarding the assays:

- One identified limitation of the *in vitro* methods is the lack of data to determine their responses to, and suitability for, non-endotoxin pyrogens that are known to be detected by the RPT.
- ECVAM validation studies focused specifically on Gram-negative endotoxin due to the unavailability of standardized, non-endotoxin pyrogens
- ***In vitro* pyrogenicity test method validation studies should evaluate an adequate sample of substances and products of the types that are intended to be tested with these methods. The list of test substances selected for inclusion in the ECVAM validation studies consists solely of marketed parenteral pharmaceuticals that have been labeled as free from detectable pyrogens. No specific rationale was provided for the selection of these test substances.**
- A recognized limitation of the *in vitro* methods is the lack of data to determine their responses to, and suitability for, non-endotoxin pyrogens that are known to be detected by the RPT.

Further testing should be conducted using a representative sample of the types of material-mediated pyrogens as are found in Annex F of ISO 10993-11:2006. When testing of this nature is completed, then the data generated would be better suited for justification of the assays acceptance in the medical device industry. Until such testing is completed and data becomes available, it would be extremely difficult to justify the use of these assays for medical devices.

Respectfully submitted by:

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FACULTY OF PHARMACEUTICAL SCIENCES
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Your ref: 71FR74533

Dear Dr Stokes,

re: Independent Scientific Peer Review Meeting on the Use of *In vitro* Pyrogenicity Testing Methods, Bethesda, MD, Feb 6th 2007 – request for comments.

In accordance with the invitation issued 12th Dec 2006, we would like to submit some comments for your consideration, specifically to the document ‘Draft ICCVAM Test Method Recommendations: *In Vitro* Pyrogenicity Test Methods’, dated 01 Dec 2006 (file PWGrec12016.pdf).

We submit these comments as independent developers of an alternative proprietary *in vitro* pyrogen test, or IVPT. The test has been developed by us at the Faculty of Pharmaceutical Sciences at the University of Copenhagen [1]. Our test differs from the five ECVAM ‘interleukin’ tests under consideration here in that it is based on the measurement of reactive oxygen species produced from terminally-differentiated cells derived from the human HL-60 promyelocytic leukemia cell line. Whilst we believe that our test has all the advantages claimed by the various ECVAM test methods over the RPT, and more besides, our comments here will be restricted to the ICCVAM evaluation of the validation status of these ECVAM tests and the draft recommendations for such test methods.

Comments to PWGrec12016:

1.1 Draft recommended test method uses

“While the scientific basis of these (ECVAM) test methods suggests that they have the capability to detect pyrogenicity produced by a wider range of pyrogens (i.e. those mediated by non-endotoxin sources), there is insufficient data to support this broader application.”

25. JANUARY 2007

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It is very clear from the current literature, and indeed from our own experience of many years working with similar assays (PBMC/IL-1 and MM6/IL-6 assays), that of the five ECVAM tests under evaluation, only the MonoMac6 test has a relevant and useful sensitivity towards non-endotoxin pyrogens. However, this property of the MonoMac6 test does not yet appear to have been validated.

Since the aim of your evaluation is to find an appropriate replacement for the RPT, and that one of the principal strengths of the RPT is that it offers the possibility of detecting pyrogens that would otherwise be missed by the BET, we offer the comment that perhaps it should be considered essential that a suitable IVPT replacement for the RPT must be validated in respect of its ability to detect relevant non-endotoxin pyrogens.

1.2 Draft recommended Future Studies

We wholeheartedly agree with the recommendation that “additional studies that include a broader range of pyrogenic materials...” be conducted if any of the five test methods under consideration are to be considered as potential replacements for the RPT.

We also strongly agree with footnote (3), that “an international standard [for non-endotoxin pyrogens]” is needed in order to demonstrate the utility of these (and other) test methods for the detection of non-endotoxin pyrogens.

We suggest that suitable sources of non-endotoxin standards for this purpose might include yeast, fungi and gram-positive bacteria e.g. *Candida albicans* and *Staphylococcus aureus* either as whole organisms or isolated components hereof as for instance LTA from *S. aureus*. We suggest these two because both pathogens are of clinical relevance.

Appendix A, 1.4.4: Similarities and Differences in the Endpoints of IPT Methods and Currently recognized Pyrogenicity Test Methods

“...the *in vitro* release of pro-inflammatory cytokines, such as IL-1 β and IL-6, is intended to predict the onset of [an inflammatory response]”

Although we do not argue against the relevance of these endpoints *per se*, we feel that we must make the comment that simple serum-level increases in either one or both of these interleukins are not sufficient in themselves to predict either an inflammatory reaction or a febrile response [2]. We should also like to point out that, although the focus here is on production of interleukins in the tests being evaluated, there are other endpoints that are just as relevant for prediction of inflammatory responses by the human immune system, indeed perhaps more so, and that one of these is the production of reactive oxygen species by macrophage- and PMN-like cells when challenged with pyrogenic materials.

Appendix A, 2.3.1: Essential Test method Components, *In Vitro* Cell Culture Conditions

Regarding the use of cryo-preserved whole blood, we appreciate that this is one possible way to avoid the need to make large numbers of willing blood donors available to testing laboratories. However, several laboratories, including our own, have experienced significant problems using cryo-preserved blood in these assays – in our case, the “cryo WB/IL-1” test, commercially obtained from Charles River Labs. Whilst the WB/IL-1 test delivered the results expected using fresh whole human blood, when we tested the same kit with cryo-preserved blood obtained from a source recommended by the manufacturers, it gave no results at all. We believe that the reason for this was that the cryo-preserved blood cells had been irretrievably damaged by the freezing process; the blood sample, thawed according to instructions, was thick and denatured with every indication of extreme cellular damage. From our discussions with others who have also tried using cryo-preserved blood in this test, we conclude that this is a not un-common problem.

Appendix A, 2.3.3.2: Positive Control Substance:

An important distinction between the BET/LAL test and the RPT is that the BET detects only endotoxin pyrogens, whereas the rabbit pyrogen test is capable of also detecting non-endotoxin pyrogens. We suggest that it should therefore be a requirement of the performance standards for any IVPT that might replace the RPT that said *in vitro* test is assessed directly for its ability to detect non-endotoxin pyrogens, as well as LPS.

We therefore suggest that the performance standards include a requirement for one or more positive control pyrogenic substances selected from a group of non-endotoxin pyrogens (perhaps those suggested in our comment to point 1.2, above), in addition to the reference standard LPS to demonstrate adequate sensitivity of the cell system to relevant pyrogens. The sensitivity of any suitable test method to these non-endotoxin pyrogens should be at least comparable to the sensitivity of the rabbit pyrogen test to these same substances.

Appendix A, 2.4: Reference Substances for In Vitro Pyrogenicity Test Methods

In line with the various comments made above, we would suggest that Reference Substances be spiked not only with Gram-negative endotoxin standards, but also non-endotoxin pyrogen standards in order to properly assess the accuracy and reliability of a proposed IVPT that should replace the RPT.

We hope that these few comments will be useful to you in the process of evaluating the validation status of the EVCAM tests, and for drafting future Performance Standards by which to determine the relevance and reliability of these and other *in vitro* test methods for the highly desirable purpose of replacing the RPT.

Yours sincerely,



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Associate Professor
University of Copenhagen
Faculty of Pharmaceutical Sciences



Michael Timm
MSc (Pharm)
University of Copenhagen
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P.S. In case this may be of interest, we have attached the most recent results obtained with our HL-60 ROS IVPT, further optimized from the test reported in [1]. The table reports the responses obtained from a wide variety of pyrogenic components. This table also contains results obtained by us for these same substances tested using the WB/IL-1 IPT (Charles River Labs), and literature data for the same substances run in the RPT.

References:

- [1] Timm, M., Hansen, E.W., Moesby, L., Christensen, J.D. (2006). Utilization of the human cell line HL-60 for chemiluminescence based detection of microorganisms and related substances. *Eur J Pharmaceutical Sciences* 27: 252-258
- [2] Blatteis, C.M. (2006). Endotoxic fever: New concepts of its regulation suggest new approaches to its management. *Pharmacology & Therapeutics* 111: 194 – 223.

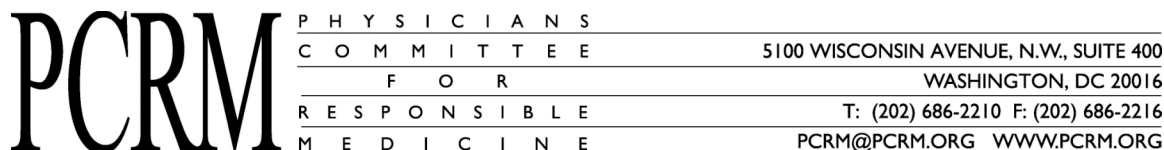
Pyrogen Test Benchmark Data: Hansen & Timm, University of Copenhagen

Positive detections by four assays evaluated for pyrogen determination

Sample	HL-60 assay	IPT assay	Rabbit pyrogen test	LAL test
Zymosan 0,5 µg/ml	+	-	-	-
Zymosan 5 µg/ml	+	+	-	-
LTA standard (0,5 EEU/ml)	+	+	-	-
<i>Candida albicans</i> 10 ⁴ yeasts/ml	+	-	-	-
<i>Candida albicans</i> 10 ⁵ yeasts/ml	+	-	-	-
<i>Saccharomyces cerevisiae</i> 10 ⁴ yeasts/ml	+	-	-	-
<i>Saccharomyces cerevisiae</i> 10 ⁵ yeasts/ml	+	-	-	-
LTA from <i>Bacillus subtilis</i> 25 ng/ml	+	(-)	-*	-
LTA from <i>Bacillus subtilis</i> 100 ng/ml	+	+	-*	-
<i>Staphylococcus aureus</i> 10 ⁵ bacteria/ml	+	-	-*	-
<i>Staphylococcus aureus</i> 10 ⁶ bacteria/ml	+	-	+*	-
<i>Bacillus subtilis</i> 10 ⁴ bacteria/ml	+	+	+*	-
<i>Bacillus subtilis</i> 10 ⁵ bacteria/ml	+	+	+*	-
<i>Salmonella typhimurium</i> 10 ³ bacteria/ml	+	(-)	-*	+
<i>Salmonella typhimurium</i> 10 ⁴ bacteria/ml	+	+	+*	+
<i>Aspergillus niger</i> spores 10 ⁵ spores/ml	+	-	-	-
<i>Aspergillus niger</i> spores 10 ⁶ spores/ml	+	-	-	-
LPS standard 5 EU/ml	+	+	+*	+
LPS standard 2,5 EU/ml	+	+	+*	+
LPS standard 1 EU/ml	+	+	+*	+
LPS standard 0,5 EU/ml	+	+	+*	+
LPS standard 0,25 EU/ml	+	-	-*	+
LPS standard 0,125 EU/ml	+	-	-*	+

(-) samples do excite a response above non stimulated control, but do not score as pyrogenic according to manufactures description.

(*) data obtained from literature.



March 12, 2007

Via e-mail to: niceatm@niehs.nih.gov

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Dear Dr. Stokes:

These comments are intended to be a follow-up to the recent NICETAM-sponsored Independent Scientific Peer Review: Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products (February 6, 2007). They are supported by the larger Animal protection community, including the more than 10 million members of the Physicians Committee for Responsible Medicine, People for the Ethical Treatment of Animals, the Humane Society of the United States, and the Doris Day Animal League.

Based on information communicated to me at this meeting, I understand that these additional comments are accepted because of limited time available during my oral public comment. I appreciate the opportunity to submit these additional comments and I urge NICETAM to take them into account when considering the Peer Review Panel's (PRP) recommendations and conclusions.

Panel Recommendations

As you know, the ICCVAM recommendations were written with the intention that the *in vitro* pyrogenicity tests (IVPTs) would replace a small subset of rabbits used in the rabbit pyrogen test (RPT), but not the Limulus amoebocyte lysate (LAL), nor rabbits used for non-endotoxin-mediated pyrogenicity testing. As you know, the PRP did not even agree with these limited recommendations. The PRP did, however, make several of their own recommendations. I was pleased to hear of some of them and hope that ICCVAM will consider putting them into place as quickly as possible (abridged below):

1. Human data on pyrogens is extensive and should be analyzed, presented, and consulted.
2. More discussions on the financial and ethical costs (including monetary values and animal numbers) associated with the RPT are needed.
3. Individual product-specific validation studies are required and may negate the need for a large validation study.

However, several of the panel's observations and recommendations seemed nonsensical, irrelevant, or inappropriate (abridged below):

1. The methods should not be called "*in vitro* pyrogen tests" because only bacterial endotoxin was evaluated.
2. The *in vivo* reference data is not adequate and/or of unknown quality.
3. The calculated "theoretical sensitivity" of the RPT data used in the validation study does not reflect current practice and regulatory use.
4. The IVPT validation data should be quantitative

5. Concordance between the IVPTs and the RPT is not demonstrated.

Considerations as to the realities of a validation study are clearly not recognized by the PRP, as reflected in its deliberations and recommendations. The validation study conducted by ECVAM used scientifically-justified *in vivo* reference data, a scientifically-justified method to calculate the theoretical sensitivity of the RPT data (as admitted by the panel), and generated data with theoretical concordance values presented. Indeed, the RPT itself does not give quantitative data, but a decision of "pyrogenic" or "not pyrogenic," while the *in vitro* methods do have this potential.

What went wrong?

Panel Selection: This meeting reflects a growing concern of the animal protection community that ICCVAM is more interested in picking new non-animal methods apart than in seriously considering them for adoption. Accepted peer review process guidelines state: "Peer reviewers should include individuals who will not be affected by the outcome of the results, but who are well-versed in the relevant experimental techniques and the specific method under review."¹ However, many of the panel members were either demonstrably biased against the IVPTs, silent, or ignorant of the validation and acceptance procedures, the PRP's role, or the ICCVAM process. Too often it seems that panelists have unreasonable expectations regarding every minute detail of the alternative methods, without a clear understanding of the limitations of the current animal-based tests. This was especially true in this meeting. Random selection of panel members from the scientific topic of interest biases every single panel towards the null hypothesis, leaving an unreasonably high barrier over which the new alternative methods cannot cross.

Charge/Question Wording: It was clear from the deliberation among the PRP that the panel members had no clear idea of their task, and were unnecessarily confused by the questions posed to them by ICCVAM. One question elicited an hour's debate over what the question actually meant. Simplification of the questions posed to the panel, as well as a pre-meeting orientation, is in order. For example, there was clearly little or no background information provided on the limitations of the animal tests. An orientation process could also help the panelists stay focused. The panel deviated too often from the task at hand into both broad and detailed scientific questions that had no bearing on the validation of the IVPTs. For example, one panel recommendation stated that an explanation should be given as to why *in vitro* responses are a better reflection of *in vivo* human responses than *in vivo* rabbit responses. While biological relevance is important, it has already been demonstrated; this recommendation has no bearing on the validation status of the assays as presented, whether it is true or not.

Validation Study Considerations

Despite public testimony given at the time of the meeting, the PRP did not take the realities of validation studies, nor this particular one, into account. First, the validation study selected a small set of pharmaceuticals and spiked them with endotoxin, because endotoxin standard is the only standard available, and the majority of febrile reactions are due to endotoxin. As is often done with animal tests, practical experience over the past couple of decades led the validation study directors to surmise that the methods would also work with non-endotoxin pyrogens, and with medical devices and blood products. Pages of data were provided to the PRP to support this conclusion. Given that the methods would require product-specific validation in the future, and limited resources for the validation study, a large, complicated validation study was not called for. Further, parallel rabbit testing, for animal welfare reasons, could not be conducted. So, the study directors designed an approach that would allow the use of historical RPT data of a comparable nature. It was determined that in order to "pass," the IVPTs would

¹ NIEHS (National Institute of Environmental Health Sciences), Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods, NIH Publication No. 97-3981, NIEHS, Research Triangle Park, North Carolina, U.S.A., 1997, section 2.4.2.6.

be validated with spiked products at a level of detection comparable with the most sensitive rabbit species. Even with these "unacceptable" sensitivity and specificity values, the IVPTs still surpassed the performance of the RPT. Comments to this effect from the study directors themselves were ignored by the PRP.

The PRP showed no tolerance for adopted tenets of the validation process that call for flexibility: "...the test validation process should be highly flexible and adapted to the specific test and its proposed use."² These procedures do not require direct comparison of *in vivo/in vitro* methods, and indeed, the BRD and other documentation submitted to the PRP contain all of the Validation Criteria listed in the above-referenced ICCVAM document.

The Way Forward

I would like to reiterate the animal protection community's initial comments, sent before the meeting:

"We therefore strongly urge ICCVAM to significantly revise its Recommendations and B[ackground] R[eview] D[ocument] to more accurately reflect the potential use of these methods as full replacements for both the LAL and the RPT. The available evidence shows that the IVPTs are fully valid for the detection of all pyrogens. We also strongly encourage ICCVAM to delete the recommendation regarding the conduct of *de novo* RPTs to further demonstrate *in vivo/in vitro* concordance."³

Our organizations stand by these initial recommendations. **However, given the PRP's final recommendations, we request that ICCVAM coordinate with the pharmaceutical and medical devices industry to conduct product-specific validation on a set of pre-selected products and devices to serve as further validation work.** Since this work will need to be conducted anyway, and would be acceptable to the Food and Drug Administration, this would be an appropriate way forward. Further delays or *de novo* validation work would result in the deaths of thousands of additional animals is not recommended.

Thank you for your attention to these comments. I can be reached at kstoick@pcrm.org or 510.834.8320 with any questions.

Sincerely,



Kristie Stoick, MPH
Physicians Committee for Responsible Medicine

² NIEHS (National Institute of Environmental Health Sciences), Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods, NIH Publication No. 97-3981, NIEHS, Research Triangle Park, North Carolina, U.S.A., 1997, sections 2.4.7 and 2.5.

³ Letter submitted to NICEATM January 26, 2007.

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Dear Dr. Stokes:

Please, find below our comments to the "Independent Peer Review Panel Report: Five *In vitro* Test Methods Proposal for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products".

Comment to A 1.2.1 Criterion 4 (page 3), also comment to A 4.6 (page 12)

The PRP discussed critically whether the right end-points had been set in the validation study regarding sensitivity (respectively detection limits) of the tests. We are wondering why the PRP did not consider the internationally accepted endotoxin limits. They are regulated in the respective monographs for endotoxin testing (Bacterial Endotoxin Test, BET) since decades in the international pharmacopoeias. The endotoxin limit for parenteral drugs intended for intravenous administration is 5 International Units endotoxin (E.U., to calibrate using the WHO Endotoxin Standard which is identical with the US Endotoxin Standard) per kilogram body weight of the patient (in the past: administration during one hour period; following the current ICH document: as a bolus injection). Exactly this endotoxin limit had been used for calculation of the detection limits in examining the involved drugs. As usual in pyrogen testing, a patient having a body weight of 70 kg (corresponding to a maximal endotoxin content of 350 E.U. of the whole volume of the given drug) had been considered for calculation. Furthermore, the WHO Endotoxin Standard had been used in the study.

Additionally, the sensitivity respectively the detection limit of Rabbit Pyrogen Test (RPT) had been considered in the study design. The sensitivity of RPT can be calculated considering the fever threshold respectively the threshold of significant temperature increase of rabbits. The most sensitive rabbit strains show a fever

threshold of 5 E.U. per kilogram body weight (see papers Hoffmann et al. 2005, Journal of Immunological Methods, Vol. 298, pp. 161-173, and Hoffmann et al. 2005, Journal of Endotoxin Research, Vol. 11, pp. 1-7). This endotoxin concentration may be contained in maximally 10 milliliter which represents the highest allowed burden for the rabbits following the animal protection laws. In consequence, the sensitivity of RPT is represented by 0.5 E.U. per milliliter (5 E.U. in 10 ml = 0.5 E.U. per ml) corresponding to 50 pg/ml. This endotoxin concentration had been used for setting the detection limits of the five *In vitro* Test methods and it is (at least) fulfilled by all tests. So the five alternative pyrogen tests meet worst case conditions of RPT and guarantee, therefore, a high safety level for the patients.

It has to be mentioned here that the endotoxin limit regulation mentioned above (5 E.U. per kg body weight of the recipient) comes directly from rabbit's sensitivity. Preparing the implementation of BET into the pharmacopoeias decades ago, the safety level of the drugs was the most important criterion. In this time, only data from the rabbit were available and, consequently, they were used for definition of endotoxin limits. This was a wise decision since the fever threshold of human beings lies in a range of 10 - 20 E.U. per kg body weight and, therefore, the safety of drugs regarding potential pyrogenicity is guaranteed. Taking into account the background of endotoxin limits, it is surprising when the expert panel used the phrasing "theoretical sensitivity" of the RPT. As demonstrated above, the calculations for the validation study reflects exactly the current practice and the regulatory use.

Comment to A 1.2.1 Criterion 5 (page 3)

The PRP stated: "The new test methods clearly take longer to produce definitive results". This statement does not consider the mandatory pre-test for RPT which has to be performed two days prior to the main test employing the same animals (i.e. RPT lasts all together not less than 48 hours). The *in vitro* tests are usually performed within less than 20 hours (i.e. incubation of the cells overnight and measuring the cytokine content in ELISA next morning). If necessary, the tests can be performed within 10 hours by shortening the cell culture to 6 hours.

Comment to A 1.2.2 (page 3)

There is a contradiction in this passage. On the one, hand it is stated: "The RPT (Rabbit Pyrogen Test) detects both endotoxin and non-endotoxin pyrogens, but the *in vitro* pyrogen tests have not been validated for non-endotoxin pyrogens. Therefore, they cannot be considered complete replacements for the RPT." On the other hand, it is stated: "The BET (Bacterial Endotoxin Test) detects endotoxin in most cases and is used instead of the RPT for this purpose." It is not understandable why the *in vitro* tests, able to detect endotoxin, cannot replace the RPT but BET, able to detect only endotoxin, can.

There is a clear need for tests able to detect non-endotoxin pyrogens (for examples regarding adverse reactions caused by non-endotoxin pyrogens, see comment to A 4.0 below, please). The PRP mentioned several times in the report that non-endotoxin pyrogens were not included in the validation study. This holds true but

there is a broad specter of publications demonstrating that *in vitro* pyrogen test methods are able to detect non-endotoxin pyrogens. This includes papers which applied the same procedure for pyrogen testing using human whole blood as used in the validation study (e.g. Hermann et al. European Journal of Immunology, 2002, Vol. 32, pp. 541-551, and Morath et al., Infection and Immunity, 2002, Vol. 70, pp. 938-944). One would appreciate if the PRP (at least) had mentioned those publications.

Comment to A 1.3.2 (page 4)

The PRP stated: "A major concern is the lack of validation of these new assays directly compared to the RPT." There were data available on several studies regarding comparison of RPT and *in vitro* pyrogen tests as used in the validation study. The first study (Spreitzer et al., Altex, 2002, Vol. 19, pp. 73-75) concerns a comparative study of Rabbit Pyrogen Test and Human Whole Blood Assay implementing 29 batches of 10 different Human Serum Albumins from 5 manufacturers. All together, 261 rabbits were included in the study. Two endotoxin spike concentrations in the range of RPT detection limit were used. There was no failure in the *in vitro* pyrogen test. Actually, the *in vitro* test appeared more sensitive than the RPT. In the second study (Andrale et al. International Journal of Pharmaceutics, 2003, Vol. 265, pp. 115-124) a broad range of parenterals (15 different drugs) were tested comparing RPT and BET with Human Whole Blood Assay and, additionally, with Human Peripheral Blood Mononuclear Cell (PBMC) Test. The two *in vitro* tests showed good agreement overall, both with each other and with BET and the RPT. The third study concerns a comparison of six different Coagulation Factor VIII Concentrates (3 lots each) in RPT and in Human Whole Blood Assay. 162 rabbits were included in the study; two different endotoxin spikes in the range of RPT detection limit were used. As in the above mentioned albumin study, no failure was seen in the *in vitro* assay. Again, the *in vitro* test appeared more sensitive as the RPT. The latter study is not published yet but, due to our knowledge, the data had been provided to the PRP.

Comment to A 3.1 (page 9)

The PRP stated: "No 'classical' examples of biological products or medical devices were included; thus, the validation of either of these categories has not been provided."

It should be mentioned that Coagulation Factor VIII concentrate had been included in the pre-validation study where it was successfully tested. This preparation could not be considered in main study because of its high price. Additionally, see the above comment to A 1.3.2, please. Human Serum Albumin and Coagulation Factor VIII concentrate belong to the 'classical' biological products.

Comment to A 3.4 (page 10)

The PRP stated: "The coding procedures were adequate for the assessment of relevance during the validation studies. However, the identity of substances used in the reproducibility analyses was not blinded (although the spike concentrations were). A reason was not given."

It is commonly known that a pharmaceutical company has to perform for any test the so called product validation in order to exclude potential interferences of the preparation with the test system (e.g. inhibition of the test by the drug). Of course, the best approach is to perform the product validation using a clean batch of the product. The latter procedure had been chosen for the validation study considering the practice in pharmaceutical industry.

Comment to A 4.0 (page 10)

The PRP stated: "... a summary of reference data demonstrating whether substances that were shown to be pyrogenic in humans either passed or failed the RPT, BET, or *in vitro* tests would have been useful." There are data published regarding adverse reactions (fever respectively pro-inflammatory reactions) in patients caused by drugs which were negative in RPT as well as in BET, but could be tested positive using *in vitro* pyrogen tests. The first event happened with a Tick Borne Encephalitis Vaccine which induced fever up to cramps and hospitalization in around 50 percent of the recipients. As mentioned above, both RPT and BET remained negative in testing the product. In contrast, this product produced positive results with blood samples of 50 percent of the donors applying the *in vitro* pyrogen test (Whole Blood Test, Fischer et al., *Altex*, 2001, Vol. 18, pp. 47-49). Another example concerns a dialysis solution which caused aseptic peritonitis in the patients (Martis et al. *Lancet*, 2005, Vol. 365, pp. 588-594). Again, both RPT and BET were negative whereas the *in vitro* pyrogen test (PBMC Test) could identify the incriminated batches. These two examples demonstrate that the *in vitro* pyrogen tests are in certain cases superior to the RPT since they are working in the 'homologous system' (i.e. human indicator cells and fever/pro-inflammatory reactions in humans).

It should be pointed out that the PRP should know the above cited cases as one of its members was in touch with both of them.

Comment to A 4.3 (page 11)

The PRP stated: "Archived records have not been audited by ECVAM or ICCVAM."

This statement is wrong as the archived records have been audited by ECVAM in PEI.

Comment to A 4.4 (page 11)

The PRP stated: "However, the PEI did not have formal GLP accreditation (refer to Section 5.5, ECVAM request for additional information)."

As the Federal Agency of Sera and Vaccines, the unit for pyrogen and endotoxin testing of the Paul Ehrlich Institute (PEI) is accredited following ISO 17025 (for the lists of accredited methods in PEI see http://www.pei.de/cIn_049/nn_162948/EN/infos-en/pu-en/11-quality-management-en/accredited-methods-en/akkreditierungen-inhalt-en.html, please).

Despite the above cited ECVAM answer, it should be mentioned here that one of the PRP members visited the PEI unit for pyrogen and endotoxin testing and knows its accreditation status.

Comment to A 5.1.1 (page 12)

The PRP stated: "Quality control (QC) testing of cell viability is not performed. Viability testing of human cells before and after incubation should be performed."

This statement does not consider how the tests are designed. It is one of the advantages of the *in vitro* pyrogen tests that additional testing of cell viability is not necessary. The functionality of cells is controlled in every test via reaction of monocytes to endotoxin controls which have to induce a defined minimum of cytokine concentration. This internal quality control gives more information on the status of the monocytes than a viability test; viability test indicates only that the cells are living whereas functionality test indicates that cells are living and able to react.

Comment to A 10.2 (page 21)

The PRP stated: "The discussion that reduction of the use of animals (i.e., rabbits) will be associated with the increased use of another animal (i.e., humans) is inadequate."

This statement seems to be far away from practice. It is commonly known that worldwide millions of people are donating blood (for example, more than 5 millions blood donations per year in Germany). One whole blood donation consists of 500 ml blood, a volume which would be theoretically sufficed for 5,000 to 50,000 whole blood pyrogen tests. Therefore, the use of human blood for pyrogen testing would lead to a marginal increase of blood donation. Blood donors are mainly volunteers offering their blood for philanthropic reasons. Donating blood for safety testing of drugs for human use is a philanthropic attitude, too.

Comment to A 11.4 (page 22)

The PRP stated: "Furthermore, the *in vitro* pyrogen test methods are dependent on the availability of donors or blood supplies, which might further restrict the frequency of which these tests can be performed."

This statement does not consider the cryo-preserved blood since it would be available at any time.

Thank you for your attention to these comments.

Sincerely,

Dr. Thomas Montag-Lessing
Dr. Ingo Spreitzer

Appendix D3
SACATM Comments: ICCVAM Evaluation of *In Vitro* Pyrogen Test Methods

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The following is excerpted from the final minutes of the SACATM meeting convened on June 12, 2007. The full meeting minutes will be available online at <http://ntp-server.niehs.nih.gov/ntpweb/index.cfm?objectid=AF6CC417-F1F6-975E-75B5F3FF7DF1CDDC>.

Overview of the ICCVAM Evaluation of *In Vitro* Pyrogen Test Methods

Dr. Richard McFarland, U.S. Food and Drug Administration (FDA), ICCVAM Pyrogenicity Working Group (PWG) Chair, presented an update on ICCVAM's ongoing evaluation of five *in vitro* human cell-based pyrogen test methods nominated for review by the European Centre for the Validation of Alternative Methods (ECVAM). Pyrogenicity is defined as an increase in body temperature following the release of pro-inflammatory cytokines [e.g., interleukin (IL)-1, IL-6, and tumor necrosis factor- α (TNF- α)] by leukocytes. Pyrogens may be found in processing and packaging materials, chemicals, or parenteral pharmaceuticals, biologicals, and medical devices. Bacterial endotoxin, a component of the outer cell wall of Gram-negative bacteria, is one of the most potent pyrogenic materials. Pyrogen testing is important to prevent the introduction of endotoxin or non-endotoxin pyrogen-contaminated products into humans or animals.

Currently there are two accepted pyrogen tests. The Rabbit Pyrogen Test (RPT), which measures a temperature rise in rabbits injected with a test substance, can detect both endotoxin and non-endotoxin pyrogens. The Bacterial Endotoxin Test (BET), also referred to as the *Limulus* Amoebocyte Lysate (LAL) Test, detects endotoxin by its ability to activate a serine-protease catalytic cascade.

In June 2005, ECVAM submitted background review documents (BRDs) on five methods for consideration by NICEATM as replacements for the RPT. The methods are:

- Human Whole Blood (WB)/Interleukin (IL)-1 *In Vitro* Pyrogen Test
- Human WB/IL-1 *In Vitro* Pyrogen Test: Application of Cryopreserved Human WB
- Human WB/IL-6 *In Vitro* Pyrogen Test
- Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test
- *In Vitro* Pyrogen Test using the monocytoid cell line, Mono Mac 6 (MM6)/IL-6

Before describing the evaluation process, Dr. McFarland listed the members of the PWG, provided a time line for the various activities connected with the evaluation process, and described the ICCVAM acceptance and validation criteria for alternative test methods.

Following a prescreen evaluation, NICEATM requested additional information and clarification from ECVAM in regard to the data provided in their BRDs. ECVAM submitted revised BRDs that addressed these requests. Subsequently, ICCVAM prepared a draft ICCVAM BRD that contained a comprehensive review of all available data and information regarding the usefulness and limitations of the five alternative *in vitro* pyrogen test methods and described the current validation status of the test methods including their relevance, reliability, scope of substances tested, and the availability of a standardized test method protocol for each test method.

The major difference among the five test methods is the cell types used; the methodology used for the test methods is very similar. Briefly, the test substance is applied to cultures of the specific human-derived cells, which are then incubated for 16-24 hr. The concentration of pro-inflammatory cytokines (e.g., IL-1 β , IL-6) is quantified via a cytokine-specific enzyme-linked immunosorbent assay (ELISA). The endotoxin activity of a test substance is calculated by comparing the induced cytokine release with that induced by the endotoxin standard.

The test methods were reviewed for their ability to detect the presence of Gram-negative endotoxin when several parenteral pharmaceuticals were spiked with the endotoxin standard at several different concentrations. The reference pharmaceuticals were considered positive for endotoxin if the endotoxin content was > 0.5 endotoxin units (EU)/mL. Differences were found in the performance of the five test methods. Based on the information contained in the BRD, ICCVAM developed draft recommendations for the use, formulated draft performance standards and draft test method protocols for each test method, and identified proposed future studies.

ICCVAM's draft recommendations on test method uses and limitations was that, based on the validation studies with a limited number of pharmaceuticals, there is sufficient information to substantiate the use of these test methods for the detection of pyrogenicity mediated by Gram-negative endotoxins in materials that are currently tested in the RPT, subject to product-specific validation to demonstrate equivalency. Further, ICCVAM's draft recommendations stated that although the five *in vitro* test methods may be capable of detecting a wider range of pyrogens than was tested, the data in the BRDs do not support this broader application. One limitation of the validation study was the lack of a direct comparison of the results for the same test substances in the proposed *in vitro* test methods versus the RPT.

ICCVAM also provided draft recommendations for performance standards for these five *in vitro* test methods for consideration by the peer review panel and for public comment; the purpose of performance standards are to ensure that any proposed mechanistically and functionally similar proposed test method meets acceptable standards. Performance standards include essential test method components based upon common structural, functional, and procedural elements that should be included in the protocol of a mechanistically and functionally similar proposed test method; recommended reference substances for evaluating the relevance and reliability of the proposed test method and the performance characteristics (relevance and reliability values) that should be met or exceeded. ICCVAM also recommended draft standardized protocols that were based on those used in the ECVAM validation study. Finally, ICCVAM recommended future studies that included the testing of a broader range of pyrogenic materials under conditions where the *in vitro* pyrogen test(s) and the RPT were run in parallel to be able to directly compare the results.

Peer Panel Report

ICCVAM and NICEATM held a peer review panel meeting on February 6, 2007, to review the five *in vitro* pyrogenicity test methods. Dr. Karen Brown, DRL Pharma and

Pair O'Doc's Enterprises, chair of the peer panel, said the task was daunting because the panel was tasked to complete the evaluation of the five *in vitro* test methods in one day. She recognized the hard work and diligence of the panel.

The charge to the peer review panel was to review the draft BRDs for completeness, assess whether each applicable criterion for validation and acceptance of the test method had been appropriately addressed, and consider whether the information in the BRD supported the draft ICCVAM recommendations for the draft standardized protocols, the draft test method performance standards, and the draft proposed future studies.

The panel concluded that the explanation in the BRD of the usefulness and limitations of the *in vitro* pyrogenicity test methods and of the description of the current validation status of these methods was sufficient. However, they identified a number of deficiencies in the BRD, which are briefly described below.

1. There were some sections where additional details would have improved the document. For example, the panel wanted information included about (1) the number of RPTs conducted per year to evaluate bacterial endotoxin, (2) the number of rabbits used for *pyrogenicity* testing per year, and (3) the costs and logistical considerations for either setting up the cell culture for the MM6 test or obtaining human blood for the other tests.
2. The rationale for selecting the test substances for evaluating the five *in vitro* test methods was flawed because it did not represent the range of products tested for bacterial endotoxin using the RPT and seven of the 10 substances were not tested in the RPT but rather in the BET. For example, no biologicals or medical devices were evaluated. The panel felt that the number of substances tested in the validation study was not adequate to evaluate whether a specific test method could replace the RPT.
3. The *in vivo* RPT reference data were limited to one strain of rabbit tested in one laboratory by one protocol using two sources of bacterial endotoxin.
4. The evaluation of the relevance of each test method was adequately demonstrated and discussed in the BRDs, but was limited by the ability to judge a positive versus a negative response based on 0.5 endotoxin units (EU)/ mL. Since samples were only spiked with bacterial endotoxin, the relevance was only demonstrated for the detection of this type of pyrogen, and there was no evaluation for the ability to detect non-endotoxin pyrogens.
5. The discussion on concordance in the RPT is speculative because there was no parallel testing with the RPT, and the RPT performance was modeled statistically.
6. The whole blood IL-1 test is inadequate because there were too many false positives and false negatives; however the IL-6 assay appeared to perform better.

It would have been more appropriate to compare these *in vitro* tests directly with the BET, since only bacterial endotoxin samples were used.

7. Test method reliability was acceptable in both within and between laboratory studies; however, a quantitative assessment of intra- and inter-laboratory variability would have been more informative. A statistical assessment providing acceptability criteria should have been performed to test the hypothesis that there were no differences among groups.
8. The assessment of test method reliability had the following deficiencies:
 - a. There was a high exclusion rate for individual runs of the whole blood IL-1 assay due to excessive variability among the four replicates.
 - b. The agreement across three validation laboratories was only 57% for the whole blood IL-1 assay.
 - c. The same subset of drugs tested for sensitivity and specificity should have been tested for reliability.

Most of the panel agreed that application of the validation criteria to determine the usefulness and limitations of these test methods to replace the RPT under conditions where the test was for the presence of Gram-negative endotoxin was adequately addressed in the BRDs.

The panel concluded that the usefulness of the test methods to detect Gram-negative endotoxin was not assessed properly to determine their concordance with the RPT or to compare their relevance with the BET. The assessment of the usefulness was limited because non-endotoxin pyrogens were not included, and the pure form of the test materials may stimulate cytokine production.

The panel agreed that the BRDs did support the proposed standardized test method protocols if the list of its inadequacies were fully addressed. The panel noted that to reduce variability, similar acceptance criteria must be used for multiple blood donors and similar exclusion rules must be used for each test method. They recommended that a more specific protocol be developed that details recruitment of human blood donors, selection criteria for donors, as well as conditions for veinipuncture.

The panel concluded that the test method performance standards were not supported by the BRD. Statements about the five methods' accuracy and reliability were not supported because two assays demonstrated false-positive results greater than 16 % and the *in vitro* test methods should have been compared to both the BET and RPT. Also, the panel thought that the small list of substances was inadequate to assess whether these test methods could replace the RPT. Test substances need to include all classes of endotoxins as well as non-endotoxin pyrogens.

The panel agreed that additional studies should be performed, and that ICCVAM should consider their comments and recommendations. They suggested (1) establishment of a

repository of clinically identified pyrogens to use in future validation studies, (2) inclusion of both endotoxin and non-endotoxin pyrogens in future validation studies, (3) prospective comparison of any *in vitro* tests with the RPT and BET, and (4) evaluation of IL-1 and IL-6 levels in the *in vitro* tests and their correlation with levels produced in rabbits exposed to similar levels of endotoxin.

Overall, the peer review panel concluded that these five test methods could be applicable for a wider range of pyrogens and test materials if they were adequately validated for such uses. It is important to recognize that, despite the panel's concerns about the performance of these five *in vitro* test methods, the FDA has a formal process for materials regulated under 21CFR610.9 (e.g., parenteral drugs) that allows drug manufacturers to qualify *in vitro* test methods for identifying Gram-negative endotoxin, on a case-by-case basis.

Public comments:

Dr. Freedman identified the written comments submitted by Physicians Committee for Responsible Medicine (PCRM).

Ms. Kristy Stoick, PCRM, said her organization submitted written comments after the peer review panel meeting. PCRM was disappointed with the ICCVAM draft recommendations and the peer review panel report. Since federal regulations specify that these methods must undergo product specific validation for pyrogenicity, she encouraged SACATM to recommend that ICCVAM help facilitate further development of these methods by companies so the regulatory community can begin to use them as soon as possible. She did not support additional *in vivo* validation studies.

Dr. Thomas Hartung, ECVAM, joined the public for this specific agenda item because of a conflict of interest as a patent holder for the methods. Three of the *in vitro* test methods were based on his research and he had coordinated the validation study prior to joining ECVAM. He was pleased that the European Pharmacopoeia will hold a peer review panel to review and accept these methods. He was disappointed with the outcome of the peer review panel meeting. He noted that pyrogenicity tests are very expensive and the approval and release of a single product can cost several hundred thousand dollars. The validation studies were set up to assess whether the new tests would outperform the old tests within a set threshold. Only 50% of the samples would be positive in the most sensitive rabbit strain. All of the *in vitro* assays have an accuracy of around 90%. He outlined six points where the BRD had been criticized.

1. ICCVAM said the BRD is deficient due to the limited data for only 10 pharmaceutical substances from the validation studies, which alone cost \$6M. The recommendations for additional studies from the peer review panel would cost between \$20-40M and they would be a waste of resources because a product-specific validation process would be required for each application. To help contain cost, the tests described in the BRD were designed to emphasize the accuracy of the method to detect pyrogens near the threshold.

2. The peer review panel did not acknowledge the difference in status of the five methodologies. Some methods are used in more than 80 laboratories while others are used infrequently; however, the same criticisms were applied to all of the methods.
3. The BRD recommended that parallel testing be conducted with the RPT. However, parallel testing in rabbits is unnecessary because these studies have been performed for 65 years using a WHO standard as a reference material. The outcome from rabbit testing is so predictable that ethically it is not warranted. Also, in the European Union, it will be impossible for ECVAM to carry out these *in vivo* tests especially as the new methodologies have shown partial concordance.
4. Endotoxins are only tested in the BET assay, and this assay has replaced the RPT for about 90% of substances; the remaining 10% of substances consist of non-endotoxin pyrogen products that interfere with the BET. He asked why the new tests have to meet higher standards than the BET, which has been endorsed for the testing of many pyrogenic products. He noted that no reference non-endotoxin pyrogens are suitable for validation purposes in rabbits and humans; therefore, inclusion of such controls is scientifically impossible.
5. High endotoxin concentrations will be detected accurately in the RPT, BET, or any of the new *in vitro* pyrogenicity assays. Hence, a concentration near 50 pg of endotoxin, which is equivalent to 0.5 EU and is the threshold for rabbits, was chosen for the assays. Additional concentrations of 100 pg and 25 pg were also selected. Even though the assays were challenged at these low concentrations, they were 90% accurate. False positives were due to spikes at half the threshold indicating that the assays are too sensitive.
6. The new assays were evaluated fairly in comparison to the limitations of the existing tests. The rabbit test, which has a number of limitations, has never been properly validated for non-endotoxin pyrogens. The BET does not detect all Gram-positive endotoxins although the new assays have shown some capability for doing so.

In conclusion, the proposed test methods for which data sets have been provided perform better than the BET and RPT. Dr. Hartung proposed that the rabbit assay be replaced with the *in vitro* assays because the RPT cannot match their performance, as reported in the BRD.

SACATM Discussion.

SACATM was asked to address questions regarding the peer review panel's conclusion and recommendations of the draft ICCVAM BRD with regard to its completeness; the panel's identification of errors or omissions; whether ICCVAM's applicable criteria for validation and acceptance of toxicological test methods were addressed; and to provide comments on the draft ICCVAM test methods recommendations, usefulness of the test

methods, the test method protocols, proposed performance standards, as well as proposed additional studies.

Dr. Barile, a lead discussant, said there was no question about the usefulness of pyrogenicity testing and the urgency and importance of validating these tests. In combination, some of these tests will contribute to the reduction of animal usage. One major deficiency of present pyrogenicity testing is that the RPT only detects about 50% of the endotoxins. Some of the proposed *in vitro* tests had false negative responses in the range of 10% while the IL-1 assay had a false negative response of 27%. These false negative responses could be due to consistently higher variability among some donors, which would be a limitation relative to a whole blood human assay. He expressed concern that the IL6 ELISA test, marketed by Novartis, is a proprietary test and he would not recommend approving a method without knowing the experimental details. He agreed with Dr. Hartung that parallel testing in rabbits was unnecessary during development of the testing methodologies. However, a comparison to RPT data is necessary so that a valid concordance or regression analysis between the *in vivo* and *in vitro* methods can be undertaken. He said samples spiked with endotoxin are not representative of real world samples such as a biological vaccine or a solubilized pharmaceutical product. There is no solubility problem associated with the testing of biological vaccines in rabbits, but insolubility is a problem in *in vitro* tests even if the test article is in suspension and this technicality must be addressed. He believes that the cell culture methods are more developed than the whole blood methods for validation purposes. A few additional studies, which address the panel's recommendations, would allow the cell culture pyrogenicity tests to receive validation status.

Dr. McClellan said he was generally pleased with the draft BRD until he heard Dr. Hartung's statement. He did not believe that the BRD is adequate nor can he compliment the peer review panel on its report. He wondered how this difference of opinion would be resolved and asked Dr. Brown to comment.

Dr. Freedman said he was confident that all of SACATM's comments would be taken into account by ICCVAM and, if necessary, ICCVAM could reconvene the expert panel.

Dr. Brown said ECVAM produced a reasonably comprehensive BRD, but the panel was not able to address all of the components of the individual *in vitro* methods because time for discussion was limited. Some of the details were missing or difficult to understand; however, she felt that given more time to discuss these methods, the panel might have been able to provide a stronger recommendation for one or more of the assays.

Personally, she felt that the MM6 assay has the greatest potential and several of the other panel members agreed. The most bothersome aspect for the panel was trying to identify the specifics of the validation protocols. She noted that for an *in vitro* assay it is critical to identify every component and every single condition of the assay completely, but this information was not provided, particularly for the MM6 test method. She was impressed with the cell culture methodology, although specifics such as cell passage levels, or how many cells are used in a test were lacking. She felt that the panel did not seem to understand cell culture methodology and its related costs. Consequently, they got side-

tracked in specifics, which hindered them from making progress and reaching conclusions.

Dr. Brown said she does not believe that it is necessary to run *in vivo* assays in parallel with the *in vitro* assays. She is unsure how one can run a regression analysis with one test that is 90% accurate and a second that is 50% accurate. She questioned whether it is necessary to validate an *in vitro* test against an animal test that is not as accurate as the *in vitro* assay itself.

Dr. McClellan said that Dr. Hartung disclosed his own potential biases, concerns, and background. He asked whether Dr. Hartung was suggesting that two of the assays should have received more attention and wondered which of the assays Dr. Hartung thought were appropriately validated and whether he might focus the panel toward those assays.

Dr. Stokes said that in the future NICEATM would set aside at least two days for a peer review meeting, so that a panel can fully understand the methodologies before they deliberate on the evaluation questions.

Dr. Qu had some comments on the panel's concern about data transformations. The panel was not sure if the data were transformed and whether or not the use of a "t" test for their analysis was appropriate. She said it is not necessary to use a "t" test even if the data are normal. A non-parametric test such as the permutation test, which does not require transformation, could be used. Dr. Qu noted also that it is important to control for false positives when doing a multiple comparison for several tests. By doing multiple comparisons, it is possible to obtain a statistically significant difference that is not biologically significant. One approach to dealing with this problem is to use a more stringent level of significance.

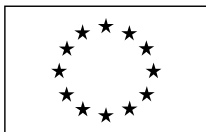
Dr. Becker welcomed the proposed longer time frame for a peer review meeting. He suggested that it might be useful to convene a meeting with a core panel of validation experts and then have subject-specific experts to address specific assays.

Appendix E

ESAC Statement on the Validity of *In Vitro* Pyrogen Tests

ESAC Statement on the Validity of *In Vitro* Pyrogen TestsE-3

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EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
European Centre for the Validation of Alternative Methods (ECVAM)

STATEMENT ON THE VALIDITY OF IN-VITRO PYROGEN TESTS

At its 24th meeting, held on 20-21 March 2006 at the European Centre for the validation of alternative methods (ECVAM), Ispra, Italy, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC)¹ unanimously endorsed the following statement:

Following a review of scientific reports and peer reviewed publications on the following range of in-vitro pyrogen tests:

1. Human Whole Blood IL-1,
2. Human Whole Blood IL-6,
3. PBMC IL-6,
4. MM6 IL-6, and
5. Human Cryopreserved Whole Blood IL-1,

it is concluded that these tests have been scientifically validated for the detection of pyrogenicity mediated by Gram-negative endotoxins, and quantification of this pyrogen, in materials currently evaluated and characterized by rabbit pyrogen tests.

These methods have the potential to satisfy regulatory requirements for the detection and quantification of these pyrogens in these materials subject to product-specific validation.

The test methods have the capacity of detecting pyrogenicity produced by a wider range of pyrogens, but the evidence compiled for, and considered within this peer review and validation process, is not sufficient to state that full scientific validation of this wider domain of applicability has been demonstrated and confirmed.

Thus, the above test methods can currently be considered as full replacements for the evaluation of materials or products where the objective is to identify and evaluate pyrogenicity produced by Gram-negative endotoxins, but not for other pyrogens.

This endorsement takes account of the dossiers prepared for peer review; the views of independent experts who evaluated the dossiers against defined validation criteria; supplementary submissions made by the Management Team; and the considered view of the Peer Review Panel appointed to oversee the process.

Thomas Hartung
Head of Unit
ECVAM
Institute for Health & Consumer Protection
Joint Research Centre
European Commission
Ispra

21 March 2006

1. The ESAC was established by the European Commission, and is composed of nominees from the EU Members States, industry, academia and animal welfare, together with representatives of the relevant Commission services.

This statement was endorsed by the following Members of the ESAC:

Prof Helmut Tritthart (Austria)
Dr Dagmar Jírová (Czech Republic)
Prof Elisabeth Knudsen (Denmark)
Dr Timo Ylikomi (Finland)
Prof André Guillouzo (France)
Dr Manfred Liebsch (Germany)
Dr Efstathios Nikolaidis (Greece)
Dr Katalin Horvath (Hungary)
Prof Michael Ryan (Ireland)
Dr Annalaura Stammati (Italy)
Dr Mykolas Maurica (Lithuania)
Prof Eric Tschirhart (Luxembourg)
Dr Jan van der Valk (The Netherlands)
Dr Dariusz Sladowski (Poland)
Prof Milan Pogačnik (Slovenia)
Dr Argelia Castaño (Spain)
Dr Patric Amcoff (Sweden)
Dr Jon Richmond (UK)
Dr Odile de Silva (COLIPA)
Dr Julia Fentem (ECETOC)
Dr Nathalie Alépée (EFPIA)
Prof Robert Combes (ESTIV)
Dr Maggy Jennings (Eurogroup for Animal Welfare)
Mr Roman Kolar (Eurogroup for Animal Welfare)

The following Commission Services and Observer Organisations were involved in the consultation process, but not in the endorsement process itself.

Mr Thomas Hartung (ECVAM; chairman)
Mr Jens Linge (ECVAM; ESAC secretary)
Mr Juan Riego Sintes (ECB)
Ms Beatrice Lucaroni (DG Research, Unit F.5)
Mr Sylvain Bintein (DG Environment, Unit C.3)
Mr Sigfried Breier (DG Enterprise, Unit F.3)
Prof Dr Constantin Mircioiu (Romania)
Dr William Stokes (NICEATM, USA)
Prof Dr Vera Rogiers (ECOPA)

Annex

The novel pyrogen tests are based on the human fever reaction. Monocytoid cells, either primary from human blood or as propagated cell lines, detect pyrogens of different chemical nature and respond by the release of inflammatory mediators such as cytokines. Since lipopolysaccharides from Gram-negative bacteria are the only type of proven pyrogen, for which an International reference material is available, the tests were standardised to detect the presence of significantly less than 0.5 Endotoxin Units of this preparation, which is considered to be the threshold level for fever induction in the most sensitive rabbit species according to pharmacopoeia test procedures.

The five tests which were sufficiently reproducible and exceeded the rabbit test with regard to sensitivity and specificity for the detection of lipopolysaccharide spiked samples, differ with regard to cell source and preparation, cryopreservation and cytokine measured. The tests have been described elsewhere (1-4). The concept of the validation study (5) and the international validation studies are available (6-7).

1. Poole, S., Thorpe, R., Meager, A., Hubbard, A.R., Gearing, A.J. (1988) Detection of pyrogen by cytokine release. *Lancet* 8577, 130.
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National Institutes of Health

Appendix B1

Draft ICCVAM Recommended Protocol for Future Studies Using the Cryopreserved Whole Blood (Cryo WB)/Interleukin-1 β (IL-1 β) Test Method

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1 **Draft ICCVAM Recommended Protocol for Future Studies Using the Cryopreserved**
2 **Whole Blood (Cryo WB)/Interleukin-1 β (IL-1 β) Test Method**

3

4

PREFACE

5

6 This proposed protocol for the detection of pyrogenicity is based on information obtained
7 from 1) The European Centre for the Validation of Alternative Methods (ECVAM) Cryo
8 WB/IL-1 β Background Review Document (BRD) presented in Appendix A of the draft
9 Interagency Coordination Committee on the Validation of Alternative Methods (ICCVAM)
10 Pyrogenicity Test Method BRD, which includes ECVAM Standard Operating Procedures
11 (SOPs) for the Cryo WB/IL-1 β test method, and 2) Information provided to the National
12 Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative
13 Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The
14 ECVAM SOPs are based on the Cryo WB/IL-1 β methodology first described by Schindler et
15 al. (2004). A table of comparison between the draft ICCVAM recommended protocol and the
16 ECVAM SOP is provided in **Table 1**. Future studies using the Cryo WB/IL-1 β test method
17 may include further characterization of the usefulness or limitations of the assay for
18 regulatory decision-making. Users should be aware that the proposed test method protocol
19 might be revised based on additional optimization and/or validation studies. ICCVAM
20 recommends that test method users routinely consult the ICCVAM/NICEATM website
21 (<http://iccvam.niehs.nih.gov/>) to ensure that the most current test method protocol is used.

22

22 **Table 1 Comparison of Draft ICCVAM Recommended Test Method Protocol**
 23 **with the ECVAM SOP for the Cryopreserved Whole Blood (Cryo**
 24 **WB)/Interleukin-1 β (IL-1 β) Pyrogen Test**

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP
Test Substance	Test neat or at minimal dilution that produces 50% to 200% of 1 EU/mL EC	Test at MVD
Incubation Plate (number of control or test groups at n=4 each)	NSC (1)	Same as ICCVAM protocol
	EC (5)	EC (2)
	TS (14)	TS (3) x EC (5) spikes
	PPC ¹ (0)	PPC (3)
	NPC ¹ (0)	NPC (3)
ELISA Plate	Includes seven point IL-1 β SC and blank in duplicate	Same as ICCVAM protocol
Decision Criteria for Interference	0.5 x Median OD ₄₅₀ ² of 1 EU/mL EC <2x Median OD ₄₅₀ of 1 EU/mL EC	Mean OD ₄₅₀ ² of PPC \geq 1.6X Mean OD ₄₅₀ of NPC
Assay Acceptability Criteria	Mean OD ₄₅₀ of 0.5 EU/mL EC \geq 1.6X Mean OD ₄₅₀ of NSC	Same as ICCVAM protocol
	Mean OD ₄₅₀ of PPC is 50% to 200% of 0.5 EU/mL EC	Same as ICCVAM protocol
	Mean OD ₄₅₀ of NSC \leq 0.15 ³	Mean OD ₄₅₀ of NSC \leq 0.10
	Not included	If one OD ₄₅₀ of the 1.0 EU/mL EC >Max, the ELISA may be repeated at reduced incubation time
	EC SC produces OD ₄₅₀ values that ascend in a sigmoidal concentration response	Same as ICCVAM protocol
	Quadratic function of IL-1 β SC $r^2 \geq$ 0.95 ³	Same as ICCVAM protocol
Decision Criteria for Pyrogenicity	OD ₄₅₀ Test sample \geq OD ₄₅₀ 0.5 EU/mL EC	Same as ICCVAM protocol

25 Abbreviations: EC = Endotoxin control; MVD = Maximum Valid Dilution; NPC = Negative Product Control;
 26 NSC = Normal saline control;

27 PPC = Positive Product Control; SC = Standard curve; TS = Test substance

28 ¹ PPC and NPC are evaluated during the interference test.

29 ² Median or mean OD₄₅₀ values are corrected (i.e., reference filter reading, if applicable, and NSC are
 30 subtracted).

31 ³ Criteria originated from PBMC SOP.

32

33

34

34 **1.0 PURPOSE AND APPLICABILITY**

35 The purpose of this protocol is to describe the procedures used to evaluate the presence of a
36 pyrogen (i.e., Gram-negative endotoxin) in a test substance. The presence of Gram-negative
37 endotoxin is detected by its ability to induce cytokine IL-1 β release from monocytoid cells in
38 human Cryo WB. The quantity of IL-1 β released is obtained using an enzyme-linked
39 immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for
40 IL-1 β . Release of IL-1 β is measured by incubation of Cryo WB with test substances or
41 controls (i.e., positive, negative). The amount of pyrogen present is determined by comparing
42 the values of endotoxin equivalents produced by cells exposed to the test substance to those
43 exposed to an internationally-harmonized Reference Standard Endotoxin (RSE)¹ or an
44 equivalent standard expressed in Endotoxin Units (EU)/mL. Based on a rabbit threshold
45 pyrogen dose of 0.5 EU/mL, which was established in a retrospective evaluation of rabbit
46 pyrogen test (RPT) data, a test substance is considered pyrogenic if it induces a level of IL-
47 1 β release equal to or greater than that induced by 0.5 EU/mL of endotoxin.

48 The focus of this protocol is on the use of the Cryo WB/IL-1 β test method, specifically for
49 the detection of Gram-negative endotoxin in parenteral pharmaceuticals. The relevance and
50 reliability for non-endotoxin pyrogens (e.g., lipoteichoic acid) has not been demonstrated in a
51 formal validation study.

52 **2.0 SAFETY AND OPERATING PRECAUTIONS**

53 All procedures for procurement of eligible blood donors and blood donations should follow
54 the regulations and procedures set forth by institutional guidelines for utilization of human
55 substances, which include but are not limited to blood, tissues, and tissue fluids. Standard
56 laboratory precautions are recommended including the use of laboratory coats, eye
57 protection, and gloves. If necessary, additional precautions required for specific study
58 substances or hazardous chemicals will be identified in the Material Safety Data Sheet
59 (MSDS).

¹ RSEs are internationally-harmonized reference standards (e.g., WHO *E. coli* Lipopolysaccharide [LPS] 94/580 [0113:H10:K-]; USP RSE Lot G3E069; FDA Lot EC-6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

60 The stop solution used in the ELISA kit is acidic and corrosive and should be handled with
61 the proper personal protective devices. If this reagent comes into contact with skin or eyes,
62 wash thoroughly with water. Seek medical attention, if necessary.

63 Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-
64 TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate
65 personal protection should be used to prevent bodily contact.

66 Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage,
67 antigenic response) and should be handled with care. Skin cuts should be covered and
68 appropriate personal protective devices should be worn. In case of contact with endotoxin,
69 immediately flush eyes or skin with water for at least 15 min. If inhaled, remove the affected
70 individual from the area and provide oxygen and/or artificial respiration as needed. Skin
71 absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

72 **3.0 MATERIALS, EQUIPMENT, AND SUPPLIES**

73 **3.1 Source of Cells**

74 Leukocytes from WB are the source of cells for cytokine production in the Cryo WB/IL-1 β
75 test method (Hartung and Wendel, 1999; Schindler et al., 2004, 2006). WB is obtained from
76 healthy human volunteers who have provided their consent according to established
77 institutional guidelines. Volunteers are expected not to have taken any drugs (e.g.,
78 prescription drugs, recreational drugs, herbal drugs) and to have been free from illness for at
79 least two weeks prior to donation.

80 The WB is processed and cryopreserved using either the Konstanz method developed at the
81 University of Konstanz (Schindler et al., 2004) or the PEI method developed at the Paul
82 Ehrlich Institute (Schindler et al., 2006).

83 **3.2 Equipment and Supplies**

84 For all steps in the protocol, excluding the ELISA procedure, the materials that will be in
85 close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be
86 sterile and free from detectable pyrogens.

- 87 3.2.1 Blood Incubation
- 88 3.2.1.1 *Equipment*
- 89 • Centrifuge
- 90 • Hood; Bio-safety, laminar flow (recommended)
- 91 • Incubator; cell culture (37 \pm 1 $^{\circ}$ C + 5% CO₂)
- 92 • Pipetter; multichannel (8- or 12-channel)
- 93 • Pipettors; single-channel adjustable (20 and 200 μ L)
- 94 • Repeating pipetter
- 95 • Vortex mixer
- 96 3.2.1.2 *Consumables*
- 97 • Centrifuge tubes; nonpyrogenic, polystyrene (15 and 50 mL)
- 98 • Combitips; repeating pipetter (1.0 and 2.5 mL)
- 99 • Needle set; Sarstedt multify, pyrogen-free, 19 mm, 21 gauge for S-Monovette
- 100 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- 101 • Reaction tubes; polystyrene (1.5 mL)
- 102 • Reservoirs; fluid
- 103 • RPMI-1640 cell culture medium
- 104 • Tips; pipetter, sterile, pyrogen-free (20 and 200 μ L)
- 105 • Tubes; Sarstedt S-Monovette, 7.5 mL, heparinized for blood collection
- 106 3.2.2 ELISA
- 107 3.2.2.1 *Equipment*
- 108 • Microplate mixer
- 109 • Microplate reader (450 nm with an optional reference filter in the range of
- 110 600-690 nm)
- 111 • Microplate washer (optional)

- 112 • Multichannel pipetter

113 3.2.2.2 *Consumables*

- 114 • Container; storage, plastic
- 115 • Deionized water; nonsterile
- 116 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- 117 • Pyrogen-free water (PFW)
- 118 • Reservoirs; fluid
- 119 • Tips; pipetter, nonsterile
- 120 • Tubes; polystyrene (12mL)

121 3.2.2.3 *ELISA Kit*

122 An ELISA that measures IL-1 β release from Cryo WB is used. A variety of IL-1 β ELISA
123 kits are commercially available and the IL-1 β ELISA procedure outlined in this protocol is
124 intended to serve as an example for using an ELISA kit. If the user prefers to prepare an in-
125 house ELISA, then additional reagents would be required. The IL-1 β ELISA should be
126 calibrated using an international reference standard (e.g., WHO 86/680) prior to use. The IL-
127 1 β cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this
128 reagent must be purchased separately. Results obtained using these products are subject to
129 the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-1 β ELISA
130 kit components may include the following:

- 131 • ELISA plates coated with anti-human IL-1 β capture antibody; monoclonal or
132 polyclonal
- 133 • Buffered wash solution
- 134 • Dilution buffer
- 135 • Enzyme-labeled detection antibody
- 136 • Human IL-1 β reference standard
- 137 • Pyrogen-free saline (PFS)

- 138 • Stop solution
- 139 • TMB/substrate solution

140 **3.3 Chemicals**

- 141 • Endotoxin (e.g., WHO *E. coli* LPS 2nd International Standard 94/580; USP
- 142 RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

143 **3.4 Solutions**

- 144 • RPMI-1640 cell culture medium

145 **4.0 ASSAY PREPARATION**

146 All test substances, endotoxin, and endotoxin-spiked solutions should be stored at 4°C.

147 **4.1 Endotoxin Standard Curve**

148 An internationally harmonized RSE or equivalent is used to generate the endotoxin standard
149 curve. The use of any other *E. coli* LPS requires calibration against a RSE using the Cryo
150 WB/IL-1 β test method.

151 A standard endotoxin curve consisting of a Normal Saline Control (NSC) and five RSE
152 concentrations (0.25, 0.50, 1.0, 2.5, and 5.0 EU/mL) are included in the incubation step (refer
153 to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard
154 curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized
155 content of the stock vial by following the instructions provided by the manufacturer (e.g., for
156 a vial containing 10,000 EU, 5 mL of PFW is added). To reconstitute the endotoxin, the stock
157 vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for 5
158 min. The stock solution is stable for 14 days when stored at 2 to 8°C. An endotoxin standard
159 curve is prepared by making serial dilutions of the stock solution in PFS as described in
160 **Table 4-1**.

161

161

162 **Table 4-1 Preparation of Endotoxin Standard Curve**

Stock Endotoxin EU/mL	μ L of Stock Endotoxin	μ L of PFS	Endotoxin Concentration EU/mL
2000 ^{1,2}	50	1950	50 ³
50	100	900	5.0
5.0	500	500	2.5
2.5	400	600	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0	0	1000	0

163 Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

164 Each stock tube should be vortexed vigorously prior to its use to make the subsequent dilution.

165 ¹ A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.166 ² The stock solution of USP RSE may be stored in aliquots and kept at -20°C for up to 6 months. Do not store
167 the endotoxin at -80°C.168 ³ This concentration is not used in the assay.

169

170 **4.2 Test Substances**

171 Liquid test substances should be tested neat or, if interference is detected (see **Section 4.2.1**),
172 diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in
173 saline, dissolved in dimethylsulfoxide (DMSO) then diluted up to 0.5% (v/v) with PFS,
174 provided that this concentration does not interfere with the assay. The test substances should
175 be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for 5 min.

176 **4.2.1 Interference Testing**

177 Interference testing must be carried out on any test sample for which no interference
178 information is available. The purpose of the interference test is to determine the lowest
179 dilution (i.e., highest concentration) of a test substance from which an endotoxin spike can be
180 detected (i.e., based on the decision criteria described in **4.2.1.2**). However, to ensure a valid
181 test, a test substance should not be diluted beyond its Maximum Valid Dilution (MVD).

182 For many marketed products, values for the MVD and the Endotoxin Limit Concentration
183 (ELC) are published in the U.S. Pharmacopeia, the European Pharmacopoeia, and/or Food
184 and Drug Administration (FDA) guidelines. However if one or both of these values are not
185 available, then calculation of the MVD is dependent on the ELC (see **Section 12.3**). If
186 unknown, the ELC can be approximated by dividing the maximum hourly dose of the

187 product by the hourly dose received per patient. For example, if a product is used at an
188 hourly dose of 100 mg per patient, then the ELC would be 350 EU/100 mg or 3.5 EU/mg.

189 4.2.1.1 Reference Endotoxin for Spiking Test Substances

190 The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent international RSE (e.g., USP
191 G3E069, FDA EC-6) is recommended for preparation of the endotoxin control (EC). If a
192 different *E. coli* LPS is used and the potency relative to the RSE is not provided, then each
193 lot must be calibrated against the RSE in the Cryo WB/IL-1 β test method. For interference
194 testing, an endotoxin standard curve (see **Section 4.1**) should be included on each plate.

195 4.2.1.2 Spiking Test Substances with Endotoxin

196 For interference testing, non-spiked and endotoxin-spiked test substances are prepared in
197 microplate wells (n=4 replicates) and an *in vitro* pyrogen test is performed. Either RPMI or a
198 fixed concentration (a concentration selected from the middle of the EC standard curve) of
199 the RSE (i.e., 1 EU/mL) in RPMI is added to the test substance in serial two-fold dilutions.
200 An illustrative example of endotoxin spiking solutions is shown in **Table 4-2**. For non-spiked
201 solutions, 200 μ L of RPMI is added to a well followed by 20 μ L of the test substance (neat or
202 at serial dilution) and 20 μ L of WB. Endotoxin-spiked solutions are prepared by adding 180
203 μ L of RPMI to each well followed by 20 μ L of the test substance (neat or at serial dilution)
204 and 20 μ L of WB. Then, 20 μ L of a 1 EU/mL solution of endotoxin in RPMI is added and
205 the well contents are mixed (see example presented in **Table 4-2**).

206

206

207 **Table 4-2 Preparation of Endotoxin-Spiked and Non-spiked Solutions for**
 208 **Determination of Test Substance Interference in the Incubation and**
 209 **ELISA Test Systems**

Sample Addition	Spiked	Non-spiked
	$\mu\text{L}/\text{well}^1$	
RPMI	180	200
Endotoxin spike solution ²	20	0
Test substance (neat and each serial dilution)	20	20
Cryo WB	20	20
Total ³	240	240

210 Abbreviations: Cryo WB = Cryopreserved whole blood

211 ¹ n=4 replicates each

212 ² Endotoxin concentration is 1.0 EU/mL in RPMI.

213 ³ A total volume of 240 μL per well is used for the incubation.

214

215

216 The lowest dilution of the test substance that yields an endotoxin spike recovery of 50% to
 217 200% in the pyrogen test is determined. The optical density (OD) values of the endotoxin-
 218 spiked and non-spiked test substances are calibrated against the endotoxin calibration curve.
 219 The resulting EU value of the non-spiked test substance is subtracted from the corresponding
 220 EU value of the endotoxin-spiked test substance at each dilution. The % recovery for each
 221 sample dilution is then determined from the endotoxin spike solution concentration set to
 222 100%. For example, consider the following interference test results in **Table 4-3**:

223 **Table 4-3 Example of Interference Data Used to Determine Sample Dilution for**
 224 **Assay**

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

225

226

227 Based on these results, the dilution of the test substance used in the *in vitro* pyrogen test
 228 would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1 EU/mL EC).

229 4.2.2 Interference with ELISA System

230 If the data obtained from the experiment in **Section 4.2.1** suggests the presence of
231 interference, then a subsequent experiment similar to that described in **Section 4.2.1** would
232 need to be performed to confirm that the test substance(s) does not directly interfere with the
233 ELISA. For this experiment, an ELISA would be performed in the absence of Cryo WB.

234 **5.0 CONTROLS**

235 **5.1 Negative Control**

236 A negative control (e.g., PFS is added instead of the test sample) is included in each
237 experiment in order to detect nonspecific changes in the test system, as well as to provide a
238 baseline for the assay endpoints.

239 **5.2 Solvent Control**

240 Solvent controls are recommended to demonstrate that the solvent is not interfering with the
241 test system when solvents other than PFS are used to dissolve test substances.

242 **5.3 Positive Control**

243 An internationally standardized EC (e.g., WHO 94/580; USP G3E069; 0.5 EU/mL) is
244 included in each experiment to verify that an appropriate response is induced.

245 **5.4 Benchmark Controls**

246 Benchmark controls may be used to demonstrate that the test method is functioning properly,
247 or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals,
248 medical device eluates) of a specific class or a specific range of responses, or for evaluating
249 the relative pyrogenic potential of a test substance. Appropriate benchmark controls should
250 have the following properties:

- 251 • consistent and reliable source(s) for the chemicals (e.g., parenteral
252 pharmaceuticals, medical device eluates)
- 253 • structural and functional similarities to the class of substance being tested
- 254 • known physical/chemical characteristics

255 • supporting data on known effects in animal models

256 • known potency in the range of response

257 **5.5 Positive Product Control (PPC)**

258 The PPC is a test substance diluted to a level that does not interfere with the test method and
259 does not exceed the MVD. The PPC is obtained by spiking a test substance with a known EC
260 (e.g., 1 EU/mL) and demonstrating that 50% to 200% of the EC is recovered.

261 **5.6 Negative Product Control (NPC)**

262 The NPC is the test substance diluted to the MVD and then spiked with PFS. It is the
263 negative control for the PPC.

264 **6.0 EXPERIMENTAL DESIGN**

265 **6.1 Incubation with Test Samples and Measurement of IL-1 β Release**

266 6.1.1 Collection of Human Blood

267 WB is obtained from healthy human volunteers who have provided their consent according
268 to established institutional guidelines. Volunteers are expected not to have taken any drugs
269 and to have been free from illness for at least two weeks prior to donation. The criteria for
270 rejection of data from donors that are low responders or that are suspect due to veracity of
271 health information is addressed in **Section 8.0**.

272 Fresh WB is drawn by venipuncture using a multily needle set and collected in heparinized
273 tubes (15 IU/mL lithium heparin). All components of the blood collection system (i.e.,
274 syringes, tubes, connecting lines) must be sterile and pyrogen-free.

275 6.1.2 Cryopreservation Procedure

276 Two methods are available for cryopreservation of blood 1) The PEI method developed at the
277 Paul Ehrlich Institute and 2) The Konstanz method developed at the University of Konstanz.

278 6.1.3 PEI Method of Cryopreservation

279 In the PEI method (Schindler et al., 2006), heparinized WB pooled from five donors is frozen
280 at -80°C in a cryoprotective phosphate buffer (Soerensen's) containing 10% (v/v) pyrogen-
281 free, clinical-grade DMSO in cryotubes.

282 6.1.3.1 *Konstanz Method of Cryopreservation*

283 In the Konstanz method (Schindler et al., 2004), pyrogen-free, clinical grade DMSO is added
284 directly to the blood of individual donors at a final concentration of 10% (v/v). The blood is
285 then pooled and 1.2 mL aliquots are placed in cryotubes. The blood is frozen in a computer-
286 controlled freezer using several cycles of programmed freezing down to -120°C. Tubes of
287 blood are then removed from the instrument and placed in liquid nitrogen.

288 6.1.3.2 *Thawing Procedure*

289 Calculate the volume of Cryo WB needed to carry out the assay (20 μ L/well or 1.92 mL/96-
290 well plate) and remove a sufficient number of aliquots from the freezer. Place the tubes in an
291 incubator at 37 \pm 1°C and allow them to thaw for 15 min. In a laminar flow hood, unscrew the
292 caps and pool the Cryo WB in a centrifuge tube. Mix the tubes by gentle inversion. **Do Not**
293 **Vortex.**

294 6.1.4 Incubation Plate

295 Test substances are prepared at a level of dilution that did not show interference with the test
296 system or for which it is known that interference does not occur. Each incubation plate can
297 accommodate an endotoxin standard curve, a NSC, and 14 test samples (see **Table 6-1**).
298

298

299 **Table 6-1 Overview of Incubation Plate Preparation in the Cryo WB/IL-1 β Test**
 300 **Method (PEI Method)**

Number of Wells	Sample	RPMI	EC	Test Sample	Cryo WB ¹	Mix the samples; incubate overnight at 37 \pm 1 $^{\circ}$ C in a humidified atmosphere with 5% CO ₂ .	Mix the samples; immediately transfer to an ELISA plate ⁴ and run ELISA or store plate at -20 $^{\circ}$ C or -80 $^{\circ}$ C.
20 ²	EC	180	20	0	40		
4	NSC	220	0	0	20		
56 ³	Test samples (1-14)	200	0	20	20		

301 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; Cryo WB = Cryopreserved whole
 302 blood

303 ¹ For the Konstanz method of cryopreservation, 20 μ L of Cryo WB is used and the volume of RPMI is adjusted
 304 to 200 μ L.

305 ² Five EC concentrations (0.25, 0.50, 1.0, 2.5, 5.0 EU/mL) in quadruplicate.

306 ³ 14 test samples (n=4) per plate.

307 ⁴ An IL-1 β standard curve is prepared in Columns 11 and 12 on the ELISA plate. Therefore, 80 wells are
 308 available for test samples and controls on the incubation plate.

309

310 6.1.5 Incubation Assay for IL-1 β Release

311 Blood samples are prepared in a microtiter plate using a laminar flow hood. All consumables
 312 and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately
 313 with a permanent marker. An overview of the incubation plate preparation is shown in **Table**
 314 **6-1**. The incubation procedure is outlined below:

315 **Step 1.** Refer to the incubation plate template presented in **Table 6-2**.

316 **Step 2.** Using a pipetter, transfer either 200 or 180 μ L of RPMI into each well (for
 317 the Konstanz or PEI method of cryopreservation, respectively – refer to **Step 5**
 318 below).

319 **Step 3.** Transfer 20 μ L of test sample into the appropriate wells as indicated in the
 320 template.

321 **Step 4.** Transfer 20 μ L of the EC (standard curve) and the NSC controls in
 322 quadruplicate into the appropriate wells according to the template.

323 **Step 5.** Transfer either 20 or 40 μ L of Cryo WB (for the Konstanz or PEI method
324 of cryopreservation, respectively) into each well and mix by gently swirling the
325 plate.

326 **Step 6.** Mix the contents of the wells thoroughly by gently pipetting up and down
327 five times using a multichannel pipetter, changing the tips between each row in
328 order to avoid cross-contamination.

329 **Step 7.** Place the covered plate in a tissue culture incubator for 10 to 24 hr at
330 $37\pm 1^{\circ}\text{C}$ in a humidified atmosphere containing 5% CO_2 .

331 **Step 8.** If using the Konstanz method, freeze the plate at -20°C or -80°C until the
332 contents of the well are completely frozen and then, thaw the plate at RT or in a
333 water bath not exceeding 37°C .

334 **Step 9.** Prior to transferring the test samples onto the ELISA plate, mix the
335 contents of the wells by pipetting up and down three times using a multichannel
336 pipetter, changing the tips between each row in order to avoid cross-contamination.

337 *Note: The aliquots may be tested immediately in the ELISA or stored at -20°C or*
338 *-80°C for testing at a later time. After transfer to the ELISA plate, freeze the*
339 *remaining aliquots at -20°C or -80°C for subsequent experiments, if necessary (see*
340 *Assay Acceptability and Decision Criteria in **Sections 8.0 and 9.0**).*

341

341

342 **Table 6-2 Incubation Plate - Sample and Control Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	Void ³	Void
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

343 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

344 ¹ EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.345 ² TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.346 ³ Columns 11 and 12 are reserved for the IL-1 β standard curve on the ELISA plate (see **Table 6-3**).

347

348

349 **6.2 ELISA to Measure IL-1 β Release**350 **6.2.1 IL-1 β Standard Curve**

351 An IL-1 β standard, supplied with the ELISA kit, is used. IL-1 β standards are typically
 352 supplied in lyophilized form and should be reconstituted according to the manufacturer's
 353 instructions. The stock solution should be diluted in RPMI to the following concentrations: 0,
 354 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL. Each well on the ELISA plate will receive
 355 100 μ L of an IL-1 β blank or standard.

356 **6.2.2 ELISA**

357 The manufacturer's instructions provided with the ELISA kit should be followed and a
 358 typical experimental design is outlined below. If the user prefers to prepare an in-house
 359 ELISA, then appropriate modification and validation of these changes would be necessary.
 360 The ELISA should be carried out at room temperature (RT) and therefore all components
 361 must be at RT prior to use. Do *not* thaw frozen specimens by heating them in a water bath. A
 362 suggested ELISA plate template is shown in **Table 6-3**, which includes a five-point EC
 363 standard curve, a NSC, an eight-point IL-1 β standard curve (0 to 4000 pg/mL), and 14 test

364 substances in quadruplicate. The EC standard curve, the NSC, and the test sample
365 supernatants are transferred directly from the incubation plate. The IL-1 β standard curve is
366 prepared as described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown
367 in **Table 6-4**.

368 **Step 1.** Add 100 μ L of enzyme-labeled detection antibody to each well.

369 **Step 2.** After pipetting up and down three times to mix the supernatant, transfer
370 100 μ L from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

371 **Step 3.** Add 100 μ L of each IL-1 β standard (0 to 4000 pg/mL) into the respective
372 wells on the ELISA plate.

373 **Step 4.** Cover the microtiter plate(s) with adhesive film and incubate for 90 min on
374 a microplate mixer at 350-400 rpm at 20 to 25°C.

375 **Step 5.** Decant and wash each well three times with 300 μ L Buffered Wash
376 Solution and then rinse three times with deionized water. Place the plates upside
377 down and tap to remove water.

378 **Step 6.** Add 200 μ L of TMB/Substrate Solution to each well and incubate at RT in
379 the dark for 15 min. If necessary, decrease the incubation time.

380 **Step 7.** Add 50 μ L of Stop Solution to each well.

381 **Step 8.** Tap the plate gently after the addition of Stop Solution to aid in mixing.

382 **Step 9.** Read the OD₄₅₀ within 15 min of adding the Stop Solution. Measurement
383 with a reference wavelength of 600-690 nm is recommended.

384

384

385 **Table 6-3 ELISA Plate - Sample and Control Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-1 β ³ 0	IL-1 β 0
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	IL-1 β 62.5	IL-1 β 62.5
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	IL-1 β 125	IL-1 β 125
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	IL-1 β 250	IL-1 β 250
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	IL-1 β 500	IL-1 β 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-1 β 1000	IL-1 β 1000
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-1 β 2000	IL-1 β 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-1 β 4000	IL-1 β 4000

386 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

387 ¹ EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.388 ² TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.389 ³ IL-1 β values in columns 11 and 12 are in pg/mL.

390

391

392 **Table 6-4 Overview of ELISA Procedure**

Enzyme-labeled Antibody (μL)	Material transfer from Incubation Plate (μL)	Incubate 90 min on a plate mixer at 350-400 rpm.	TMB/Substrate Solution (μL)	Incubate 15 min at RT in dark.	Stop Solution (μL)	Read optical density at 450 nm with a 600-690 nm wavelength reference filter.
100	100		200		50	

393 Abbreviations: RT = Room temperature

394

395 **7.0 EVALUATION OF TEST RESULTS**396 **7.1 OD Measurements**

397 The OD of each well is obtained by reading the samples in a standard microplate

398 spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD₄₅₀) with

399 a 600 to 690 nm reference filter (recommended). OD₄₅₀ values are used to determine assay
400 acceptability and in the decision criteria for the detection of endotoxin in a test substance (see
401 **Sections 8.0** and **9.0**).

402 **8.0 CRITERIA FOR AN ACCEPTABLE TEST**

403 Obtain the PPC and the corresponding NPC by interference testing of a test substance in the
404 presence and absence of a fixed quantity of endotoxin (i.e., 1.0 EU/mL) in quadruplicate. An
405 EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6
406 standard curve should be included in each ELISA as shown in the template presented in
407 **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- 408 • The quadratic function of the IL-1 β standard curve produces an $r \geq 0.95$ and
409 the OD₄₅₀ of the blank control is below 0.15.
- 410 • The endotoxin standard curve produces OD₄₅₀ values that ascend in a
411 sigmoidal concentration response.

412 **9.0 DATA INTERPRETATION/DECISION CRITERIA**

413 **9.1 Decision Criteria for Determination of Pyrogenicity**

414 The *t*-test is used to compare the data of a test sample against the data of the EC (0.5 EU/mL)
415 that is performed in parallel. If this test results in a significant *p*-value (i.e., smaller than 1%),
416 then the sample is considered to be non-pyrogenic, and as pyrogenic if otherwise (Hoffmann
417 et al., 2005), as long as the assay acceptability criteria in **Section 8.0** has been met.

418 **10.0 STUDY REPORT**

419 The test report should include the following information:

420 *Test Substances and Control Substances*

- 421 • Name of test substance
- 422 • Purity and composition of the substance or preparation
- 423 • Physicochemical properties (e.g., physical state, water solubility)

- 424 • Treatment of the test/control substances prior to testing (e.g., vortexing,
425 sonication, warming, resuspension solvent)

426 *Justification of the In Vitro Test Method and Protocol Used*

427 *Test Method Integrity*

- 428 • The procedure used to ensure the integrity (i.e., accuracy and reliability) of the
429 test method over time
- 430 • If the test method employs proprietary components, documentation on the
431 procedure used to ensure their integrity from “lot-to-lot” and over time
- 432 • The procedures that the user may employ to verify the integrity of the
433 proprietary components

434 *Criteria for an Acceptable Test*

- 435 • Acceptable concurrent positive control ranges based on historical data
- 436 • Acceptable negative control data

437 *Test Conditions*

- 438 • Cell system used
- 439 • Calibration information for the spectrophotometer used to read the ELISA
- 440 • Details of test procedure
- 441 • Description of any modifications of the test procedure
- 442 • Reference to historical data of the model
- 443 • Description of evaluation criteria used

444 *Results*

- 445 • Tabulation of data from individual test samples

446 *Description of Other Effects Observed*

447 *Discussion of the Results*

448 *Conclusion*

449 *A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies*

- 450 • This statement should indicate all inspections made during the study and the
451 dates any results were reported to the Study Director. This statement should
452 also confirm that the final report reflects the raw data.

453 If GLP-compliant studies are performed, then additional reporting requirements provided in
454 the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be
455 followed.

456

456

457 **11.0 REFERENCES**

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486

487 **12.0 TERMINOLOGY AND FORMULA**488 **12.1 Assay Sensitivity (λ)**

489 For an *in vitro* cell-based assay, the variable λ is defined as the lowest statistically significant
490 point on the standard endotoxin concentration-response curve and represents the relative
491 sensitivity of the test method for the detection of endotoxin (i.e., level of detection).

492 **12.2 Endotoxin Control (EC)**

493 The EC is incubated with Cryo WB and serves as the positive control for the experiment. The
494 results should be compared to historical values to insure that it provides a known level of
495 cytokine release relative to the NSC.

496 **12.3 Endotoxin Limit Concentration (ELC)**

497 The ELC is the maximum allowable concentration of endotoxin for a particular product and
498 is expressed in EU per volume (mL) or weight (mg). The ELC is defined by the FDA or
499 specified in the USP². It is calculated as the product of K/M, where:

500 K is the threshold pyrogen dose for parenteral use in rabbits or humans (5.0 EU/kg). At an
501 injection volume of 10 mL/kg, K is equal to 0.5 EU/mL.

502 M is the larger of the rabbit dose or the maximum human dose administered in one hour as
503 defined below and varies with test substance³.

504 **12.4 Maximum Valid Dilution (MVD)**

505 The MVD is the maximum dilution of a test substance that can be tolerated in a test system
506 without exceeding the ELC, if the test substance must be diluted as a result of assay
507 interference. Dilutions beyond the MVD would not be valid for endotoxin detection in the

² ELC values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, Pharmacopoeial guidelines, FDA publications).

³ Values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, pharmacopoeial guidelines, FDA publications).

508 test system. Calculation of the MVD is dependent on whether or not the ELC for a test
509 substance is published. When the ELC is known, the MVD is:

$$510 \quad \text{MVD} = (\text{ELC} \times \text{Product Potency [PP]})/\lambda$$

511 As an example, for "Cyclophosphamide Injection," the ELC is 0.17 EU/mg, PP is 20
512 mg/mL, and the assay sensitivity is 0.1 EU/mL. The calculated MVD would be 34. The test
513 substance can be diluted no more than 1:34 prior to testing.

514 If the ELC is not known, the MVD is:

$$515 \quad \text{MVD} = \text{PP}/\text{Minimum Valid Concentration (MVC)}$$

$$516 \quad \text{where, MVC} = (\lambda \times \text{M})/\text{K}$$

517 where, M is the maximum human dose

518 As an example, for "Cylophosphamide Injection," the PP is 20 mg/mL, M is 30
519 mg/kg, and assay sensitivity is 0.1 EU/mL. The calculated MVC is 0.6 mg/mL and the MVD
520 is 33.3. The test substance can be diluted no more than 1:33 in the assay prior to testing.

521 **12.5 Negative Product Control (NPC)**

522 The NPC is a test sample to which PFS is added. The NPC is the baseline for determination
523 of cytokine release relative to the endotoxin-spiked PPC.

524 **12.6 Negative Saline Control (NSC)**

525 The NSC is Cryo WB (in RPMI) incubated with PFS (used for dilution of test substance) and
526 is used as the blank.

527 **12.7 Parenteral Threshold Pyrogen Dose (K)**

528 The value K represents the threshold pyrogen dose for parenteral products for rabbits and
529 humans. Based on experimental data, K is fixed at 5.0 EU/kg. For intrathecal products, K is
530 0.2 EU/kg.

531 **12.8 Positive Product Control (PPC)**

532 The PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an
533 amount of endotoxin equal to that which produces $\frac{1}{2}$ the maximal increase in OD from the

534 endotoxin standard curve) to insure that the test system is capable of endotoxin detection in
535 the product as diluted in the assay.

536 **12.9 Product Potency (PP)**

537 The concentration for a test substance is the PP typically expressed as $\mu\text{g/mL}$ or mg/mL .

538 **12.10 Rabbit Pyrogen Test Dose or Maximum Human Dose (M)**

539 The variable M represents the rabbit test dose or the maximum human dose in 1 hr. The
540 variable M is expressed in mg/kg and varies with the test substance. For
541 radiopharmaceuticals, M should be adjusted to account for product activity (radioactive
542 decay) at time administration. An average human standard weight of 70 kg is used for the
543 calculation. If a pediatric dose should be used and it is higher than the adult dose, then it
544 should be used in the formula.

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Appendix B2

**Draft ICCVAM Recommended Protocol for Future Studies Using the Mono Mac 6
(MM6)/Interleukin-6 (IL-6) Test Method**

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39 **Table 1 Comparison of Draft ICCVAM Recommended Test Method Protocol with the ECVAM SOP for the Mono Mac**
 40 **6 (MM6)/Interleukin-6 (IL-6) Pyrogen Test Method**

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP	ECVAM Validation SOP
Test Substance	Test neat or at minimal dilution that produces no interference	Same as ICCVAM protocol	Test at MVD
Incubation Plate (number of control or test groups at n=4 each)	NSC (1)	Same as ICCVAM protocol	Same as ICCVAM protocol
	EC (5)	Same as ICCVAM protocol	Same as ICCVAM protocol
	TS (14)	Same as ICCVAM protocol	TS (2) x EC (5) spikes
	PPC ¹ (0)	Same as ICCVAM protocol	PPC (2)
	NPC ¹ (0)	Same as ICCVAM protocol	NPC (2)
	PC (0)	Same as ICCVAM protocol	PC (1)
	NC (0)	Same as ICCVAM protocol	NC (1)
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Same as ICCVAM protocol	Same as ICCVAM protocol
Decision Criteria for Interference	0.5 x Median OD ₄₅₀ ² of 1 EU/mL EC <2x Median OD ₄₅₀ of 1 EU/mL EC	Same as ICCVAM protocol	Not applicable (tested at MVD)
Assay Acceptability Criteria	Mean OD ₄₅₀ ² of PPC is 50% to 200% of 0.5 EU/mL EC	Same as ICCVAM protocol	Same as ICCVAM protocol
	Mean OD ₄₅₀ of NSC ≤0.15	Same as ICCVAM protocol	Mean OD ₄₅₀ of NSC ≤0.20
	Not included	Outliers rejected using Dixon's text (p≥0.5)	Outliers rejected using Dixon's text (p≥0.5)
	Not included	Mean OD ₄₅₀ EC > NSC (2SD with n-1 weighting)	Mean OD ₄₅₀ PC > LOQ ³
	Quadratic function of IL-6 SC r ² ≥0.95	Same as ICCVAM protocol	Same as ICCVAM protocol
	EC SC produces OD ₄₅₀ values that ascend in a sigmoidal concentration response	Same as ICCVAM protocol	Mean OD ₄₅₀ of each EC > Mean OD ₄₅₀ of next lower EC concentration (minimum of 4 data points needed for valid SC)
Decision Criteria for Pyrogenicity	Mean OD ₄₅₀ ² of TS > Mean OD ₄₅₀ of 0.5 EU/mL EC	EC SC data transformed to 4-parameter logistical model by an in-house program and the linear mean square is calculated. TS pyrogen content is compared with the ELC ⁴ using confidence limits for significance. Dixon's test is used to reject outliers.	EC SC data transformed to 4-parameter logistical model by an in-house program and the linear mean square is calculated. TS pyrogen content is compared with the ELC ⁴ using confidence limits for significance. Dixon's test is used to reject outliers.

41 Abbreviations: EC = Endotoxin control; ELC = Endotoxin Limit Concentration; LOQ = Limit of Quantification; MVD = Maximum Valid Dilution; NC =
42 Negative Control; NPC = Negative Product Control; NSC = Normal saline control; PC = Positive control; PPC = Positive Product Control; SC = Standard curve;
43 SD = Standard Deviation; TS = Test substance
44 ¹ PPC and NPC are evaluated during the interference test.
45 ² Median or mean OD₄₅₀ values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).
46 ³ LOQ is the mean OD₄₅₀ of the NSC + 10xSD mean OD₄₅₀ of the NSC.
47 ⁴ Where unknown, the ELC is calculated (e.g., Based on a rabbit sensitivity of 5 EU/kg, for a product injected at 10 mL/kg, the detection limit is 5 EU/10 mL/kg
48 or 0.5 EU/mL/kg, or an ELC of 0.5 EU/mL).
49
50

51 **1.0 PURPOSE AND APPLICABILITY**

52 The purpose of this protocol is to describe the procedures used to evaluate the presence of a
53 pyrogen (i.e., Gram-negative endotoxin) in a test substance. The presence of Gram-negative
54 endotoxin is detected by its ability to induce cytokine IL-6 release from MM6 cells. The
55 quantity of IL-6 released is obtained using an enzyme-linked immunosorbent assay (ELISA)
56 that includes monoclonal or polyclonal antibodies specific for IL-6. Release of this cytokine
57 is measured by incubation of the MM6 cells with test substances or controls (i.e., positive,
58 negative). The amount of pyrogen present is determined by comparing the values of
59 endotoxin equivalents produced by MM6 cells exposed to the test substance to those exposed
60 to an internationally-harmonized Reference Standard Endotoxin (RSE)¹ or an equivalent
61 standard expressed in Endotoxin Units (EU)/mL. Based on a rabbit threshold pyrogen dose of
62 0.5 EU/mL, which was established in a retrospective evaluation of rabbit pyrogen test (RPT)
63 data, a test substance is considered pyrogenic if it induces a level of IL-6 release equal to or
64 greater than 0.5 EU/mL.

65 The focus of this protocol is on the use of the MM6/IL-6 test method specifically for the
66 detection of Gram-negative endotoxin in parenteral pharmaceuticals. The relevance and
67 reliability for non-endotoxin pyrogens (e.g., lipoteichoic acid) has not been demonstrated in a
68 formal validation study.

69 **2.0 SAFETY AND OPERATING PROCEDURES**

70 All procedures for procurement of eligible blood donors and blood donations should follow
71 the regulations and procedures set forth by institutional guidelines for utilization of human
72 substances, which include but are not limited to blood, tissues, and tissue fluids. Standard
73 laboratory precautions are recommended, including the use of laboratory coats, eye

¹ RSEs are internationally-harmonized reference standards (e.g., WHO *E. coli* Lipopolysaccharide [LPS] 94/580 [0113:H10:K-]; USP RSE Lot G3E069; FDA Lot EC-6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

74 protection, and gloves. If necessary, additional precautions required for specific study
75 substances or hazardous chemicals will be identified in the Material Safety Data Sheet
76 (MSDS).

77 The stop solution used in the ELISA kit is acidic and corrosive and should be handled with
78 the proper personal protective devices. If this reagent comes into contact with skin or eyes,
79 wash thoroughly with water. Seek medical attention, if necessary.

80 Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5,
81 5'-TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate
82 personal protection should be used to prevent bodily contact.

83 Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage,
84 antigenic response) and should be handled with care. Skin cuts should be covered and
85 appropriate personal protective devices should be worn. In case of contact with endotoxin,
86 immediately flush eyes or skin with water for at least 15 min. If inhaled, remove the affected
87 individual from the area and provide oxygen and/or artificial respiration as needed. Skin
88 absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

89 **3.0 MATERIALS, EQUIPMENT AND SUPPLIES**

90 **3.1 Source of Cells**

91 The MM6 cell line is a human monocytic cell line originally described by Professor H.W.L.
92 Ziegler-Heitbrock (Institute for Immunology, University of Munich, Munich, Germany). A
93 Master Cell Bank and a Working Cell Bank have been established at the National Institute
94 for Biological Standards and Control (NIBSC), from which the MM6 cells can be purchased.

95 **3.2 Equipment and Supplies**

96 For all steps in the protocol, excluding the ELISA procedure, the materials that will be in
97 close contact with samples (e.g., pipet tips, containers, solutions) should be sterile and free
98 from detectable pyrogens.

- 99 3.2.1 Utilization of MM6 cells
- 100 3.2.1.1 Equipment
- 101 • Centrifuge
- 102 • Hemacytometer
- 103 • Hood; Bio-safety, laminar flow (recommended)
- 104 • Incubator; cell culture ($37\pm 1^{\circ}\text{C} + 5\% \text{CO}_2$)
- 105 • Inverted Microscope
- 106 • pH meter
- 107 • Pipetter; multichannel (8- or 12-channel)
- 108 • Pipettors; single-channel adjustable (20, 200, and 1000 μL)
- 109 • Repeating pipetter
- 110 • Vortex mixer
- 111 • Water bath
- 112 3.2.1.2 *Consumables*
- 113 • Centrifuge tubes; nonpyrogenic, polystyrene (15 and 50 mL)
- 114 • Cryotubes; screw-cap (2 mL)
- 115 • Combitips; repeating pipetter (1.0 and 2.5 mL)
- 116 • Filters; sterile, 0.22 μm
- 117 • Flasks; tissue culture
- 118 • Phosphate buffered saline (PBS); sterile
- 119 • Pipets; sterile, glass
- 120 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- 121 • Reaction tubes; polystyrene (1.5 mL)
- 122 • Reservoirs; fluid

- 123 • RPMI-1640 cell culture medium; supplemented as described in **Section 4.3** to
124 yield RPMI-Complete (RPMI-C)
- 125 • Tips; pipetter, sterile, pyrogen-free (20 and 200 μ L)
- 126 • Tubes; polystyrene

127 3.2.2 ELISA

128 3.2.2.1 *Equipment*

- 129 • Microplate mixer
- 130 • Microplate reader (450 nm with an optional reference filter in the range of
131 540-590 nm)
- 132 • Microplate washer (optional)
- 133 • Multichannel pipetter

134 3.2.2.2 *Consumables*

- 135 • Container; storage, plastic
- 136 • Deionized water; nonsterile
- 137 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- 138 • Pyrogen-free water (PFW)
- 139 • Reservoirs; fluid
- 140 • Tips; pipetter, nonsterile
- 141 • Tubes; polystyrene (12 mL)

142 3.2.2.3 *ELISA Kit*

143 An ELISA that measures IL-6 release from MM6 cells is used. A variety of IL-6 ELISA kits
144 are commercially available and the IL-6 ELISA procedure outlined in this protocol is
145 intended to serve as an example for using an ELISA kit. If the user prefers to prepare an in-
146 house ELISA, then additional reagents would be required. The IL-6 ELISA should be
147 calibrated using an IL-6 international reference standard (e.g., WHO 89/548) prior to use.
148 The IL-6 cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this

149 reagent must be purchased separately. Results obtained using these products are subject to
150 the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA
151 kit components may include the following:

- 152 • ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or
153 polyclonal
- 154 • Buffered wash solution
- 155 • Dilution buffer
- 156 • Enzyme-labeled detection antibody
- 157 • Human IL-6 reference standard
- 158 • Pyrogen-free saline (PFS)
- 159 • Stop solution
- 160 • TMB/substrate solution

161 **3.3 Chemicals**

- 162 • Endotoxin (e.g., WHO *E. coli* LPS 2nd International Standard 94/580; USP
163 RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

164 **3.4 Solutions**

- 165 • RPMI-C cell culture medium

166 **4.0 ASSAY PREPARATION**

167 All test substances, endotoxin, and endotoxin-spiked solutions should be stored at 4°C.

168 **4.1 Endotoxin Standard Curve**

169 An internationally harmonized RSE or equivalent is used to generate the endotoxin standard
170 curve. The use of any other *E. coli* LPS requires calibration against a RSE using the
171 MM6/IL-6 test method.

172 A standard endotoxin curve consisting of a Normal Saline Control (NSC) and five RSE
173 concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) are included in the incubation step

174 (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin
 175 standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the
 176 lyophilized content of the stock vial by following the instructions provided by the
 177 manufacturer (e.g., for a vial containing 10,000 EU, 5 mL of PFW is added). To reconstitute
 178 the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in
 179 a bath sonicator for 5 min. The stock solution is stable for 14 days when stored at 2 to 8°C.
 180 An endotoxin standard curve is prepared by making serial dilutions of the stock solution in
 181 PFS as described in **Table 4-1**.

182 **Table 4-1 Preparation of Endotoxin Standard Curve**

Stock Endotoxin EU/mL	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000 ^{1,2}	20	1980	20 ³
20	100	900	2.0
2.0	500	500	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0	0	1000	0

183 Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

184 Each stock tube should be vortexed vigorously prior to its use to make the subsequent dilution.

185 ¹ A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

186 ² The stock solution of USP RSE may be stored in aliquots and kept at -20°C for up to 6 months. Do not store
 187 the endotoxin at -80°C.

188 ³ This concentration is not used in the assay.

189

190 **4.2 Test Substances**

191 Liquid test substances should be tested neat or, if interference is detected (see **Section 4.2.1**),
 192 diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in
 193 saline, dissolved in DMSO then diluted up to 0.5% (v/v) with PFS provided that this
 194 concentration does not interfere with the assay. The test substances should be vortexed
 195 vigorously for at least 30 min or sonicated in a bath sonicator for 5 min.

196 **4.2.1 Interference Testing**

197 Interference testing must be carried out on any test sample for which no interference
 198 information is available. The purpose of the interference test is to determine the lowest
 199 dilution (i.e., highest concentration) of a test substance from which an endotoxin spike can be

200 detected (i.e., based on the decision criteria described in **Section 4.2.1.2**). However, to ensure
201 a valid test, a test substance should not be diluted beyond its Maximum Valid Dilution
202 (MVD).

203 For many marketed products, values for the MVD and the Endotoxin Limit Concentration
204 (ELC) are published in the U.S. Pharmacopeia, the European Pharmacopoeia, and/or Food
205 and Drug Administration (FDA) guidelines. However, if one or both of these values are not
206 available, then calculation of the MVD is dependent on the ELC (see **Section 12.3**). If
207 unknown, the ELC can be approximated by dividing the maximum hourly dose of the
208 product by the hourly dose received per patient. For example, if a product is used at an
209 hourly dose of 100 mg per patient, then the ELC would be 350 EU/100 mg or 3.5 EU/mg.

210 4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

211 The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent international RSE (e.g., USP
212 G3E069, FDA EC-6) is recommended for preparation of the endotoxin control (EC). If a
213 different *E. coli* LPS is used and the potency relative to the RSE is not provided, then each
214 lot must be calibrated against the RSE in the MM6/IL-6 test method. For interference testing,
215 an endotoxin standard curve (see **Section 4.1**) should be included on each plate.

216 4.2.1.2 *Spiking Test Substances with Endotoxin*

217 For interference testing, non-spiked and endotoxin-spiked test substances are prepared in
218 microplate wells (n=4 replicates) and an *in vitro* pyrogen test is performed. Either RPMI-C or
219 a fixed concentration (a concentration selected from the middle of the EC standard curve) of
220 the RSE (i.e., 1 EU/mL) in RPMI-C is added to the test substance in serial two-fold dilutions.
221 An illustrative example of endotoxin spiking solutions is shown in **Table 4-2**. For non-spiked
222 solutions, 150 μ L of RPMI-C and 50 μ L of the test substance (neat or at serial dilution) are
223 added to a well. Then, 50 μ L of MM6 cells are added and the well contents mixed are mixed.
224 Endotoxin-spiked solutions are prepared by adding 100 μ L of RPMI-C, 50 μ L of the test
225 substance (neat or at serial dilution), and 50 μ L of an endotoxin spike solution (1.0 EU/mL).
226 MM6 cells (50 μ L) are then added and the well contents are mixed (see example presented in
227 **Table 4-2**).

228

229 **Table 4-2 Preparation of Endotoxin Spiked and Non-Spiked Solutions for**
 230 **Determination of Test Substance Interference in the Incubation and**
 231 **ELISA Test Systems**

Sample Addition	Spiked	Non-spiked
	μL/well ¹	
RPMI-C (containing 2% FCS)	100	150
Endotoxin spike solution ²	50	0
Test substance (neat and each serial dilution)	50	50
MM6 cells ³	50	50
Total ⁴	250	250

232 Abbreviations: MM6 cells = Mono Mac 6 cells

233 ¹ n=4 replicates each

234 ² Endotoxin concentration is 1.0 EU/mL in RPMI-C.

235 ³ MM6 cells are resuspended in RPMI-C (2.5×10^6 cells/mL).

236 ⁴ A total volume of 250 μL per well is used for the incubation.

237

238 The lowest dilution of the test substance that yields an endotoxin spike recovery of 50% to
 239 200% in the pyrogen test is determined. The optical density (OD) values of the endotoxin-
 240 spiked and non-spiked test substances are calibrated against the endotoxin calibration curve.
 241 The resulting EU value of the non-spiked test substance is subtracted from the corresponding
 242 EU value of the endotoxin-spiked test substance at each dilution. The % recovery for each
 243 sample dilution is then determined from the endotoxin spike solution concentration set to
 244 100%. For example, consider the following interference test results in **Table 4-3**:

245 **Table 4-3 Example of Interference Data Used to Determine Sample Dilution for**
 246 **Assay**

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

247

248 Based on these results, the dilution of the test substance used in the *in vitro* pyrogen test
 249 would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1.0 EU/mL EC).

250 4.2.2 Interference with ELISA

251 If the data obtained from the experiment in **Section 4.2.1** suggests the presence of
 252 interference, then a subsequent experiment similar to that described in **Section 4.2.1** would

253 need to be performed to confirm that the test substance(s) does not directly interfere with the
254 ELISA. For this experiment, an ELISA would be performed in the absence of MM6 cells.

255 4.3 Cell Culture Medium

256 MM6 cells are maintained in RPMI-C. For use in the ELISA procedure, the concentration of
257 fetal calf serum (FCS) is reduced to 2%. Each medium is prepared and stored as described by
258 the manufacturer.

259 4.3.1 RPMI-C

- 260 • Bovine insulin; 0.23 IU/mL
- 261 • FCS; heat-inactivated (50 mL or a 10% final concentration)
- 262 • HEPES buffer; 20 mM
- 263 • L-Glutamine; 2 mM
- 264 • MEM non-essential amino acids; 0.1 mM
- 265 • Oxaloacetic acid; 1 mM
- 266 • Penicillin/streptomycin (10,000 IU/mL penicillin, 10 mg/mL streptomycin)
- 267 • RPMI-1640 medium (500 mL)
- 268 • Sodium pyruvate; 1 mM

269 4.3.2 Starting a Culture of MM6 Cells

270 To initiate a culture of MM6 cells, remove a vial of the primary stock from liquid nitrogen.
271 Thaw the vial on ice. Gently mix and transfer the cells to a 50 mL centrifuge tube and add 10
272 mL of RPMI-C. Centrifuge at 100 x g for 5 min at room temperature (RT). Remove the
273 supernatant and resuspend the cells in ice-cold RPMI-C. Centrifuge at 100 x g for 5 min at
274 RT. Remove the supernatant and resuspend the MM6 cells in 2 mL of RPMI-C. Add 8 mL of
275 RPMI-C to a 25 cm² tissue culture flask and transfer the cell suspension to the flask. Place
276 the flasks in a cell culture incubator and maintain the cells at 37±1°C + 5% CO₂.

277 4.3.3 Propagation of MM6 Cells

278 To propagate the MM6 cells, transfer the MM6 cells to a 50 mL tube. Centrifuge at 100 x g
279 for 8 min at RT. Remove the supernatant, resuspend the cell pellet in 4 mL of RPMI-C, and
280 gently pipet up and down to mix. Transfer an aliquot of the cell suspension to new tissue
281 culture flasks and add fresh RPMI-C to obtain a final concentration of 2×10^5 cells/mL.
282 Place the flasks in a cell culture incubator and maintain the cells at $37 \pm 1^\circ\text{C} + 5\% \text{CO}_2$.

283 4.3.4 Preparation of a MM6 Cell Bank

284 To initiate a bank of MM6 cells, centrifuge the cell culture(s) at 100 x g for 8 min at 4°C .
285 Remove the supernatant and resuspend the cells in FBS at 4°C . Adjust the cell concentration
286 to 4×10^6 cells/mL and store on ice for 10 min. Add an equal volume of ice-cold FBS
287 containing 10% dimethylsulfoxide (DMSO) drop-wise to the cell suspension (final
288 concentration is 2×10^6 cells/mL with 5% DMSO). Transfer the cell suspension to sterile,
289 pyrogen-free cryotubes (1 mL/tube). Place the tubes in a well-insulated polystyrene box and
290 store at -80°C or below for greater than 48 hr and then transfer to a liquid nitrogen container.

291 **5.0 CONTROLS**

292 **5.1 Negative Control**

293 A negative control (e.g., RPMI-C) is included in each experiment in order to detect
294 nonspecific changes in the test system, as well as to provide a baseline for the assay
295 endpoints.

296 **5.2 Solvent Control**

297 Solvent controls are recommended to demonstrate that the solvent is not interfering with the
298 test system when solvents other than PFS are used to dissolve test substances.

299 **5.3 Positive Control**

300 An internationally standardized EC (e.g., WHO 94/580; USP G3E069; 0.5 EU/mL) is
301 included in each experiment to verify that an appropriate response is induced.

302 **5.4 Benchmark Control**

303 Benchmark controls may be used to demonstrate that the test method is functioning properly,
304 or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals,
305 medical device eluates) of a specific class or a specific range of responses, or for evaluating
306 the relative pyrogenic potential of a test substance. Appropriate benchmark controls should
307 have the following properties:

- 308 • consistent and reliable source(s) for the chemicals (e.g., parenteral
309 pharmaceuticals, medical device eluates)
- 310 • structural and functional similarities to the class of the substance being tested
- 311 • known physical/chemical characteristics
- 312 • supporting data on known effects in animal models
- 313 • known potency in the range of response

314 **5.5 Positive Product Control (PPC)**

315 The PPC is a test substance diluted to a level that does not interfere with the test method and
316 does not exceed the MVD. The PPC is obtained by spiking a test substance with a known EC
317 (e.g., 1 EU/mL) and demonstrating that 50% to 200% of the EC is recovered.

318 **5.6 Negative Product Control (NPC)**

319 The NPC is the test substance diluted to the MVD and then spiked with PFS. It is the
320 negative control for the PPC.

321 **6.0 EXPERIMENTAL DESIGN**

322 **6.1 Incubation with Test Samples and Measurement of IL-6 Release**

323 6.1.1 Preparation of MM6 Cells for the Incubation Plate

324 To perform an ELISA on the following day, obtain 30 to 50 mL of MM6 cell suspension
325 from propagation flasks and centrifuge at 100 x g for 8 min at RT. Remove the supernatant,
326 resuspend the cell pellet in 3-5 mL of RPMI-C (containing 2% FCS for all ELISA
327 procedures), and gently pipet up and down to mix. Transfer aliquots of cells to new culture

328 flasks and add RPMI-C to obtain a concentration of 4×10^5 cells/mL. Place the flasks in a cell
 329 culture incubator and maintain the cells at $37 \pm 1^\circ\text{C} + 5\% \text{CO}_2$. In general, each 96-well assay
 330 plate requires approximately 10 mL of cell suspension at 2.5×10^6 cells/mL.

331 6.1.2 Incubation Plate

332 Test substances are prepared at a level of dilution that did not show interference with the test
 333 system or for which it is known that interference does not occur. Each incubation plate can
 334 accommodate an endotoxin standard curve, a NSC, and 14 test substances (see **Table 6-1**).

335 **Table 6-1 Overview of Incubation Plate Preparation in the MM6/IL-6 Test Method**

Number of Wells	Sample	RPMI-C	EC	Test Sample	MM6 ¹	Mix the samples; incubate overnight at $37 \pm 1^\circ\text{C}$ in a humidified atmosphere with 5% CO_2 .	Mix the samples; immediately transfer to an ELISA plate ⁴ and run ELISA or store plate at -20°C or -80°C .
		μL					
20 ²	EC	100	50	0	100		
4	NSC	150	0	0	100		
56 ³	Test samples (1-14)	100	0	50	100		

336 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; MM6 = Mono Mac 6 cell line

337 ¹ MM6 cell concentration is 2.5×10^6 cells/mL.

338 ² Five EC concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) in quadruplicate.

339 ³ 14 test samples (n=4 each) per plate.

340 ⁴ An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate. Therefore, 80 wells are
 341 available for test samples and controls on the incubation plate.

342

343 6.1.3 Incubation Assay for IL-6 Release

344 **Step 1.** Refer to the suggested incubation plate template presented in **Table 6-2**.

345 **Step 2.** Using a pipetter, transfer 100 μL of RPMI-C into each well.

346 **Step 3.** Transfer 50 μL of test sample into the appropriate wells as indicated in the
 347 template.

348 **Step 4.** Transfer 50 μL of the EC (standard curve) and the NSC controls in
 349 quadruplicate into the appropriate wells according to the template.

350 **Step 5.** Transfer 100 μL of a well-mixed MM6 cell suspension into each well.

351 **Step 6.** Place the covered plate in a tissue culture incubator for 16 to 24 hr at
 352 37±1°C in a humidified atmosphere containing 5% CO₂.

353 **Step 7.** Remove 150 µL of the supernatant from each well, without disrupting the
 354 cells, and transfer to a new 96-well ‘transfer’ plate for the IL-6 ELISA.

355 **Table 6-2 Incubation Plate - Sample and Control Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	Void ³	Void
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

356 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

357 ¹ EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.

358 ² TS number (e.g., TS 1) represents an arbitrary sequence for individual test substances.

359 ³ Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).

360
 361

362 6.2 ELISA to Measure IL-6 Release

363 6.2.1 IL-6 Standard Curve

364 An IL-6 standard supplied with the ELISA kit is used. IL-6 standards are typically supplied
365 in lyophilized form and should be reconstituted according to the manufacturer's instructions.
366 The stock solution should be diluted in RPMI-C to the following concentrations: 0, 62.5, 125,
367 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 μ L. Each well on the
368 ELISA plate will receive 50 μ L of an IL-6 blank or standard.

369 6.2.2 ELISA

370 The manufacturer's instructions provided with the ELISA kit should be followed and a
371 typical experimental design is outlined below. If the user prefers to prepare an in-house
372 ELISA, then appropriate modifications and validation of these changes would be necessary.
373 The ELISA should be carried out at RT and therefore all components must be at RT prior to
374 use. Do *not* thaw frozen specimens by heating them in a water bath. A suggested ELISA
375 plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, a NSC,
376 an eight-point IL-6 standard curve (0 to 4000 pg/mL), and 14 test substances in
377 quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are
378 transferred directly from the incubation plate. The IL-6 standard curve is prepared as
379 described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-**
380 **4**.

381 **Step 1.** After pipetting up and down very carefully three times (avoid detachment
382 of the adherent MM6 cells) to mix the supernatant, transfer 50 μ L from each well
383 of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

384 **Step 2.** Add 50 μ L of each IL-6 standard (0 to 4000 pg/mL) into the respective
385 wells on the ELISA plate.

386 **Step 3.** Add 200 μ L of the enzyme-labeled detection antibody (neat as supplied, or
387 diluted, if necessary) to each of the wells.

388 **Step 4.** Cover the microtiter plate(s) with adhesive film and incubate for 2 to 3 hr
389 at 20 to 25°C.

390 **Step 5.** Decant and wash each well three times with 300 μ L Buffered Wash
 391 Solution and then rinse three times with deionized water. Place the plates upside
 392 down and tap to remove water.

393 **Step 6.** Add 200 μ L of TMB/Substrate Solution to each well and incubate at RT in
 394 the dark for 15 min. If necessary, decrease the incubation time.

395 **Step 7.** Add 50 μ L of Stop Solution to each well.

396 **Step 8.** Tap the plate gently after the addition of Stop Solution to aid in mixing.

397 **Step 9.** Read the OD₄₅₀ within 15 min of adding the Stop Solution. Measurement
 398 with a reference wavelength of 540 to 590 nm is recommended.

399

400 **Table 6-3 ELISA Plate - Sample and Control Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6 ³ 0	IL-6 0
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	IL-6 500	IL-6 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-6 1000	IL-6 1000
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-6 2000	IL-6 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-6 4000	IL-6 4000

401 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

402 ¹ EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.

403 ² TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

404 ³ IL-6 values in columns 11 and 12 are in pg/mL.

405

406

407 **Table 6-4 Overview of ELISA Procedure**

Material transfer from Incubation Plate (μL)	Enzyme-labeled Antibody (μL)	Cover the Incubation Plate and incubate for 2 to 3 hr at 20-25°C.	TMB/Substrate Solution (μL)	Incubate 15 min at RT in dark.	Stop Solution (μL)	Read optical density at 450 nm with a 540-590 nm wavelength reference filter.
50	200		200		50	

408 RT = Room temperature

409

410

411 **7.0 EVALUATION OF TEST RESULTS**412 **7.1 OD Measurements**

413 The OD of each well is obtained by reading the samples in a standard microplate
 414 spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD_{450}) with
 415 a 540 to 590 nm reference filter (recommended). OD_{450} values are used to determine assay
 416 acceptability and in the decision criteria for the detection of endotoxin in a test substance (see
 417 **Sections 8.0 and 9.0**).

418 **8.0 CRITERIA FOR AN ACCEPTABLE TEST**

419 Obtain the PPC and the corresponding NPC by interference testing of a test substance in the
 420 presence and absence of a fixed quantity of endotoxin (i.e., 1 EU/mL) in quadruplicate. An
 421 EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6
 422 standard curve should be included in each ELISA as shown in the template presented in
 423 **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- 424 • The quadratic function of the IL-6 standard curve produces an $r \geq 0.95$ and the
 425 OD_{450} of the blank control is below 0.15.
- 426 • The endotoxin standard curve produces OD_{450} values that ascend in a
 427 sigmoidal concentration response.

428 **9.0 DATA INTERPRETATION/DECISION CRITERIA**

429 **9.1 Decision Criteria for Determination of Pyrogenicity**

430 The *t*-test is used to compare the data of a test sample against the data of the EC (0.5 EU/mL)
431 that is performed in parallel. If this test results in a significant *p*-value (i.e., smaller than 1%),
432 then the sample is considered to be non-pyrogenic, and as pyrogenic if otherwise (Hoffmann
433 et al., 2005), as long as the assay acceptability criteria in **Section 8.0** has been met.

434 **10.0 STUDY REPORT**

435 The test report should include the following information:

436 *Test Substances and Control Substances*

- 437
- 438 • Name of test substance
 - 439 • Purity and composition of the substance or preparation
 - 440 • Physicochemical properties (e.g., physical state, water solubility)
 - 441 • Treatment of the test/control substances prior to testing (e.g., vortexing,
442 sonication, warming, resuspension solvent)

442 *Justification of the In Vitro Test Method and Protocol Used*

443 *Test Method Integrity*

- 444
- 445 • The procedure used to ensure the integrity (i.e., accuracy and reliability) of the
446 test method over time
 - 447 • If the test method employs proprietary components, documentation on the
448 procedure used to ensure their integrity from “lot-to-lot” and over time
 - 449 • The procedures that the user may employ to verify the integrity of the
450 proprietary components

450 *Criteria for an Acceptable Test*

- 451
- 452 • Acceptable concurrent positive control ranges based on historical data
 - Acceptable negative control data

453 *Test Conditions*

- 454 • Cell system used
- 455 • Calibration information for the spectrophotometer used to read the ELISA
- 456 • Details of test procedure used
- 457 • Description of any modifications of the test procedure
- 458 • Reference to historical data of the model
- 459 • Description of evaluation criteria used

460 *Results*

- 461 • Tabulation of data from individual test samples

462 *Description of Other Effects Observed*463 *Discussion of the Results*464 *Conclusion*465 *A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies*

- 466 • This statement should indicate all inspections made during the study and the
467 dates any results were reported to the Study Director. This statement should
468 also confirm that the final report reflects the raw data.

469 If GLP-compliant studies are performed, then additional reporting requirements provided in
470 the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be
471 followed.

472

472 **11.0 REFERENCES**

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- 490

490 **12.0 TERMINOLOGY AND FORMULA**

491 **12.1 Assay Sensitivity (λ)**

492 For an *in vitro* cell-based assay, the variable λ is defined as the lowest statistically significant
493 point on the standard endotoxin concentration-response curve and represents the relative
494 sensitivity of the test method for the detection of endotoxin (i.e., level of detection).

495 **12.2 Endotoxin Control (EC)**

496 The EC is incubated with MM6 cells and serves as the positive control for the experiment.
497 The results should be compared to historical values to insure that it provides a known level of
498 cytokine release relative to the NSC.

499 **12.3 Endotoxin Limit Concentration (ELC)**

500 The ELC is the maximum allowable concentration of endotoxin for a particular product and
501 is expressed in EU per volume (mL) or weight (mg). The ELC is defined by the FDA or
502 specified in the USP². It is calculated as the product of K/M, where:

503 K is the threshold pyrogen dose for parenteral use in rabbits or humans (5.0 EU/kg). At an
504 injection volume of 10 mL/kg, K is equal to 0.5 EU/mL.

505 M is the larger of the rabbit dose or the maximum human dose administered in one hour as
506 defined below and varies with test substance³.

507 **12.4 Maximum Valid Dilution (MVD)**

508 The MVD is the maximum dilution of a test substance that can be tolerated in a test system
509 without exceeding the ELC, if the test substance must be diluted as a result of assay
510 interference. Dilutions beyond the MVD would not be valid for endotoxin detection in the

² ELC values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, Pharmacopoeial guidelines, FDA publications).

³ Values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, pharmacopoeial guidelines, FDA publications).

511 test system. Calculation of the MVD is dependent on whether or not the ELC for a test
512 substance is published. When the ELC is known, the MVD is:

513
$$\text{MVD} = (\text{ELC} \times \text{Product Potency [PP]})/\lambda$$

514 As an example, for "Cyclophosphamide Injection," the ELC is 0.17 EU/mg, PP is 20
515 mg/mL, and the assay sensitivity is 0.1 EU/mL. The calculated MVD would be 34. The test
516 substance can be diluted no more than 1:34 prior to testing.

517 If the ELC is not known, the MVD is:

518
$$\text{MVD} = \text{PP}/\text{Minimum Valid Concentration (MVC)}$$

519 where, $\text{MVC} = (\lambda \times \text{M})/\text{K}$

520 where, M is the maximum human dose

521 As an example, for "Cyclophosphamide Injection," the PP is 20 mg/mL, M is 30
522 mg/kg, and assay sensitivity is 0.1 EU/mL. The calculated MVC is 0.6 mg/mL and the MVD
523 is 33.3. The test substance can be diluted no more than 1:33 in the assay prior to testing.

524 **12.5 Negative Product Control (NPC)**

525 The NPC is a test sample to which PFS is added. The NPC is the baseline for determination
526 of cytokine release relative to the endotoxin-spiked PPC.

527 **12.6 Negative Saline Control (NSC)**

528 The NSC is MM6 cells (in RPMI-C) incubated with PFS (used for dilution of test substance)
529 and is used as the blank.

530 **12.7 Parenteral Threshold Pyrogen Dose (K)**

531 The value K represents the threshold pyrogen dose for parenteral products for rabbits and
532 humans. Based on experimental data, K is fixed at 5.0 EU/kg. For intrathecal products, K is
533 0.2 EU/kg.

534 **12.8 Positive Product Control (PPC)**

535 The PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an
536 amount of endotoxin equal to that which produces ½ the maximal increase in OD from the

537 endotoxin standard curve) to insure that the test system is capable of endotoxin detection in
538 the product as diluted in the assay.

539 **12.9 Product Potency (PP)**

540 The concentration for a test substance is the PP typically expressed as $\mu\text{g/mL}$ or mg/mL .

541 **12.10 Rabbit Pyrogen Test Dose or Maximum Human Dose (M)**

542 The variable M represents the rabbit test dose or the maximum human dose in 1 hr. The
543 variable M is expressed in mg/kg and varies with the test substance. For
544 radiopharmaceuticals, M should be adjusted to account for product activity (radioactive
545 decay) at time administration. An average human standard weight of 70 kg is used for the
546 calculation. If a pediatric dose should be used and it is higher than the adult dose, then it
547 should be used in the formula.

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Appendix B3

Draft ICCVAM Recommended Protocol for Future Studies Using the Peripheral Blood Mononuclear Cell (PBMC)/Interleukin-6 (IL-6) Test Method

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1 **Draft ICCVAM Recommended Protocol for Future Studies Using the Peripheral Blood**
2 **Mononuclear Cell (PBMC)/Interleukin-6 (IL-6) Test Method**

3

4

PREFACE

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6 This proposed protocol for the detection of pyrogenicity is based on information obtained
7 from 1) The European Centre for the Validation of Alternative Methods (ECVAM)
8 PBMC/IL-6 Background Review Document (BRD) presented in Appendix A of the draft
9 Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)
10 Pyrogenicity Test Method BRD, which includes ECVAM Standard Operating Procedures
11 (SOPs) for the PBMC/IL-6 test method, and 2) Information provided to the National
12 Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative
13 Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The
14 ECVAM SOPs are based on various test methods that use human PBMCs to detect cytokine
15 production as a measure of pyrogenicity (Bleeker et al., 1994; Dinarello et al., 1984; Poole et
16 al., 2003). A table of comparison between the draft ICCVAM recommended protocol and the
17 ECVAM SOPs is provided in **Table 1**. Future studies using the PBMC/IL-6 test method may
18 include further characterization of the usefulness or limitations of the assay for regulatory
19 decision-making. Users should be aware that the proposed test method protocol might be
20 revised based on additional optimization and/or validation studies. ICCVAM recommends
21 that test method users routinely consult the ICCVAM/NICEATM website
22 (<http://iccvam.niehs.nih.gov/>) to ensure that the most current test method protocol is used.

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39 **Table 1 Comparison of Draft Recommended Test Method Protocol with the ECVAM SOP for the Peripheral Blood**
 40 **Mononuclear Cell (PBMC)/Interleukin-6 (IL-6) Pyrogen Test**

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP/ ECVAM Catch-Up Validation SOP
Test Substance	Test neat or at minimal dilution that produces no interference	Test at MVD during validation - otherwise similar to ICCVAM protocol
Incubation Plate (number of control or test groups at n=4 each)	NSC (1)	Same as ICCVAM protocol
	EC (5)	Same as ICCVAM protocol
	TS (14)	Same as ICCVAM protocol
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Same as ICCVAM protocol
Decision Criteria for Interference	$0.5 \times \text{Median OD}_{450}^1$ of 1 EU/mL EC $< 2 \times \text{Median OD}_{450}$ of 1 EU/mL EC	$0.5 \times \text{Median OD}_{450}$ of 0.25 EU/mL ³ EC $< 2 \times \text{Median OD}_{450}$ of 0.25 EU/mL EC
Assay Acceptability Criteria	Mean OD ₄₅₀ of PPC is 50% to 200% of 0.5 EU/mL EC	Mean OD ₄₅₀ of PPC is 50% to 200% of 0.25 EU/mL ² EC
	Mean OD ₄₅₀ of NSC ≤ 0.15	Same as ICCVAM protocol
	Quadratic function of IL-6 SC $r^2 \geq 0.95$	Same as ICCVAM protocol
	EC SC produces OD ₄₅₀ values that ascend in a sigmoidal concentration response	Same as ICCVAM protocol
	High responder (i.e., > 200 pg/mL IL-6) or low responder (i.e., Mean OD ₄₅₀ for 1 EU/mL EC is significantly less than that for 1000 pg/mL IL-6) blood donors may be excluded (limited to 1 of 4 donors). One of four donors may be excluded or the test repeated with four additional donors.	Same as ICCVAM protocol
Decision Criteria for Pyrogenicity	Mean corrected OD ₄₅₀ of TS > Mean corrected OD ₄₅₀ of 0.5 EU/mL EC	EC SC data transformed to 4-parameter logistical model by an in-house program and the linear mean square is calculated. TS pyrogen content is compared with the ELC ³ using confidence limits for significance. The preparation being examined must pass the test with blood from three separate donors. Dixon's test is used to reject outliers.

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP/ ECVAM Catch-Up Validation SOP
	<p><i>Decision Level 1</i> Passes if all are negative Fails if 2 or more are positive If one is positive, repeat with 4 more donors</p> <p><i>Decision Level 2</i> Passes if 1/8 are positive Fails if 2 or more of 8 are positive</p>	<p>Same as ICCVAM protocol</p>
	<p>Not included</p>	<p>Limit test is run to determine whether or not a TS after correction and dilution contains < 0.5 EU/mL of endotoxin</p>

41 Abbreviations: EC = Endotoxin control; ELC = Endotoxin Limit Concentration; MVD = Maximum Valid Dilution; NSC = Normal saline control; PPC =
 42 Positive Product Control; SC = Standard curve; TS = Test substance

43 ¹ Median or mean OD₄₅₀ values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

44 ² Value of 0.25 EU/mL is arbitrary - should correspond to a concentration in the middle of the SC for the EC.

45 ³ Where unknown, the ELC is calculated (e.g., Based on a rabbit sensitivity of 5 EU/kg, for a product injected at 10 mL/kg, the detection limit is 5 EU/10 mL/kg
 46 or 0.5 EU/mL/kg, or an ELC of 0.5 EU/mL).

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56 1.0 PURPOSE AND APPLICABILITY

57 The purpose of this protocol is to describe the procedures used to evaluate the presence of a
58 pyrogen (i.e., Gram-negative endotoxin) in a test substance. The presence of Gram-negative
59 endotoxin is detected by its ability to induce cytokine IL-6 release from human PBMCs. The
60 quantity of IL-6 released is obtained using an enzyme-linked immunosorbent assay (ELISA)
61 that includes monoclonal or polyclonal antibodies specific for IL-6. Release of this cytokine
62 is measured by incubation of PBMCs with test substances or controls (i.e., positive,
63 negative). The amount of pyrogen present is determined by comparing the values of
64 endotoxin equivalents produced by PBMCs exposed to the test substance to those exposed to
65 an internationally-harmonized Reference Standard Endotoxin (RSE)¹ or an equivalent
66 standard expressed in Endotoxin Units (EU)/mL.

67 The focus of this protocol is on the use of the PBMC/IL-6 test method specifically for the
68 detection of Gram-negative endotoxin in parenteral pharmaceuticals. The relevance and
69 reliability for non-endotoxin pyrogens (e.g., lipoteichoic acid) has not been demonstrated in a
70 formal validation study.

71 2.0 SAFETY AND OPERATING PRECAUTIONS

72 All procedures for procurement of eligible blood donors and blood donations should follow
73 the regulations and procedures set forth by institutional guidelines for utilization of human
74 substances, which include but are not limited to blood, tissues, and tissue fluids. Standard
75 laboratory precautions are recommended including the use of laboratory coats, eye
76 protection, and gloves. If necessary, additional precautions required for specific study
77 substances or hazardous chemicals will be identified in the Material Safety Data Sheet
78 (MSDS).

¹ RSEs are internationally-harmonized reference standards (e.g., WHO *E. coli* Lipopolysaccharide [LPS] 94/580 [0113:H10:K-]; USP RSE Lot G3E069; FDA Lot EC-6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

79 The stop solution used in the ELISA kit is acidic and corrosive and should be handled with
80 the proper personal protective devices. If this reagent comes into contact with skin or eyes,
81 wash thoroughly with water. Seek medical attention, if necessary.

82 Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5,
83 5'- TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate
84 personal protection should be used to prevent bodily contact.

85 Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage,
86 antigenic response) and should be handled with care. Skin cuts should be covered and
87 appropriate personal protective devices should be worn. In case of contact with endotoxin,
88 immediately flush eyes or skin with water for at least 15 min. If inhaled, remove the affected
89 individual from the area and provide oxygen and/or artificial respiration as needed. Skin
90 absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

91 **3.0 MATERIALS, EQUIPMENT, AND SUPPLIES**

92 **3.1 Source of Cells**

93 PBMCs from fresh WB are the source of cells for cytokine production in the PBMC/IL-6 test
94 method (Poole et al., 2003). WB is obtained from healthy human volunteers who have
95 provided their consent according to established institutional guidelines. Volunteers are
96 expected not to have taken any drugs (e.g., prescription drugs, recreational drugs, herbal
97 drugs) and to have been free from illness for at least two weeks prior to donation.

98 PBMCs are isolated from WB using density gradient centrifugation (refer to **Section 6.1.1.1**).
99 The isolated PBMC suspension may be used directly in the PBMC/IL-6 test assay (**Section**
100 **6.1.3**) or frozen for later use (**Section 6.1.1.3**).

101 **3.2 Equipment and Supplies**

102 For all steps in the protocol, excluding the ELISA procedure, the materials that will be in
103 close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be
104 sterile and free from detectable pyrogens.

105 3.2.1 Preparation of PBMCs106 3.2.1.1 *Equipment*

- 107 • Centrifuge
- 108 • Hood; Bio-safety, laminar flow (recommended)
- 109 • Incubator; cell culture (37±1°C + 5% CO₂)
- 110 • Lymphoprep™
- 111 • Pipetter; multichannel (8- or 12-channel)
- 112 • Pipettors; single-channel adjustable (20, 200, and 1000 µL)
- 113 • Repeating pipetter
- 114 • Vortex mixer

115 3.2.1.2 *Consumables*

- 116 • Centrifuge tubes; nonpyrogenic, polystyrene (15 and 50 mL)
- 117 • Combitips; repeating pipetter (2.5 and 5.0 mL)
- 118 • Cryotubes; screw-cap, 2 mL
- 119 • Filters; sterile, 0.22 µm
- 120 • Needle set; Sarstedt multifly, pyrogen-free, 19 mm, 21 gauge for S-Monovette
- 121 • Phosphate buffered saline (PBS); sterile
- 122 • Pipets; serological, sterile (5, 10, and 25 mL)
- 123 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- 124 • Reaction tubes; polystyrene (1.5 mL)
- 125 • Reservoirs; fluid
- 126 • RPMI-1640 cell culture medium; supplemented with the following reagents to
- 127 yield RPMI-Complete (RPMI-C)
 - 128 ○ FCS; heat-inactivated (5 mL or a 1% final concentration)
 - 129 ○ L-Glutamine; 2 mM

- 130 ○ Penicillin/streptomycin (10,000 IU/mL penicillin, 10 mg/mL
- 131 streptomycin)
- 132 • Syringes; sterile (100 µL and 30 mL)
- 133 • Tips; pipetter, sterile, pyrogen-free (20, 200, and 1000 µL)
- 134 • Tubes; Sarstedt S-Monovette, 7.5 mL, heparinized for blood collection

135 3.2.2 ELISA

136 3.2.2.1 *Equipment*

- 137 • Microplate mixer
- 138 • Microplate reader (450 nm with an optional reference filter in the range of
- 139 540-590 nm)
- 140 • Microplate washer (optional)
- 141 • Multichannel pipetter

142 3.2.2.2 *Consumables*

- 143 • Container; storage, plastic
- 144 • Deionized water; nonsterile
- 145 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- 146 • Pyrogen-free water (PFW)
- 147 • Reservoirs; fluid
- 148 • Tips; pipetter, nonsterile
- 149 • Tubes; polystyrene (12 mL)

150 3.2.2.3 *ELISA Kit*

151 An ELISA that measures IL-6 release from PBMCs is used. A variety of IL-6 ELISA kits are
152 commercially available and the IL-6 ELISA procedure outlined in this protocol is intended to
153 serve as an example for using an ELISA kit. If the user prefers to prepare an in-house
154 ELISA, then additional reagents would be required. The IL-6 ELISA should be calibrated
155 using an IL-6 international reference standard (e.g., WHO 89/548) prior to use. The IL-6

156 cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this reagent
157 must be purchased separately. Results obtained using these products are subject to the assay
158 acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA kit
159 components may include the following:

- 160 • ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or
161 polyclonal
- 162 • Buffered wash solution
- 163 • Dilution buffer
- 164 • Enzyme-labeled detection antibody
- 165 • Human IL-6 reference standard
- 166 • Pyrogen-free saline (PFS)
- 167 • Stop solution
- 168 • TMB/substrate solution

169 **3.3 Chemicals**

- 170 • Endotoxin (e.g., WHO *E. coli* LPS 2nd International Standard 94/580; USP
171 RSE *E. coli* LPS Lot G3E069; USP RSE *E. Coli* Lot G; FDA *E. coli* Lot EC6)

172 **3.4 Solutions**

- 173 • RPMI-C cell culture medium

174 **4.0 ASSAY PREPARATION**

175 All test substances, endotoxin, and endotoxin-spiked solutions should be stored at 4°C.

176 **4.1 Endotoxin Standard Curve**

177 An internationally harmonized RSE or equivalent is used to generate the endotoxin standard
178 curve. The use of any other *E. coli* LPS requires calibration against a RSE using the
179 PBMC/IL-6 test method.

180 A standard endotoxin curve consisting of a Normal Saline Control (NSC) and five RSE
 181 concentrations (0.063, 0.125, 0.25, 0.50, and 1.0 EU/mL) are included in the incubation step
 182 (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin
 183 standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the
 184 lyophilized content of the stock vial by following the instructions provided by the
 185 manufacturer (e.g., for a vial containing 10,000 EU, 5 mL of PFW is added). To reconstitute
 186 the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in
 187 a bath sonicator for 5 min. The stock solution is stable for 14 days when stored at 2 to 8°C.
 188 An endotoxin standard curve is prepared by making serial dilutions of the stock solution in
 189 PFS as described in **Table 4-1**.

190 **Table 4-1 Preparation of Endotoxin Standard Curve**

Stock Endotoxin EU/mL	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000 ^{1,2}	20	1980	20 ³
20	100	1900	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0.125	500	500	0.063
0	0	1000	0

191 Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

192 Each stock tube should be vortexed vigorously prior to its use to make the subsequent dilution.

193 ¹ A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

194 ² The stock solution of USP RSE may be stored in aliquots and kept at -20°C for up to 6 months. Do not store
 195 the endotoxin at -80°C.

196 ³ This concentration is not used in the assay.

197

198 **4.2 Test Substances**

199 Liquid test substances should be tested neat or, if interference is detected (see **Section 4.2.1**),
 200 diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in
 201 saline, dissolved in dimethylsulfoxide (DMSO) then diluted up to 0.5% (v/v) with PFS
 202 provided that this concentration does not interfere with the assay. The test substances should
 203 be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for 5 min.

204 4.2.1 Interference Testing

205 Interference testing must be carried out on any test sample for which no interference
206 information is available. The purpose of the interference test is to determine the lowest
207 dilution (i.e., highest concentration) of a test substance from which an endotoxin spike can be
208 detected (i.e., based on the decision criteria described in **Section 4.2.1.2**). However, to ensure
209 a valid test, a test substance should not be diluted beyond its Maximum Valid Dilution
210 (MVD).

211 For many marketed products, values for the MVD and the Endotoxin Limit Concentration
212 (ELC) are published in the U.S. Pharmacopeia, the European Pharmacopoeia, and/or Food
213 and Drug Administration (FDA) guidelines. However if one or both of these values are not
214 available, then calculation of the MVD is dependent on the ELC (see **Section 12.3**). If
215 unknown, the ELC can be approximated by dividing the maximum hourly dose of the
216 product by the hourly dose received per patient. For example, if a product is used at an
217 hourly dose of 100 mg per patient, then the ELC would be 350 EU/100 mg or 3.5 EU/mg.

218 4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

219 The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent international RSE (e.g., USP
220 G3E069, FDA EC-6) is recommended for preparation of the endotoxin control (EC). If a
221 different *E. coli* LPS is used and the potency relative to the RSE is not provided, then each
222 lot must be calibrated against the RSE in the PBMC/IL-6 test method. For interference
223 testing, an endotoxin standard curve (see **Section 4.1**) should be included on each plate.

224 4.2.1.2 *Spiking Test Substances with Endotoxin*

225 For interference testing, non-spiked and endotoxin-spiked test substances are prepared in
226 microplate wells (n=4 replicates) and an *in vitro* pyrogen test is performed. Either RPMI-C or
227 a fixed concentration (a concentration selected from the middle of the EC standard curve) of
228 the RSE (i.e., 0.25 EU/mL) in RPMI-C is added to the test substance in serial two-fold
229 dilutions. An illustrative example of endotoxin spiking solutions is shown in **Table 4-2**. For
230 non-spiked solutions, 150 µl of RPMI-C is added to a well followed by 50 µl of the test
231 substance (neat or at serial dilution) and 50 µL of PBMCs and the well contents are mixed.
232 Endotoxin-spiked solutions are prepared by adding 100 µL of RPMI-C to each well followed

233 by 50 μL of the test substance (neat or at serial dilution) and 50 μL of an endotoxin spike
 234 solution (0.25 EU/mL). Finally, 50 μL of PBMCs are added and the well contents are mixed
 235 (see example presented in **Table 4-2**).

236 **Table 4-2 Preparation of Endotoxin-Spiked and Non-spiked Solutions for**
 237 **Determination of Test Substance Interference in the Incubation and**
 238 **ELISA Test Systems**

Sample Addition	Spiked	Non-spiked
	$\mu\text{L}/\text{well}^1$	
RPMI-C	100	150
Endotoxin spike solution ²	50	0
Test substance (neat and each serial dilution)	50	50
PBMCs ³	50	50
Total ⁴	250	250

239 Abbreviations: PBMC = Peripheral blood mononuclear cells

240 ¹ n=4 replicates each

241 ² Endotoxin concentration is 0.25 EU/mL in RPMI-C.

242 ³ PBMCs are resuspended in RPMI-C (1×10^6 cells/mL).

243 ⁴ A total volume of 250 μL per well is used for the incubation.

244

245 The lowest dilution of the test substance that yields an endotoxin spike recovery of 50% to
 246 200% in the pyrogen test is determined. The optical density (OD) values of the endotoxin-
 247 spiked and non-spiked test substances are calibrated against the endotoxin calibration curve.
 248 The resulting EU value of the non-spiked test substance is subtracted from the corresponding
 249 EU value of the endotoxin-spiked test substance at each dilution. The % recovery for each
 250 sample dilution is then determined from the endotoxin spike solution concentration set to
 251 100%. For example, consider the following interference test results in **Table 4-3**:

252 **Table 4-3 Example of Interference Data Used to Determine Sample Dilution for**
 253 **Assay**

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

254

255 Based on these results, the dilution of the test substance used in the *in vitro* pyrogen test
 256 would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 0.25 EU/mL EC).

257 4.2.2 Interference with ELISA

258 If the data obtained from the experiment in **Section 4.2.1** suggests the presence of
259 interference, then a subsequent experiment similar to that described in **Section 4.2.1** would
260 need to be performed to confirm that the test substance(s) does not directly interfere with the
261 ELISA. For this experiment, an ELISA would be performed in the absence of PBMCs.

262 **5.0 CONTROLS**

263 **5.1 Negative Control**

264 A negative control (e.g., RPMI-C) is included in each experiment in order to detect
265 nonspecific changes in the test system, as well as to provide a baseline for the assay
266 endpoints.

267 **5.2 Solvent Control**

268 Solvent controls are recommended to demonstrate that the solvent is not interfering with the
269 test system when solvents other than PFS are used to dissolve test substances.

270 **5.3 Positive Control**

271 An internationally standardized EC (e.g., WHO 94/580; USP G3E069; 0.5 EU/mL) is
272 included in each experiment to verify that an appropriate response is induced.

273 **5.4 Benchmark Control**

274 Benchmark controls may be used to demonstrate that the test method is functioning properly,
275 or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals,
276 medical device eluates) of a specific class or a specific range of responses, or for evaluating
277 the relative pyrogenic potential of a test substance. Appropriate benchmark controls should
278 have the following properties:

- 279 • consistent and reliable source(s) for the chemicals (e.g., parenteral
280 pharmaceuticals, medical device eluates)
- 281 • structural and functional similarities to the class of the substance being tested
- 282 • known physical/chemical characteristics

- 283 • supporting data on known effects in animal models
- 284 • known potency in the range of response

285 **5.5 Positive Product Control (PPC)**

286 The PPC is a test substance diluted to a level that does not interfere with the test method and
287 does not exceed the MVD. The PPC is obtained by spiking a test substance with a known EC
288 (e.g., 0.25 EU/mL) and demonstrating that 50% to 200% of the EC is recovered.

289 **5.6 Negative Product Control (NPC)**

290 The NPC is the test substance diluted to the MVD and then spiked with PFS. It is the
291 negative control for the PPC.

292 **6.0 EXPERIMENTAL DESIGN**

293 **6.1 Incubation with Test Samples and Measurement of IL-6 Release**

294 6.1.1 Collection of Human Blood

295 WB is obtained from healthy human volunteers who have provided their consent according
296 to established institutional guidelines. Volunteers are expected not to have taken any drugs
297 and to have been free from illness for at least two weeks prior to donation. The criteria for
298 rejection of data from donors that are low responders or that are suspect due to veracity of
299 health information is addressed in **Section 8.0**.

300 Fresh WB is drawn by venipuncture using a multily needle set and collected in heparinized
301 tubes (15 IU/mL lithium heparin). All components of the blood collection system (i.e.,
302 syringes, tubes, connecting lines) must be sterile and pyrogen-free. Blood should be stored at
303 room temperature (RT) and must be used within 4 hr. Prior to use in the assay, the collection
304 tubes should be gently inverted 1 to 2 times. **Do Not Vortex**.

305 6.1.1.1 *Isolation of PBMCs from WB*

306 PBMCs must be isolated within two hours of WB collection using Lymphoprep™. The
307 isolation procedure described below is a modification of the manufacturer's instructions as
308 outlined in the ECVAM SOPs for the PBMC/IL-6 test method.

309 To each 15 mL of heparinized WB, add 15 mL of PBS and a sterile 10 mL pipet into the
310 tube. Add 20 mL of Lymphoprep™ to each of the two tubes, through the inserted pipet, to
311 form a lower, denser, layer. The tubes are then centrifuged at 340 x g for 45 min at room RT.
312 After centrifugation, a white band of PBMCs should be visible at approximately the 25 mL
313 graduation mark on the tube. Carefully remove the uppermost 9 mL from each tube and
314 discard. If cryopreservation of PBMCs is to be performed (see **Section 6.1.1.3**), then transfer
315 the layer to a new 50 mL tube for preparing a cryoprotective solution. The remaining
316 supernatant above the PBMC band should be aspirated and discarded. Using a 10 mL pipet,
317 transfer the PBMC layer to a new 50 mL centrifuge tube.

318 6.1.1.2 *Washing PBMCs*

319 The PBMCs are resuspended in a total volume of 50 mL of PBS and centrifuged at 340 x g
320 for 15 min. The supernatant is poured off and the cellular sediment resuspended in 10 mL of
321 PBS by pipetting up and down several times with a serological pipet. Adjust the total volume
322 in each tube to 50 mL with PBS and centrifuge at 340 x g for 10 min for a second time. After
323 centrifugation, the PBMCs are resuspended with 15 mL of RPMI-C and the two aliquots of
324 PBMCs are pooled into a single tube. Cell counts (expressed per mL) are determined using a
325 hemacytometer. If the cell count is above $1.2 \times 10^6/\text{mL}$, the cell suspension should be diluted
326 to 1×10^6 cell/mL in RPMI-C. This suspension must be used in the PBMC/IL-6 assay within
327 4 hr from the time of WB collection.

328 6.1.1.3 *Procedure for Cryopreservation and Thawing of PBMCs*

329 To freeze the PBMCs, prepare a cryoprotective solution by adding 2 mL of pyrogen-free
330 DMSO to the 18 mL of supernatant collected in the centrifugation procedure outlined in
331 **Section 6.1.1.1**. Cool the cryoprotective solution to between 2 and 8°C. Centrifuge the
332 isolated PBMCs as instructed in **Section 6.1.1.2** and then add 6 mL of the chilled
333 cryoprotective solution to the cell sediment. Pool the cell suspensions from the same donor
334 and transfer 1.0 mL aliquots to appropriately labeled screw-cap cryotubes. The cryotubes are
335 placed in a styrofoam box for thermal insulation and slowly frozen to -80°C. After 72 hr, the
336 tubes can be transferred to liquid nitrogen for prolonged storage.

337 To thaw the cryopreserved PBMCs, take two tubes (each at 1.0 mL) from a single donor and
338 submerge in a water bath at $37 \pm 1^\circ\text{C}$. After thawing, the cell suspensions are pooled in a

339 single 50 mL centrifuge tube and RPMI-C is added to give a total volume of 40 mL. The
 340 PMBCs are centrifuged at 340 x g for 10 min, the supernatant removed, and the cells
 341 resuspended in 10 mL of RPMI-C. Viability may be tested and should yield greater than 95%
 342 cell survival.

343 6.1.2 Incubation Plate

344 Test substances are prepared at a level of dilution that did not show interference with the test
 345 system or for which it is known that interference does not occur. Each incubation plate can
 346 accommodate an endotoxin standard curve, a NSC, and 14 test samples (see **Table 6-1**).

347 **Table 6-1 Overview of Incubation Plate Preparation in the PBMC/IL-6 Test**
 348 **Method**

Number of Wells	Sample	RPMI-C	EC	Test Sample	PBMCs	Mix the samples; incubate overnight at 37±1°C in a humidified atmosphere with 5% CO ₂ .	Mix the samples; immediately transfer to an ELISA plate ³ and run ELISA or store plate at -20°C or -80°C.
		µL					
20 ¹	EC	100	50	0	100		
4	NSC	150	0	0	100		
56 ²	Test samples (1-14)	100	0	50	100		

349 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; PBMC = Peripheral blood
 350 mononuclear cell

351 ¹ Five EC concentrations (0.063, 0.125, 0.25, 0.50, and 1.0 EU/mL) in quadruplicate.

352 ² 14 test samples (n=4 each) per plate.

353 ³ An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate. Therefore, 80 wells are
 354 available for test samples and controls on the incubation plate.

355

356 6.1.3 Incubation Assay for IL-6 Release

357 PBMC samples are prepared in a microtiter plate using a laminar flow hood. All
 358 consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled
 359 appropriately with a permanent marker. An overview of the incubation plate preparation is
 360 shown in **Table 6-1**. The incubation procedure is outlined below:

361 **Step 1.** Refer to the suggested incubation plate template presented in **Table 6-2**.

362 **Step 2.** Using a pipetter, transfer 100 µL of RPMI-C into each well.

363 **Step 3.** Transfer 50 μ L of test sample into the appropriate wells as indicated in the
364 template.

365 **Step 4.** Transfer 50 μ L of the EC (standard curve) and the NSC controls in
366 quadruplicate into the appropriate wells according to the template.

367 **Step 5.** Transfer 100 μ L of a well-mixed PBMC suspension into each well and mix
368 by gently swirling the plate.

369 **Step 6.** Mix the contents of the wells thoroughly by pipetting up and down several
370 times using a multichannel pipetter, changing the tips between each row in order to
371 avoid cross-contamination.

372 **Step 7.** Place the covered plate in a tissue culture incubator for 16 to 24 hr at
373 $37\pm 1^\circ\text{C}$ in a humidified atmosphere containing 5% CO_2 .

374 **Step 8.** Prior to transferring the test samples onto the ELISA plate, mix the
375 contents of the wells by pipetting up and down using a multichannel pipetter,
376 changing the tips between each row in order to avoid cross-contamination.

377 **Table 6-2 Incubation Plate - Sample and Control Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 1.0	EC 1.0	EC 1.0	EC 1.0	TS3	TS3	TS3	TS3	TS11	TS11	Void ³	Void
B	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.063	EC 0.063	EC 0.063	EC 0.063	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

378 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

379 ¹ EC value (e.g., EC 1.0) represents the endotoxin concentration in EU/mL.

380 ² TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

381 ³ Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).

382

383

384 **6.2 ELISA to Measure IL-6 Release**385 6.2.1 IL-6 Standard Curve

386 An IL-6 standard supplied with the ELISA kit is used. IL-6 standards are typically supplied
387 in lyophilized form and should be reconstituted according to the manufacturer's instructions.
388 The stock solution should be diluted in RPMI-C to the following concentrations: 0, 62.5, 125,
389 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 μ L. Each well on the
390 ELISA plate will receive 50 μ L of an IL-6 blank or standard.

391 6.2.2 ELISA

392 The manufacturer's instructions provided with the ELISA kit should be followed and a
393 typical experimental design is outlined below. If the user prefers to prepare an in-house
394 ELISA, then appropriate modifications and validation of these changes would be necessary.
395 The ELISA should be carried out at RT and therefore all components must be at RT prior to
396 use. Do *not* thaw frozen specimens by heating them in a water bath. A suggested ELISA
397 plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, a NSC,
398 an eight-point IL-6 standard curve (0 to 4000 pg/mL), and 14 test substances in
399 quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are
400 transferred directly from the incubation plate. The IL-6 standard curve is prepared as
401 described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-**
402 **4**.

403 **Step 1.** After pipetting up and down very carefully three times (avoid detachment
404 of the adherent PBMCs) to mix the supernatant, transfer 50 μ L from each well of
405 the Incubation Plate (A1-10; H1-10) to the ELISA plate.

406 **Step 2.** Add 50 μ L of each IL-6 standard (0 to 4000 pg/mL) into the respective
407 wells on the ELISA plate.

408 **Step 3.** Add 200 μ L of the enzyme-labeled detection antibody (neat as supplied, or
409 diluted, if necessary) to each of the wells.

410 **Step 4.** Cover the microtiter plate(s) with adhesive film and incubate for 2 to 3 hr
411 at 20 to 25°C.

412 **Step 5.** Decant and wash each well three times with 300 µL Buffered Wash
413 Solution and then rinse three times with deionized water. Place the plates upside
414 down and tap to remove water.

415 **Step 6.** Add 200 µL of TMB/Substrate Solution to each well and incubate at RT in
416 the dark for 15 min. If necessary, decrease the incubation time.

417 **Step 7.** Add 50 µL of Stop Solution to each well.

418 **Step 8.** Tap the plate gently after the addition of Stop Solution to aid in mixing.

419 **Step 9.** Read the OD₄₅₀ within 15 min of adding the Stop Solution. Measurement
420 with a reference wavelength of 540 to 590 nm is recommended.

421

421

422 **Table 6-3 ELISA Plate - Sample and Control Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 1.0	EC 1.0	EC 1.0	EC 1.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6 ³ 0	IL-6 0
B	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.063	EC 0.063	EC 0.063	EC 0.063	TS7	TS7	TS7	TS7	TS13	TS13	IL-6 500	IL-6 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-6 1000	IL-6 1000
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-6 2000	IL-6 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-6 4000	IL-6 4000

423 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

424 ¹ EC value (e.g., EC 1.0) represents the endotoxin concentration in EU/mL.425 ² TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.426 ³ IL-6 values in columns 11 and 12 are in pg/mL.

427

428

429 **Table 6-4 Overview of ELISA Procedure**

Material transfer from Incubation Plate (μL)	Enzyme-labeled Antibody (μL)	Cover the Incubation Plate and incubate for 2 to 3 hr at 20-25°C.	TMB/Substrate Solution (μL)	Incubate 15 min at RT in dark.	Stop Solution (μL)	Read optical density at 450 nm with a 540-590 nm wavelength reference filter.
50	200		200		50	

430 Abbreviations: RT = Room temperature

431

432

432

433 **7.0 EVALUATION OF TEST METHODS**434 **7.1 OD Measurements**

435 The OD of each well is obtained by reading the samples in a standard microplate
436 spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD₄₅₀) with
437 a 540 to 590 nm reference filter (recommended). OD₄₅₀ values are used to determine assay
438 acceptability and in the decision criteria for the detection of endotoxin in a test substance (see
439 **Sections 8.0 and 9.0**).

440 **8.0 CRITERIA FOR AN ACCEPTABLE TEST**

441 Obtain the PPC and the corresponding NPC by interference testing of a test substance in the
442 presence and absence of a fixed quantity of endotoxin (i.e., 0.25 EU/mL) in quadruplicate.
443 An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-
444 6 standard curve should be included in each ELISA as shown in the template presented in
445 **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- 446 • The quadratic function of the IL-6 standard curve produces an $r \geq 0.95$ and the
447 OD₄₅₀ of the blank control is below 0.15.
- 448 • The endotoxin standard curve produces OD₄₅₀ values that ascend in a
449 sigmoidal concentration response.

450 Blood donors are considered to be low responders if their mean OD₄₅₀ value obtained for 1
451 EU/mL EC is significantly below the mean OD₄₅₀ value obtained for 1000 pg/mL IL-6.

452 Blood donors who produce a mean OD₄₅₀ value for the NSC that is significantly above the
453 mean OD₄₅₀ value at 500 pg/mL IL-6 are considered to be high responders. Low and high
454 responders should be excluded from analysis. Each test must be performed with PBMCs
455 from four different donors. From a set of four donors, a maximum of one donor may be
456 excluded from the assessment; otherwise, the test must be repeated with four different
457 donors.

458 9.0 DATA INTERPRETATION/DECISION CRITERIA

459 9.1 Decision Criteria for Determination of Pyrogenicity

460 The validity of the endotoxin standard curve should be calculated using a four-parameter
461 logistic model. If necessary to satisfy the model, endotoxin concentrations may be modified.

462 Calculate the mean OD₄₅₀ values of all of the replicates in each experimental group. Calibrate
463 the mean OD₄₅₀ value for each test substance using the endotoxin standard curve and
464 document the estimated endotoxin concentration. Multiply the estimated endotoxin
465 concentration by the dilution factor, if necessary. This value represents the pyrogenicity of
466 the sample in terms of endotoxin equivalents for that particular donor. The *t*-test is used to
467 compare the data of a test sample against the data of the EC (0.5 EU/mL) that is performed in
468 parallel. If this test results in a significant *p*-value (i.e., smaller than 1%), then the sample is
469 considered to be non-pyrogenic, and as pyrogenic if otherwise (Hoffmann et al., 2005), as
470 long as the assay acceptability criteria in **Section 8.0** has been met.

471 9.1.1 Decision Level 1

- 472 • If all donors show a negative reaction, then the product passes.
- 473 • If two or more donors show a positive reaction, then the product fails.
- 474 • If one donor shows a positive reaction, then an additional test with four donors
475 has to be performed (go to Decision Level 2).

476 9.1.2 Decision Level 2

- 477 • If out of 6 to 8 donors, only one donor shows a positive reaction, then the
478 product passes.
- 479 • In any other case, the product fails.

480 10.0 STUDY REPORT

481 The test report should include the following information:

482 *Test Substances and Control Substances*

- 483 • Name of test substance

- 484 • Purity and composition of the substance or preparation
- 485 • Physicochemical properties (e.g., physical state, water solubility)
- 486 • Treatment of the test/control substances prior to testing (e.g., vortexing,
- 487 sonication, warming, resuspension solvent)

488 *Justification of the In Vitro Test Method and Protocol Used*

489 *Test Method Integrity*

- 490 • The procedure used to ensure the integrity (i.e., accuracy and reliability) of the
- 491 test method over time
- 492 • If the test method employs proprietary components, documentation on the
- 493 procedure used to ensure their integrity from “lot-to-lot” and over time
- 494 • The procedures that the user may employ to verify the integrity of the
- 495 proprietary components

496 *Criteria for an Acceptable Test*

- 497 • Acceptable concurrent positive control ranges based on historical data
- 498 • Acceptable negative control data

499 *Test Conditions*

- 500 • Cell system used
- 501 • Calibration information for the spectrophotometer used to read the ELISA
- 502 • Details of test procedure used
- 503 • Description of any modifications of the test procedure
- 504 • Reference to historical data of the model
- 505 • Description of evaluation criteria used

506 *Results*

- 507 • Tabulation of data from individual test samples

508 *Description of Other Effects Observed*

509 *Discussion of the Results*

510 *Conclusion*

511 *A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies*

- 512 • This statement should indicate all inspections made during the study and the
513 dates any results were reported to the Study Director. This statement should
514 also confirm that the final report reflects the raw data.

515 If GLP-compliant studies are performed, then additional reporting requirements provided in
516 the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be
517 followed.

518

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542

542 **12.0 TERMINOLOGY AND FORMULA**

543 **12.1 Assay Sensitivity (λ)**

544 For an *in vitro* cell-based assay, the variable λ is defined as the lowest statistically significant
545 point on the standard endotoxin concentration-response curve and represents the relative
546 sensitivity of the test method for the detection of endotoxin (i.e., level of detection).

547 **12.2 Endotoxin Control (EC)**

548 The EC is incubated with PBMCs and serves as the positive control for the experiment. The
549 results should be compared to historical values to insure that it provides a known level of
550 cytokine release relative to the NSC.

551 **12.3 Endotoxin Limit Concentration (ELC)**

552 The ELC is the maximum allowable concentration of endotoxin for a particular product and
553 is expressed in EU per volume (mL) or weight (mg). The ELC is defined by the FDA or
554 specified in the USP². It is calculated as the product of K/M, where:

555 K is the threshold pyrogen dose for parenteral use in rabbits or humans (5.0 EU/kg). At an
556 injection volume of 10 mL/kg, K is equal to 0.5 EU/mL.

557 M is the larger of the rabbit dose or the maximum human dose administered in one hour as
558 defined below and varies with test substance³.

559 **12.4 Maximum Valid Dilution (MVD)**

560 The MVD is the maximum dilution of a test substance that can be tolerated in a test system
561 without exceeding the ELC, if the test substance must be diluted as a result of assay
562 interference. Dilutions beyond the MVD would not be valid for endotoxin detection in the

² ELC values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, Pharmacopoeial guidelines, FDA publications).

³ Values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, pharmacopoeial guidelines, FDA publications).

563 test system. Calculation of the MVD is dependent on whether or not the ELC for a test
564 substance is published. When the ELC is known, the MVD is:

565
$$\text{MVD} = (\text{ELC} \times \text{Product Potency [PP]})/\lambda$$

566 As an example, for "Cyclophosphamide Injection," the ELC is 0.17 EU/mg, PP is 20
567 mg/mL, and the assay sensitivity is 0.1 EU/mL. The calculated MVD would be 34. The test
568 substance can be diluted no more than 1:34 prior to testing.

569 If the ELC is not known, the MVD is:

570
$$\text{MVD} = \text{PP}/\text{Minimum Valid Concentration (MVC)}$$

571 where, $\text{MVC} = (\lambda \times \text{M})/\text{K}$

572 where, M is the maximum human dose

573 As an example, for "Cylophosphamide Injection," the PP is 20 mg/mL, M is 30
574 mg/kg, and assay sensitivity is 0.1 EU/mL. The calculated MVC is 0.6 mg/mL and the MVD
575 is 33.3. The test substance can be diluted no more than 1:33 in the assay prior to testing.

576 **12.5 Negative Product Control (NPC)**

577 The NPC is a test sample to which PFS is added. The NPC is the baseline for determination
578 of cytokine release relative to the endotoxin-spiked PPC.

579 **12.6 Negative Saline Control (NSC)**

580 The NSC is PBMCs (in RPMI-C) incubated with PFS (used for dilution of test substance)
581 and is used as the blank.

582 **12.7 Parenteral Threshold Pyrogen Dose (K)**

583 The value K represents the threshold pyrogen dose for parenteral products for rabbits and
584 humans. Based on experimental data, K is fixed at 5.0 EU/kg. For intrathecal products, K is
585 0.2 EU/kg.

586 **12.8 Positive Product Control (PPC)**

587 The PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an
588 amount of endotoxin equal to that which produces $\frac{1}{2}$ the maximal increase in OD from the

589 endotoxin standard curve) to insure that the test system is capable of endotoxin detection in
590 the product as diluted in the assay.

591 **12.9 Product Potency (PP)**

592 The concentration for a test substance is the PP typically expressed as $\mu\text{g/mL}$ or mg/mL .

593 **12.10 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)**

594 The variable M represents the rabbit test dose or the maximum human dose in 1 hr. The
595 variable M is expressed in mg/kg and varies with the test substance. For
596 radiopharmaceuticals, M should be adjusted to account for product activity (radioactive
597 decay) at time administration. An average human standard weight of 70 kg is used for the
598 calculation. If a pediatric dose should be used and it is higher than the adult dose, then it
599 should be used in the formula.

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Appendix B4

**Draft ICCVAM Recommended Protocol for Future Studies Using the Whole Blood
(WB)/Interleukin-1 β (IL-1 β) Test Method**

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37 **Table 1 Comparison of Draft ICCVAM Recommended Test Method Protocol with the ECVAM SOP for the Whole**
 38 **Blood (WB)/Interleukin-1 β (IL-1 β) Pyrogen Test Method**

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP	ECVAM Validation SOP
Test Substance	Test neat or at minimal dilution that produces no interference	Same as ICCVAM protocol	Test at MVD
Incubation Plate (number of control or test groups at n=4 each)	NSC (1)	Same as ICCVAM protocol	Same as ICCVAM protocol
	EC (5)	Same as ICCVAM protocol	EC (2)
	TS (14)	TS (25)	EC spikes (3 x 5)
	PPC ¹ (0)	Same as ICCVAM protocol	PPC (3)
	NPC ¹ (0)	Same as ICCVAM protocol	NPC (3)
	LTAC (0)	LTAC (1)	Same as ICCVAM protocol
ELISA Plate	Includes seven point IL-1 β SC and blank in duplicate	Same as ICCVAM protocol	Same as ICCVAM protocol
Decision Criteria for Interference	0.5 x Median OD ₄₅₀ ² of 1 EU/mL EC <2x Median OD ₄₅₀ of 1 EU/mL EC	0.5 x Median OD ₄₅₀ of 0.5 EU/mL EC <2x Median OD ₄₅₀ of 0.5 EU/mL EC	Not applicable (tested at MVD)
Assay Acceptability Criteria	Mean OD ₄₅₀ of 0.5 EU/mL EC \geq 1.6X Mean OD ₄₅₀ of NSC	Same as ICCVAM protocol	Same as ICCVAM protocol
	Mean OD ₄₅₀ of PPC is 50% to 200% of 0.5 EU/mL EC	Same as ICCVAM protocol	Not specified
	Mean OD ₄₅₀ of NSC \leq 0.15	Same as ICCVAM protocol	Same as ICCVAM protocol
	Quadratic function of IL-1 β SC $r^2 \geq$ 0.95	Same as ICCVAM protocol	Same as ICCVAM protocol
	EC SC produces OD ₄₅₀ values that ascend in a sigmoidal concentration response	Same as ICCVAM protocol	Same as ICCVAM protocol
Decision Criteria for Pyrogenicity	OD ₄₅₀ Test sample \geq OD ₄₅₀ 0.5 EU/mL	Same as ICCVAM protocol	Same as ICCVAM protocol

39 Abbreviations: EC = Endotoxin control; LTAC = Lipoteichoic acid (LTA) control; MVD = Maximum Valid Dilution; NPC = Negative Product Control; NSC =
 40 Normal saline control; PPC = Positive Product Control; SC = Standard curve; TS = Test substance

41 ¹ PPC and NPC are evaluated during the interference test.

42 ² Median or mean OD₄₅₀ values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

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64 **1.0 PURPOSE AND APPLICABILITY**

65 The purpose of this protocol is to describe the procedures used to evaluate the presence of a
66 pyrogen (i.e., Gram-negative endotoxin) in a test substance. The presence of Gram-negative
67 endotoxin is detected by its ability to induce cytokine IL-1 β release from monocytoid cells in
68 human WB. The quantity of IL-1 β released is obtained using an enzyme-linked
69 immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for
70 IL-1 β . Release of IL-1 β is measured by incubation of WB with test substances or controls
71 (i.e., positive, negative). The amount of pyrogen present is determined by comparing the
72 values of endotoxin equivalents produced by WB cells exposed to the test substance to those
73 exposed to an internationally-harmonized Reference Standard Endotoxin (RSE)¹ or an
74 equivalent standard expressed in Endotoxin Units (EU)/mL. Based on a rabbit threshold
75 pyrogen dose of 0.5 EU/mL, which was established in a retrospective evaluation of rabbit
76 pyrogen test (RPT) data, a test substance is considered pyrogenic if it induces a level of IL-
77 1 β release equal to or greater than 0.5 EU/mL.

78 The focus of this protocol is on the use of the WB/IL-1 β test method, specifically for the
79 detection of Gram-negative endotoxin in parenteral pharmaceuticals. The relevance and
80 reliability for non-endotoxin pyrogens (e.g., lipoteichoic acid) has not been demonstrated in a
81 formal validation study.

82 **2.0 SAFETY AND OPERATING PRECAUTIONS**

83 All procedures for procurement of eligible blood donors and blood donations should follow
84 the regulations and procedures set forth by institutional guidelines for utilization of human
85 substances, which include but are not limited to blood, tissues, and tissue fluids. Standard
86 laboratory precautions are recommended including the use of laboratory coats, eye
87 protection, and gloves. If necessary, additional precautions required for specific study
88 substances or hazardous chemicals will be identified in the Material Safety Data Sheet
89 (MSDS).

¹ RSEs are internationally-harmonized reference standards (e.g., WHO *E. coli* Lipopolysaccharide [LPS] 94/580 [0113:H10:K-]; USP RSE Lot G3E069; FDA Lot EC-6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

90 The stop solution used in the ELISA kit is acidic and corrosive and should be handled with
91 the proper personal protective devices. If this reagent comes into contact with skin or eyes,
92 wash thoroughly with water. Seek medical attention, if necessary.

93 Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-
94 TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate
95 personal protection should be used to prevent bodily contact.

96 Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage,
97 antigenic response) and should be handled with care. Skin cuts should be covered and
98 appropriate personal protective devices should be worn. In case of contact with endotoxin,
99 immediately flush eyes or skin with water for at least 15 min. If inhaled, remove the affected
100 individual from the area and provide oxygen and/or artificial respiration as needed. Skin
101 absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

102 **3.0 MATERIALS, EQUIPMENT, AND SUPPLIES**

103 **3.1 Source of Cells**

104 Leukocytes from WB are the source of cells for cytokine production in the WB/IL-1 β test
105 method (Hartung and Wendel, 1996; Schindler et al., 2006). WB is obtained from healthy
106 human volunteers who have provided their consent according to established institutional
107 guidelines. Volunteers are expected not to have taken any drugs (e.g., prescription drugs,
108 recreational drugs, herbal drugs) and to have been free from illness for at least two weeks
109 prior to donation.

110 **3.2 Equipment and Supplies**

111 For all steps in the protocol, excluding the ELISA procedure, the materials that will be in
112 close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be
113 sterile and free from detectable pyrogens.

114 3.2.1 Blood Incubation

115 3.2.1.1 *Equipment*

- 116 • Centrifuge

- 117 • Hood; Bio-safety, laminar flow (recommended)
- 118 • Incubator; cell culture (37 \pm 1 $^{\circ}$ C + 5% CO₂)
- 119 • Pipetter; multichannel (8- or 12-channel)
- 120 • Pipettors; single-channel adjustable (20 and 200 μ L)
- 121 • Repeating pipetter
- 122 • Vortex mixer

- 123 3.2.1.2 *Consumables*
- 124 • Centrifuge tubes; nonpyrogenic, polypropylene (15 and 50 mL)
- 125 • Combitips; repeating pipetter (1.0 and 2.5 mL)
- 126 • Needle set; Sarstedt multifyly, pyrogen-free, 19 mm, 21 gauge for S-Monovette
- 127 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- 128 • Reaction tubes; polypropylene (1.5 mL)
- 129 • Reservoirs; fluid
- 130 • Tips; pipetter, sterile, pyrogen-free (20 and 200 μ L)
- 131 • Tubes; Sarstedt S-Monovette, 7.5 mL, heparinized for blood collection

- 132 3.2.2 ELISA
- 133 3.2.2.1 *Equipment*
- 134 • Microplate mixer
- 135 • Microplate reader (450 nm with an optional reference filter in the range of
- 136 600-690 nm)
- 137 • Microplate washer (optional)
- 138 • Multichannel pipetter

- 139 3.2.2.2 *Consumables*
- 140 • Container; storage, plastic

- 141 • Deionized water; nonsterile
- 142 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- 143 • Pyrogen-free water (PFW)
- 144 • Reservoirs; fluid
- 145 • Tips; pipetter, nonsterile
- 146 • Tubes; polystyrene (12mL)

147 3.2.2.3 *ELISA Kit*

148 An ELISA that measures IL-1 β release from Cryo WB is used. A variety of IL-1 β ELISA
149 kits are commercially available and the IL-1 β ELISA procedure outlined in this protocol is
150 intended to serve as an example for using an ELISA kit. If the user prefers to prepare an in-
151 house ELISA, then additional reagents would be required. The IL-1 β ELISA should be
152 calibrated using an IL-1 β international reference standard (e.g., WHO 86/680) prior to use.
153 The IL-1 β cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this
154 reagent must be purchased separately. Results obtained using these products are subject to
155 the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-1 β ELISA
156 kit components may include the following:

- 157 • ELISA plates coated with anti-human IL-1 β capture antibody; monoclonal or
158 polyclonal
- 159 • Buffered wash solution
- 160 • Dilution buffer
- 161 • Enzyme-labeled detection antibody
- 162 • Human IL-1 β reference standard
- 163 • Pyrogen-free saline (PFS)
- 164 • Stop solution
- 165 • TMB/substrate solution

166 **3.3 Chemicals**

- 167 • Endotoxin (e.g., WHO *E. coli* LPS 2nd International Standard 94/580; USP
168 RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

169 **3.4 Solutions**

170 ELISA solutions are listed in **Section 3.2.**

171 **4.0 ASSAY PREPARATION**

172 All test substances, endotoxin, and endotoxin-spiked solutions should be stored at 4°C.

173 **4.1 Endotoxin Standard Curve**

174 An internationally harmonized RSE or equivalent is used to generate the endotoxin standard
175 curve. The use of any other *E. coli* LPS requires calibration against a RSE using the WB/IL-
176 1 β test method.

177 A standard endotoxin curve consisting of a Normal Saline Control (NSC) and five RSE
178 concentrations (0.25, 0.50, 1.0, 2.5, and 5.0 EU/mL) are included in the incubation step (refer
179 to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard
180 curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized
181 content of the stock vial by following the instructions provided by the manufacturer (e.g., for
182 a vial containing 10,000 EU, 5 mL of PFW is added). To reconstitute the endotoxin, the stock
183 vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for 5
184 min. The stock solution is stable for 14 days when stored at 2 to 8°C. An endotoxin standard
185 curve is prepared by making serial dilutions of the stock solution in PFS as described in
186 **Table 4-1.**

187

187

188 **Table 4-1 Preparation of Endotoxin Standard Curve**

Stock Endotoxin EU/mL	μ L of Stock Endotoxin	μ L of PFS	Endotoxin Concentration EU/mL
2000 ^{1,2}	50	1950	50 ³
50	100	900	5.0
5.0	500	500	2.5
2.5	500	500	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0	0	1000	0

189 Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

190 Each stock tube should be vortexed prior to its use to make the subsequent dilution.

191 ¹ A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.192 ² The stock solution of USP RSE may be stored in aliquots and kept at -20°C for up to 6 months. Do not store
193 the endotoxin at -80°C.194 ³ This concentration is not used in the assay.

195

196 **4.2 Test Substances**

197 Liquid test substances should be tested neat or, if interference is detected (see **Section 4.2.1**),
198 diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in
199 saline, dissolved in dimethylsulfoxide (DMSO) then diluted up to 0.5% (v/v) with PFS,
200 provided that this concentration of DMSO does not interfere with the assay. The test
201 substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator
202 for 5 min.

203 **4.2.1 Interference Testing**

204 Interference testing must be carried out on any test sample for which no interference
205 information is available. The purpose of the interference test is to determine the lowest
206 dilution (i.e., highest concentration) of a test substance from which an endotoxin spike can be
207 detected (i.e., based on the decision criteria described in **Section 4.2.1.2**. However, to ensure
208 a valid test, a test substance should not be diluted beyond its Maximum Valid Dilution
209 (MVD).

210 For many marketed products, values for the MVD and the Endotoxin Limit Concentration
211 (ELC) are published in the U.S. Pharmacopeia, the European Pharmacopoeia, and/or Food
212 and Drug Administration (FDA) guidelines. However if one or both of these values are not

213 available, then calculation of the MVD is dependent on the ELC (see **Section 12.3**). If
214 unknown, the ELC can be approximated by dividing the maximum hourly dose of the
215 product by the hourly dose received per patient. For example, if a product is used at an
216 hourly dose of 100 mg per patient, then the ELC would be 350 EU/100 mg or 3.5 EU/mg.

217 4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

218 The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent international RSE (e.g., USP
219 G3E069, FDA EC-6) is recommended for preparation of the endotoxin control (EC). If a
220 different *E. coli* LPS is used and the potency relative to the RSE is not provided, then each
221 lot must be calibrated against the RSE in the WB/IL-1 β test method. For interference testing,
222 an endotoxin standard curve (see **Section 4.1**) should be included on each plate.

223 4.2.1.2 *Spiking Test Substances with Endotoxin*

224 For interference testing, non-spiked and endotoxin-spiked test substances are prepared in
225 microplate wells (n=4 replicates) and an *in vitro* pyrogen test is performed. Either PFS or a
226 fixed concentration (a concentration selected from the middle of the EC standard curve) of
227 the RSE (i.e., 1 EU/mL) in PFS is added to the test substance in serial two-fold dilutions. An
228 illustrative example of endotoxin-spiking solutions is shown in **Table 4-2**. For non-spiked
229 solutions, 200 μ L of PFS is added to a well followed by 20 μ L each of the test substance
230 (neat or at serial dilution) and 20 μ L of WB. Endotoxin-spiked solutions are prepared by
231 adding 180 μ L of PFS to each well followed by 20 μ L of the test substance (neat or at serial
232 dilution) and 20 μ L of WB. Then, 20 μ L of a 1 EU/mL solution of endotoxin in PFS is added
233 and the well contents are mixed (see example presented in **Table 4-2**).

234

234

235 **Table 4-2 Preparation of Endotoxin-spiked and Non-spiked Solutions for**
 236 **Determination of Test Substance Interference in the Incubation and**
 237 **ELISA Test Systems**

Sample Addition	Spiked	Non-spiked
	$\mu\text{L}/\text{well}^1$	
Pyrogen-free saline	180	200
Endotoxin spike solution ²	20	0
Test substance (neat and each serial dilution)	20	20
WB	20	20
Total ³	240	240

238 Abbreviations: WB = Whole blood

239 ¹ n=4 replicates each

240 ² Endotoxin concentration is 1 EU/mL in PFS.

241 ³ A total volume of 240 μL per well is used for the incubation.

242

243

244 The lowest dilution of the test substance that yields an endotoxin spike recovery of 50% to
 245 200% in the pyrogen test is determined. The optical density (OD) values of the endotoxin-
 246 spiked and non-spiked test substances are calibrated against the endotoxin calibration curve.
 247 The resulting EU value of the non-spiked test substance is subtracted from the corresponding
 248 EU value of the endotoxin-spiked test substance at each dilution. The % recovery for each
 249 sample dilution is then determined from the endotoxin spike solution concentration set to
 250 100%. For example, consider the following interference test results in **Table 4-3**:

251 **Table 4-3 Example of Interference Data Used to Determine Sample Dilution for**
 252 **Assay**

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

253

254 Based on these results, the dilution of the test substance used in the *in vitro* pyrogen test
 255 would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1 EU/mL EC).

256 4.2.2 Interference with ELISA

257 If the data obtained from the experiment in **Section 4.2.1** suggests the presence of
258 interference, then a subsequent experiment similar to that described in **Section 4.2.1** would
259 need to be performed to confirm that the test substance(s) does not directly interfere with the
260 ELISA. For this experiment, an ELISA would be performed in the absence of PBMCs.

261 **5.0 CONTROLS**

262 **5.1 Negative Control**

263 A negative control (e.g., PFS is added instead of the test sample) is included in each
264 experiment in order to detect nonspecific changes in the test system, as well as to provide a
265 baseline for the assay endpoints.

266 **5.2 Solvent Control**

267 Solvent controls are recommended to demonstrate that the solvent is not interfering with the
268 test system when solvents other than PFS are used to dissolve test substances.

269 **5.3 Positive Control**

270 An internationally standardized EC (e.g., WHO 94/580; USP G3E069; 0.5 EU/mL) is
271 included in each experiment to verify that an appropriate response is induced.

272 **5.4 Benchmark Controls**

273 Benchmark controls may be used to demonstrate that the test method is functioning properly,
274 or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals,
275 medical device eluates) of a specific class or a specific range of responses, or for evaluating
276 the relative pyrogenic potential of a test substance. Appropriate benchmark controls should
277 have the following properties:

- 278 • consistent and reliable source(s) for the chemicals (e.g., parenteral
279 pharmaceuticals, medical device eluates)
- 280 • structural and functional similarities to the class of substance being tested
- 281 • known physical/chemical characteristics

- 282 • supporting data on known effects in animal models
- 283 • known potency in the range of response

284 **5.5 Positive Product Control (PPC)**

285 The PPC is a test substance diluted to a level that does not interfere with the test method and
286 does not exceed the MVD. The PPC is obtained by spiking a test substance with a known EC
287 (e.g., 1 EU/mL) and demonstrating that 50% to 200% of the EC is recovered.

288 **5.6 Negative Product Control (NPC)**

289 The NPC is the test substance diluted to the MVD and then spiked with PFS. It is the
290 negative control for the PPC.

291 **6.0 EXPERIMENTAL DESIGN**

292 **6.1 Incubation with Test Samples and Measurement of IL-1 β Release**

293 6.1.1 Collection of Human Blood

294 WB is obtained from healthy human volunteers who have provided their consent according
295 to established institutional guidelines. Volunteers are expected not to have taken any drugs
296 and to have been free from illness for at least two weeks prior to donation. The criteria for
297 rejection of data from donors that are low responders or that are suspect due to veracity of
298 health information is addressed in **Section 8.0**.

299 Fresh WB is drawn by venipuncture using a multily needle set and collected in heparinized
300 tubes (15 IU/mL lithium heparin). All components of the blood collection system (i.e.,
301 syringes, tubes, connecting lines) must be sterile and pyrogen-free. Blood should be stored at
302 room temperature (RT) and must be used within 4 hr. Prior to use in the assay, the collection
303 tubes should be gently inverted 1 to 2 times. **Do Not Vortex**.

304 6.1.2 Incubation Plate

305 Test substances are prepared at a level of dilution that did not show interference with the test
306 system or for which it is known that interference does not occur. Each incubation plate can
307 accommodate an endotoxin standard curve, a NSC, and 14 test samples (see **Table 6-1**).

308

308

309 **Table 6-1 Overview of Incubation Plate Preparation in the WB/IL-1 β Test Method**

Number of Wells	Sample	PFS	EC	Test Sample	WB	Mix the samples; incubate overnight at 37 \pm 1 $^{\circ}$ C in a humidified atmosphere with 5% CO ₂ .	Mix the samples; immediately transfer to an ELISA plate ³ and run ELISA or store plate at -20 $^{\circ}$ C or -80 $^{\circ}$ C.
20 ¹	EC	200	20	0	20		
4	NSC	220	0	0	20		
56 ²	Test samples (1-14)	200	0	20	20		

310 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; PFS = Pyrogen-free saline; WB =
 311 Whole blood

312 ¹ Five EC concentrations (0.25, 0.50, 1.0, 2.5, and 5.0 EU/mL) in quadruplicate.

313 ² 14 test samples (n=4) per plate.

314 ³ An IL-1 β standard curve is prepared in Columns 11 and 12 on the ELISA plate. Therefore, 80 wells are
 315 available for test samples and controls on the incubation plate.

316

317

318 6.1.3 Incubation Assay for IL-1 β Release

319 Blood samples are prepared in a microtiter plate using a laminar flow hood. All consumables
 320 and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately
 321 with a permanent marker. An overview of the incubation plate preparation is shown in **Table**
 322 **6-1**. The incubation procedure is outlined below:

323 **Step 1.** Refer to the incubation plate template presented in **Table 6-2**.

324 **Step 2.** Using a pipetter, transfer 200 μ L of PFS into each well.

325 **Step 3.** Transfer 20 μ L of test sample into the appropriate wells as indicated in the
 326 template.

327 **Step 4.** Transfer 20 μ L of the EC (standard curve) and the NSC controls in
 328 quadruplicate into the appropriate wells according to the template.

329 **Step 5.** Transfer 20 μ L of WB into each well and mix by gently swirling the plate.

330 **Step 6.** Mix the contents of the wells thoroughly by gently pipetting up and down
 331 five times using a multichannel pipetter, changing the tips between each row to
 332 avoid cross-contamination.

333 **Step 7.** Place the covered plate in a tissue culture incubator for 10 to 24 hr at
334 37 \pm 1 $^{\circ}$ C in a humidified atmosphere containing 5% CO₂.

335 **Step 8.** Prior to transferring the test samples onto the ELISA plate, mix the
336 contents of the wells by pipetting up and down three times using a multichannel
337 pipetter, changing the tips between each row to avoid cross-contamination.

338 *Note: The aliquots may be tested immediately in the ELISA or stored at -20 $^{\circ}$ C or*
339 *-80 $^{\circ}$ C for testing at a later time. After transfer to the ELISA plate, freeze the*
340 *remaining aliquots at -20 $^{\circ}$ C or -80 $^{\circ}$ C for subsequent experiments, if necessary (see*
341 *Assay Acceptability and Decision Criteria in **Sections 8.0 and 9.0**).*

342 **Table 6-2 Incubation Plate - Sample and Control Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	Void ³	Void
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

343 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

344 ¹ EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.

345 ² TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

346 ³ Columns 11 and 12 are reserved for the IL-1 β standard curve on the ELISA plate (see **Table 6-3**).

347
348

349 **6.2 ELISA to Measure IL-1 β Release**

350 **6.2.1 IL-1 β Standard Curve**

351 An IL-1 β standard, supplied with the ELISA kit, is used. IL-1 β standards are typically
352 supplied in lyophilized form and should be reconstituted according to the manufacturer's
353 instructions. The stock solution should be diluted in PFS to the following concentrations: 0,

354 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL. Each well on the ELISA plate will receive
355 100 μ L of an IL-1 β blank or standard.

356 6.2.2 ELISA

357 The manufacturer's instructions provided with the ELISA kit should be followed and a
358 typical experimental design is outlined below. If the user prefers to prepare an in-house
359 ELISA, then appropriate modifications and validation of these changes would be necessary.
360 The ELISA should be carried out at RT and therefore all components must be at RT prior to
361 use. Do *not* thaw frozen specimens by heating them in a water bath. A sample ELISA plate
362 template is shown in **Table 6-3**, which includes a five-point EC standard curve, a NSC, an
363 eight-point IL-1 β standard curve (0 to 4000 pg/mL), and 14 test substances in quadruplicate.
364 The EC standard curve, the NSC, and the test sample supernatants are transferred directly
365 from the incubation plate. The IL-1 β standard curve is prepared as described in **Section**
366 **6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4**.

367 Immediately prior to the ELISA procedure, dilute or mix any assay components according to
368 the manufacturer's instructions.

369 **Step 1.** Add 100 μ L of enzyme-labeled detection antibody to each well.

370 **Step 2.** After pipetting up and down three times to mix the supernatant, transfer
371 100 μ L from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

372 **Step 3.** Add 100 μ L of each IL-1 β standard (0 to 4000 pg/mL) into the respective
373 wells on the ELISA plate.

374 **Step 4.** Cover the microtiter plate(s) with adhesive film and incubate for 90 min on
375 a microplate mixer at 350-400 rpm at 20 to 25°C.

376 **Step 5.** Decant and wash each well five to six times with 300 μ L Buffered Wash
377 Solution per well and then rinse three times with deionized water. Place the plates
378 upside down and tap to remove the wash solution.

379 **Step 6.** Add 200 μ L of TMB/Substrate Solution to each well and incubate at RT in
380 the dark for 10 to 15 min. If necessary, decrease the incubation time.

381 **Step 7.** Add 50 μ L of Stop Solution to each well.

382 **Step 8.** Tap the plate gently after the addition of Stop Solution to aid in mixing.

383 **Step 9.** Read the OD₄₅₀ within 15 min of adding the Stop Solution. Measurement

384 with a reference wavelength of 600-690 nm is recommended.

385 **Table 6-3 ELISA Plate - Sample and Control Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-1 β ³ 0	IL-1 β 0
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	IL-1 β 62.5	IL-1 β 62.5
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	IL-1 β 125	IL-1 β 125
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	IL-1 β 250	IL-1 β 250
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	IL-1 β 500	IL-1 β 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-1 β 1000	IL-1 β 1000
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-1 β 2000	IL-1 β 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-1 β 4000	IL-1 β 4000

386 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

387 ¹ EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.

388 ² TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

389 ³ IL-1 β values in columns 11 and 12 are in pg/mL.

390

391

392 **Table 6-4 Overview of ELISA Procedure**

Enzyme-labeled Antibody (μL)	Material transfer from Incubation Plate (μL)	Incubate 90 min on a plate mixer at 350-400 rpm.	TMB/Substrate Solution (μL)	Incubate 15 min at RT in the dark.	Stop Solution (μL)	Read optical density at 450 nm with a 600-690 nm wavelength reference filter.
100	100		200		50	

393 Abbreviations: RT = Room temperature

394

395

396 7.0 EVALUATION OF TEST RESULTS

397 7.1 OD Measurements

398 The OD of each well is obtained by reading the samples in a standard microplate
399 spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD₄₅₀) with
400 a 600 to 690 nm reference filter (recommended). OD₄₅₀ values are used to determine assay
401 acceptability and in the decision criteria for the detection of endotoxin in a test substance (see
402 **Sections 8.0** and **9.0**).

403 8.0 CRITERIA FOR AN ACCEPTABLE TEST

404 Obtain the PPC and the corresponding NPC by interference testing of a test substance in the
405 presence and absence of a fixed quantity of endotoxin (i.e., 1 EU/mL) in quadruplicate. An
406 EC (five-point standard curve) and a NSC should be included in each experiment. An IL-1 β
407 standard curve should be included in each ELISA as shown in the template presented in
408 **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- 409 • The quadratic function of the IL-1 β standard curve produces an $r^2 \geq 0.95$ and
410 the OD₄₅₀ of the blank control is below 0.15.
- 411 • The endotoxin standard curve produces OD₄₅₀ values that ascend in a
412 sigmoidal concentration response.

413 9.0 DATA INTERPRETATION/DECISION CRITERIA

414 9.1 Decision Criteria for Determination of Pyrogenicity

415 The *t*-test is used to compare the data of a test sample against the data of the EC (0.5 EU/mL)
416 that is performed in parallel. If this test results in a significant *p*-value (i.e., smaller than 1%),
417 then the sample is considered to be non-pyrogenic, and as pyrogenic if otherwise (Hoffmann
418 et al., 2005), as long as the assay acceptability criteria in **Section 8.0** has been met.

419 10.0 STUDY REPORT

420 The test report should include the following information:

421 *Test Substances and Control Substances*

- 422 • Name of test substance
- 423 • Purity and composition of the substance or preparation
- 424 • Physicochemical properties (e.g., physical state, water solubility)
- 425 • Treatment of the test/control substances prior to testing (e.g., vortexing,
- 426 sonication, warming, resuspension solvent)

427 *Justification of the In Vitro Test Method and Protocol Used*

428 *Test Method Integrity*

- 429 • The procedure used to ensure the integrity (i.e., accuracy and reliability) of the
- 430 test method over time
- 431 • If the test method employs proprietary components, documentation on the
- 432 procedure used to ensure their integrity from “lot-to-lot” and over time
- 433 • The procedures that the user may employ to verify the integrity of the
- 434 proprietary components

435 *Criteria for an Acceptable Test*

- 436 • Acceptable concurrent positive control ranges based on historical data
- 437 • Acceptable negative control data

438 *Test Conditions*

- 439 • Cell system used
- 440 • Calibration information for the spectrophotometer used to read the ELISA
- 441 • Details of test procedure
- 442 • Description of any modifications of the test procedure
- 443 • Reference to historical data of the model
- 444 • Description of evaluation criteria used

445 *Results*

- 446 • Tabulation of data from individual test samples

447 *Description of Other Effects Observed*

448 *Discussion of the Results*

449 *Conclusion*

450 *A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies*

451 • This statement should indicate all inspections made during the study and the
452 dates any results were reported to the Study Director. This statement should
453 also confirm that the final report reflects the raw data.

454 If GLP-compliant studies are performed, then additional reporting requirements provided in
455 the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be
456 followed.

457 **11.0 REFERENCES**

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484 **12.0 TERMINOLOGY AND FORMULA**

485 **12.1 Assay Sensitivity (λ)**

486 For an *in vitro* cell-based assay, the variable λ is defined as the lowest statistically significant
487 point on the standard endotoxin concentration-response curve and represents the relative
488 sensitivity of the test method for the detection of endotoxin (i.e., level of detection).

489 **12.2 Endotoxin Control (EC)**

490 The EC is incubated with WB and serves as the positive control for the experiment. The
491 results should be compared to historical values to insure that it provides a known level of
492 cytokine release relative to the NSC.

493 **12.3 Endotoxin Limit Concentration (ELC)**

494 The ELC is the maximum allowable concentration of endotoxin for a particular product and
495 is expressed in EU per volume (mL) or weight (mg). The ELC is defined by the FDA or
496 specified in the USP². It is calculated as the product of K/M, where:

497 K is the threshold pyrogen dose for parenteral use in rabbits or humans (5.0 EU/kg). At an
498 injection volume of 10 mL/kg, K is equal to 0.5 EU/mL.

499 M is the larger of the rabbit dose or the maximum human dose administered in one hour as
500 defined below and varies with test substance³.

501 **12.4 Maximum Valid Dilution (MVD)**

502 The MVD is the maximum dilution of a test substance that can be tolerated in a test system
503 without exceeding the ELC, if the test substance must be diluted as a result of assay
504 interference. Dilutions beyond the MVD would not be valid for endotoxin detection in the

² ELC values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, Pharmacopoeial guidelines, FDA publications).

³ Values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, Pharmacopoeial guidelines, FDA publications).

505 test system. Calculation of the MVD is dependent on whether or not the ELC for a test
506 substance is published. When the ELC is known, the MVD is:

507
$$\text{MVD} = (\text{ELC} \times \text{Product Potency [PP]})/\lambda$$

508 As an example, for "Cyclophosphamide Injection," the ELC is 0.17 EU/mg, PP is 20
509 mg/mL, and the assay sensitivity is 0.1 EU/mL. The calculated MVD would be 34. The test
510 substance can be diluted no more than 1:34 prior to testing.

511 If the ELC is not known, the MVD is:

512
$$\text{MVD} = \text{PP}/\text{Minimum Valid Concentration (MVC)}$$

513 where, $\text{MVC} = (\lambda \times \text{M})/\text{K}$

514 where, M is the maximum human dose

515 As an example, for "Cylophosphamide Injection," the PP is 20 mg/mL, M is 30
516 mg/kg, and assay sensitivity is 0.1 EU/mL. The calculated MVC is 0.6 mg/mL and the MVD
517 is 33.3. The test substance can be diluted no more than 1:33 in the assay prior to testing.

518 **12.5 Negative Product Control (NPC)**

519 The NPC is a test sample to which PFS is added. The NPC is the baseline for determination
520 of cytokine release relative to the endotoxin-spiked Positive Product Control (PPC).

521 **12.6 Negative Saline Control (NSC)**

522 The NSC is WB and incubated with PFS (used for dilution of test substance) and is used as
523 the blank.

524 **12.7 Non-intrathecal Threshold Pyrogen Dose (K)**

525 The value K represents the threshold pyrogen dose for parenteral products for rabbits and
526 humans. Based on experimental data, K is fixed at 5.0 EU/kg. For intrathecal products, K is
527 0.2 EU/kg.

528 **12.8 Positive Product Control (PPC)**

529 The PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an
530 amount of endotoxin equal to that which produces $\frac{1}{2}$ the maximal increase in OD from the

531 endotoxin standard curve) to insure that the test system is capable of endotoxin detection in
532 the product as diluted in the assay.

533 **12.9 Product Potency (PP)**

534 The concentration for a test substance is the PP typically expressed as $\mu\text{g/mL}$ or mg/mL .

535 **12.10 Rabbit Pyrogen Test Dose or Maximum Human Dose (M)**

536 The variable M represents the rabbit test dose or the maximum human dose in 1 hr. The
537 variable M is expressed in mg/kg and varies with the test substance. For
538 radiopharmaceuticals, M should be adjusted to account for product activity (radioactive
539 decay) at time administration. An average human standard weight of 70 kg is used for the
540 calculation. If a pediatric dose should be used and it is higher than the adult dose, then it
541 should be used in the formula.

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Appendix B5

Draft ICCVAM Recommended Protocol for Future Studies Using the Whole Blood (WB)/Interleukin-6 (IL-6) Test Method

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38 **Table 1 Comparison of Draft ICCVAM Recommended Test Method Protocol with the ECVAM SOP for the Whole**
 39 **Blood (WB)/Interleukin-6 (IL-6) Pyrogen Test Method**

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP
Test Substance	Test neat or at minimal dilution that produces no interference	Same as ICCVAM protocol
Incubation Plate (number of control or test groups at n=4 each)	NSC (1)	Same as ICCVAM protocol
	EC (5)	Same as ICCVAM protocol
	TS (14)	Same as ICCVAM protocol
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Same as ICCVAM protocol
Decision Criteria for Interference	0.5 x Median OD ₄₅₀ ¹ of 1 EU/mL EC <2x Median OD ₄₅₀ of 1 EU/mL EC	0.5 x Median OD ₄₅₀ of 0.5 EU/mL EC <2x Median OD ₄₅₀ of 0.5 EU/mL EC
Assay Acceptability Criteria	Mean OD ₄₅₀ of 0.5 EU/mL EC ≥1.6X Mean OD ₄₅₀ of NSC	Same as ICCVAM protocol
	Mean OD ₄₅₀ of PPC is 50% to 200% of 0.5 EU/mL EC	Same as ICCVAM protocol
	Mean OD ₄₅₀ of NSC ≤0.15	Same as ICCVAM protocol
	Quadratic function of IL-6 SC r ² ≥0.95	Same as ICCVAM protocol
	Not included	EC SC satisfies ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures Methodology; ICH Q2B, Nov 1996
	EC SC produces OD ₄₅₀ values that ascend in a sigmoidal concentration response	Same as ICCVAM protocol
	High responder blood donors (i.e., >200 pg/mL IL-6) may be excluded (need at least 3 donors)	High responder blood donors (i.e., >200 pg/mL IL-6) may be excluded (need at least 3 donors)
Not included	Wilcoxon rank-sum test used to show that at least 3 of 4 replicates at each increasing EC concentration are higher relative to the next lowest concentration	
Decision Criteria for Pyrogenicity	Mean OD ₄₅₀ of TS > Mean corrected OD ₄₅₀ of 0.5 EU/mL EC	EC SC data transformed to logit responses by in house program and the linear mean square calculated. TS pyrogen content is compared with the ELC ² using confidence limits. The preparation being examined must pass the test with blood from three separate donors
	Not included	Limit test is run to determine whether or not a

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP
		TS after correction and dilution contains < 0.5 EU/mL of endotoxin

40 Abbreviations: EC = Endotoxin control; ELC = Endotoxin Limit Concentration; MVD = Maximum Valid Dilution; NSC = Normal saline control; PPC =
 41 Positive Product Control; SC = Standard curve; TS = Test substance

42 ¹ Median or mean OD₄₅₀ values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

43 ² Where unknown, the ELC is calculated (e.g., Based on a rabbit sensitivity of 5 EU/kg, for a product injected at 10 mL/kg, the detection limit is 5 EU/10 mL/kg
 44 or 0.5 EU/mL/kg, or an ELC of 0.5 EU/mL).

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47 **1.0 PURPOSE AND APPLICABILITY**

48 The purpose of this protocol is to describe the procedures used to evaluate the presence of a
49 pyrogen (i.e., Gram-negative endotoxin) in a test substance. The presence of Gram-negative
50 endotoxin is detected by its ability to induce cytokine IL-6 release from monocytoid cells in
51 WB. The quantity of IL-6 released is obtained using an enzyme-linked immunosorbent assay
52 (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-6. Release of this
53 cytokine is measured by incubation of WB with test substances or controls (i.e., positive,
54 negative). The amount of pyrogen present is determined by comparing the values of
55 endotoxin equivalents produced by cells exposed to the test substance to those exposed to an
56 internationally-harmonized Reference Standard Endotoxin (RSE)¹ or an equivalent standard
57 expressed in Endotoxin Units (EU)/mL. Based on a rabbit threshold pyrogen dose of 0.5
58 EU/mL, which was established in a retrospective evaluation of rabbit pyrogen test (RPT)
59 data, a test substance is considered pyrogenic if it induces a level of IL-6 release equal to or
60 greater than 0.5 EU/mL.

61 The focus of this protocol is on the use of the WB/IL-6 test method, specifically for the
62 detection of Gram-negative endotoxin in parenteral pharmaceuticals. The relevance and
63 reliability for non-endotoxin pyrogens (e.g., lipoteichoic acid) has not been demonstrated in a
64 formal validation study.

65 **2.0 SAFETY AND OPERATING PRECAUTIONS**

66 All procedures for procurement of eligible blood donors and blood donations should follow
67 the regulations and procedures set forth by institutional guidelines for utilization of human
68 substances, which include but are not limited to blood, tissues, and tissue fluids. Standard
69 laboratory precautions are recommended including the use of laboratory coats, eye
70 protection, and gloves. If necessary, additional precautions required for specific study
71 substances or hazardous chemicals will be identified in the Material Safety Data Sheet
72 (MSDS).

¹ RSEs are internationally-harmonized reference standards (e.g., WHO *E. coli* Lipopolysaccharide [LPS] 94/580 [0113:H10:K-]; USP RSE Lot G3E069; FDA Lot EC-6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

73 The stop solution used in the ELISA kit is acidic and corrosive and should be handled with
74 the proper personal protective devices. If this reagent comes into contact with skin or eyes,
75 wash thoroughly with water. Seek medical attention, if necessary.

76 Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-
77 TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate
78 personal protection should be used to prevent bodily contact.

79 Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage,
80 antigenic response) and should be handled with care. Skin cuts should be covered and
81 appropriate personal protective devices should be worn. In case of contact with endotoxin,
82 immediately flush eyes or skin with water for at least 15 min. If inhaled, remove the affected
83 individual from the area and provide oxygen and/or artificial respiration as needed. Skin
84 absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

85 **3.0 MATERIALS, EQUIPMENT, AND SUPPLIES**

86 **3.1 Source of Cells**

87 Leukocytes from fresh WB are the source of cells for cytokine production in the WB/IL-6
88 test method (Hartung and Wendel, 1996; Pool et al., 1998; Schindler et al., 2006). WB is
89 obtained from healthy human volunteers who have provided their consent according to
90 established institutional guidelines. Volunteers are expected not to have taken any drugs
91 (e.g., prescription drugs, recreational drugs, herbal drugs) and to have been free from illness
92 for at least two weeks prior to donation.

93 **3.2 Equipment and Supplies**

94 For all steps in the protocol, excluding the ELISA procedure, the materials that will be in
95 close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be
96 sterile and free from detectable pyrogens.

97

97

98 3.2.1 Blood Incubation99 3.2.1.1 *Equipment*

- 100 • Centrifuge
- 101 • Hood; Bio-safety, laminar flow (recommended)
- 102 • Incubator; cell culture (37±1°C + 5% CO₂)
- 103 • Pipetter, multichannel (8- or 12-channel)
- 104 • Pipettors, single-channel adjustable (20 and 200 µL)
- 105 • Repeating pipetter
- 106 • Vortex mixer

107 3.2.1.2 *Consumables*

- 108 • Centrifuge tubes; nonpyrogenic, polypropylene (15 and 50 mL)
- 109 • Combitips; repeating pipetter (1.0 and 2.5 mL)
- 110 • Needle set; Sarstedt multify, pyrogen-free, 19 mm, 21 gauge for S-Monovette
- 111 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- 112 • Reaction tubes; polypropylene (1.5 mL)
- 113 • Reservoirs; fluid
- 114 • Tips; pipetter, sterile, pyrogen-free (20 and 200 µL)
- 115 • Tubes; Sarstedt S-Monovette, 7.5 mL, heparinized for blood collection

116 3.2.2 ELISA117 3.2.2.1 *Equipment*

- 118 • Microplate mixer
- 119 • Microplate reader (450 nm with an optional reference filter in the range of
- 120 540-590 nm)

121 • Microplate washer (optional)

122 • Multichannel pipetter

123 3.2.2.2 *Consumables*

124 • Container; storage, plastic

125 • Deionized water; nonsterile

126 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture

127 • Pyrogen-free water (PFW)

128 • Reservoirs; fluid

129 • Tips; pipetter, nonsterile

130 • Tubes; polypropylene (12 mL)

131 3.2.2.3 *ELISA Kit*

132 An ELISA that measures IL-6 release from WB is used. A variety of IL-6 ELISA kits are
133 commercially available and the IL-6 ELISA procedure outlined in this protocol is intended to
134 serve as an example for using an ELISA kit. If the user prefers to prepare an in-house
135 ELISA, then additional reagents would be required. The IL-6 ELISA should be calibrated
136 using an IL-6 international reference standard (e.g., WHO 89/548) prior to use. The IL-6
137 cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore this reagent
138 must be purchased separately. Results obtained using these products are subject to the assay
139 acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA kit
140 components may include the following:

141 • ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or
142 polyclonal

143 • Buffered wash solution

144 • Dilution buffer

145 • Enzyme-labeled detection antibody

146 • Human IL-6 reference standard

- 147 • Pyrogen-free saline (PFS)
- 148 • Stop solution
- 149 • TMB/substrate solution

150 **3.3 Chemicals**

- 151 • Endotoxin (e.g., WHO *E. coli* LPS 2nd International Standard 94/580; USP
- 152 RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

153 **3.4 Solutions**

154 ELISA solutions are listed in **Section 3.2**.

155 **4.0 ASSAY PREPARATION**

156 All test substances and endotoxin-spiked solutions should be stored at 4°C.

157 **4.1 Endotoxin Standard Curve**

158 An internationally harmonized RSE or equivalent is used to generate the endotoxin standard
159 curve. The use of any other *E. coli* LPS requires calibration against a RSE using the WB/IL-6
160 test method.

161 A standard endotoxin curve consisting of a Normal Saline Control (NSC) and five RSE
162 concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) are included in the incubation step
163 (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin
164 standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the
165 lyophilized content of the stock vial by following the instructions provided by the
166 manufacturer (e.g., for a vial containing 10,000 EU, 5 mL of PFW is added). To reconstitute
167 the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in
168 a bath sonicator for 5 min. The stock solution is stable for 14 days when stored at 2 to 8°C.
169 An endotoxin standard curve is prepared by making serial dilutions of the stock solution in
170 PFS as described in **Table 4-1**.

171

171

172 **Table 4-1 Preparation of Endotoxin Standard Curve**

Stock Endotoxin EU/mL	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration in Tube EU/mL
2000 ^{1,2}	20	1980	20 ³
20	100	900	2.0
2.0	500	500	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0	0	1000	0

173 Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

174 Each stock tube should be vortexed prior to its use to make the subsequent dilution.

175 ¹ A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.176 ² The stock solution of USP RSE may be stored in aliquots and kept at -20°C for up to 6 months. Do not store
177 the endotoxin at -80°C.178 ³ This concentration is not used in the assay.

179

180 **4.2 Test Substances**

181 Liquid test substances should be tested neat or, if interference is detected (see **Section 4.2.1**),
182 diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in
183 saline, dissolved in dimethylsulfoxide (DMSO) then diluted up to 0.5% (v/v) with PFS
184 provided that this concentration of DMSO does not interfere with the assay. The test
185 substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator
186 for 5 min.

187 **4.2.1 Interference Testing**

188 Interference testing must be carried out on any test sample for which no interference
189 information is available. The purpose of the interference test is to determine the lowest
190 dilution (i.e., highest concentration) of a test substance from which an endotoxin spike can be
191 detected (i.e., based on the decision criteria in **Section 4.2.1.2**). However, to ensure a valid
192 test, a test substance should not be diluted beyond its Maximum Valid Dilution (MVD).

193 For many marketed products, values for the MVD and the Endotoxin Limit Concentration
194 (ELC) are published in the U.S. Pharmacopeia, the European Pharmacopoeia, and/or Food
195 and Drug Administration (FDA) guidelines. However if one or both of these values are not

196 available, then calculation of the MVD is dependent on the ELC (see **Section 12.3**). If
197 unknown, the ELC can be approximated by dividing the maximum hourly dose of the
198 product by the hourly dose received per patient. For example, if a product were used at an
199 hourly dose of 100 mg per patient, then the ELC would be 350 EU/100 mg or 3.5 EU/mg.

200 4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

201 The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent international RSE (e.g., USP
202 G3E069, FDA EC-6) is recommended for preparation of the endotoxin control (EC). If a
203 different *E. coli* LPS is used and the potency relative to the RSE is not provided, then each
204 lot must be calibrated against the RSE in the WB/IL-6 test method. For interference testing,
205 an endotoxin standard curve (see **Section 4.1**) should be included on each plate.

206 4.2.1.2 *Spiking Test Substances with Endotoxin*

207 For interference testing, non-spiked and endotoxin-spiked test substances are prepared in
208 microplate wells (n=4 replicates) and an *in vitro* pyrogen test is performed. Either PFS or a
209 fixed concentration (a concentration selected from the middle of the EC standard curve) of
210 the RSE (i.e., 1 EU/mL) in PFS is added to the test substance in serial two-fold dilutions. An
211 illustrative example of endotoxin spiking dilutions is shown in **Table 4-2**. For non-spiked
212 solutions, 50 µL of PFS is added to a well followed by 50 µL of WB and mixed by inversion.
213 Then, 50 µL of the test substance (neat or at serial dilution) is added followed by 100 µL of
214 PFS and the well contents are mixed. Endotoxin-spiked solutions are prepared by adding 50
215 µL of PFS to each well followed by 50 µL of WB and mixed by inversion. Then, 50 µL of
216 the test substance (neat or at serial dilution), 50 µL of an endotoxin spike solution (1.0
217 EU/mL), and 50 µL of PFS are added and the well contents are mixed (see example
218 presented in **Table 4-2**).

219

219

220 **Table 4-2 Preparation of Endotoxin-spiked and Non-spiked Solutions for**
 221 **Determination of Test Substance Interference in the Incubation and**
 222 **ELISA Test Systems**

Sample Addition	Spiked	Unspiked
	μL/well ¹	
Pyrogen-free saline (total volume added)	100 ²	150 ²
Endotoxin spike solution ³	50	0
Test substance (neat and each serial dilution)	50	50
WB	50	50
Total ⁴	250	250

223 Abbreviations: WB = Whole blood

224 ¹ n=4 replicates each

225 ² 50 μL of WB and 50 μL of PFS are added to each well and mixed by inversion prior to the addition of the
 226 remaining components and volume of PFS.

227 ³ Endotoxin concentration is 1.0 EU/mL in PFS.

228 ⁴ A total volume of 250 μL per well is used for the incubation.

229

230

231

232 The lowest dilution of the test substance that yields an endotoxin spike recovery of 50% to
 233 200% in the pyrogen test is determined. The optical density (OD) values of the endotoxin-
 234 spiked and non-spiked test substances are calibrated against the endotoxin calibration curve.
 235 The resulting EU value of the non-spiked test substance is subtracted from the corresponding
 236 EU value of the endotoxin-spiked test substance at each dilution. The % recovery for each
 237 sample dilution is then determined from the endotoxin spike solution concentration set to
 238 100%. For example, consider the following interference test results in **Table 4-3**:

239 **Table 4-3 Example of Interference Data Used to Determine Sample Dilution for**
 240 **Assay**

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

241

242

243 Based on these results, the dilution of the test substance used in the *in vitro* pyrogen test
 244 would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1 EU/mL EC).

245 4.2.2 Interference with ELISA

246 If the data obtained from the experiment in **Section 4.2.1** suggests the presence of
247 interference, then a subsequent experiment similar to that described in **Section 4.2.1** would
248 need to be performed to confirm that the test substance(s) does not directly interfere with the
249 ELISA. For this experiment, an ELISA would be performed in the absence of PBMCs.

250 **5.0 CONTROLS**

251 **5.1 Negative Control**

252 A negative control (e.g., PFS) is included in each experiment in order to detect nonspecific
253 changes in the test system, as well as to provide a baseline for the assay endpoints.

254 **5.2 Solvent Control**

255 Solvent controls are recommended to demonstrate that the solvent is not interfering with the
256 test system when solvents other than PFS are used to dissolve test substances.

257 **5.3 Positive Control**

258 An internationally standardized EC (e.g., WHO 94/580; USP G3E069; 0.5 EU/mL) is
259 included in each experiment to verify that an appropriate response is induced.

260 **5.4 Benchmark Controls**

261 Benchmark controls may be used to demonstrate that the test method is functioning properly,
262 or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals,
263 medical device eluates) of a specific class or a specific range of responses, or for evaluating
264 the relative pyrogenic potential of a test substance. Appropriate benchmark controls should
265 have the following properties:

- 266 • consistent and reliable source(s) for the chemicals (e.g., parenteral
267 pharmaceuticals, medical device eluates)
- 268 • structural and functional similarities to the class of the substance being tested
- 269 • known physical/chemical characteristics
- 270 • supporting data on known effects in animal models

- 271 • known potency in the range of response

272 **5.5 Positive Product Control (PPC)**

273 The PPC is a test substance diluted to a level that does not interfere with the test method and
274 does not exceed the MVD. The PPC is obtained by spiking a test substance with a known EC
275 (e.g., 1 EU/mL) and demonstrating that 50% to 200% of the EC is recovered.

276 **5.6 Negative Product Control (NPC)**

277 The NPC is the test substance diluted to the MVD and then spiked with PFS. It is the
278 negative control for the PPC.

279 **6.0 EXPERIMENTAL DESIGN**

280 **6.1 Incubation with Test Samples and Measurement of IL-6 Release**

281 6.1.1 Collection of Human Blood

282 WB is obtained from healthy human volunteers who have provided their consent according
283 to established institutional guidelines. Volunteers are expected not to have taken any drugs
284 and to have been free from illness for at least two weeks prior to donation. The criteria for
285 rejection of data from donors that are low responders or that are suspect due to veracity of
286 health information is addressed in **Section 8.0**.

287 Fresh WB is drawn by venipuncture using a multify needle set and collected in heparinized
288 tubes (15 IU/mL lithium heparin). All components of the blood collection system (i.e.,
289 syringes, tubes, connecting lines) must be sterile and pyrogen-free. Blood should be stored at
290 room temperature (RT) and must be used within 4 hr. Prior to use in the assay, the collection
291 tubes should be gently inverted 1-2 times. **Do Not Vortex**.

292 6.1.2 Incubation Plate

293 Test substances are prepared at a level of dilution that did not show interference with the test
294 system or for which it is known that interference does not occur. Each incubation plate can
295 accommodate an endotoxin standard curve, a NSC, and 14 test substances (see Table **6-1**).

296 **Table 6.1 Overview of Incubation Plate Preparation in the WB/IL-6 Test Method**

Number of Wells	Sample	PFS	EC	Test Sample	WB	Mix the samples; incubate overnight at 37±1°C in a humidified atmosphere with 5% CO ₂ .	Mix the samples; immediately transfer to an ELISA plate ³ and run ELISA or store plate at -20°C or -80°C.
		µL					
20 ¹	EC	200	20	0	20		
4	NSC	220	0	0	20		
56 ²	Test samples (1-14)	200	0	20	20		

297 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; PFS = Pyrogen-free saline WB =
 298 Whole blood

299 ¹ Five EC concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) in quadruplicate.

300 ² 14 test samples (n=4 each) per plate.

301 ³ An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate. Therefore, 80 wells are
 302 available for test samples and controls on the incubation plate.

303

304 6.1.3 Incubation Assay for IL-6 Release

305 Blood samples are prepared in a microtiter plate using a laminar flow hood. All consumables
 306 and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately
 307 with a permanent marker. An overview of the incubation plate preparation is shown in **Table**
 308 **6-1**. The incubation procedure is outlined below:

- 309 • **Step 1.** Refer to the incubation plate template presented in **Table 6-2**.
- 310 • **Step 2.** Using a pipetter, transfer 150 µL of PFS into each well.
- 311 • **Step 3.** Transfer 50 µL of test sample into the appropriate wells as indicated in
 312 the template.
- 313 • **Step 4.** Transfer 50 µL of the EC (standard curve) and the NSC controls in
 314 quadruplicate into the appropriate wells according to the template.
- 315 • **Step 5.** Transfer 50 µL of WB into each well and mix by gently swirling the
 316 plate.
- 317 • **Step 6.** Mix the contents of the wells thoroughly by gently pipetting up and
 318 down several times using a multichannel pipetter, changing the tips between
 319 each row in order to avoid cross-contamination.

320 • **Step 7.** Place the covered plate in a tissue culture incubator for 10 to 24 hr at
321 37±1°C in a humidified atmosphere containing 5% CO₂.

322 • **Step 8.** Prior to transferring the test samples onto the ELISA plate, mix the
323 contents of the wells by pipetting up and down using a multichannel pipetter,
324 changing the tips between each row in order to avoid cross-contamination.

325 *Note: The aliquots may be tested immediately in the ELISA or stored at -20°C or*
326 *-80°C for testing at a later time. After transfer to the ELISA plate, freeze the*
327 *remaining aliquots at -20°C or -80°C for subsequent experiments, if necessary (see*
328 *Assay Acceptability and Decision Criteria in **Sections 8.0 and 9.0**).*

329

329

330 **Table 6-2 Incubation Plate - Sample and Control Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	Void ³	Void
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

331 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

332 ¹ EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.333 ² TS number (e.g., TS 1) represents an arbitrary sequence for individual test substances.334 ³ Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).

335

336

337 **6.2 ELISA to Measure IL-6 Release**338 **6.2.1 IL-6 Standard Curve**339 An IL-6 standard, supplied with the ELISA kit, is used. IL-6 standards are typically supplied
340 in lyophilized form and should be reconstituted according to the manufacturer's instructions.

341 The stock solution should be diluted in PFS to the following concentrations: 0, 62.5, 125,

342 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 µL. Each well on the

343 ELISA plate will receive 100 µL of an IL-6 blank or standard.

344 **6.2.2 ELISA**345 The manufacturer's instructions provided with the ELISA kit should be followed and a
346 typical experimental design is outlined below. If the user prefers to prepare an in-house

347 ELISA, then appropriate modifications and validation of these changes would be necessary.

348 The ELISA should be carried out at RT and therefore all components must be at RT prior to

349 use. Do *not* thaw frozen specimens by heating them in a water bath. A suggested ELISA350 plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, a NSC,

351 an eight-point IL-6 standard curve (0 to 4000 pg/mL), and 14 test substances in
352 quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are
353 transferred directly from the incubation plate. The IL-6 standard curve is prepared as
354 described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-**
355 **4**.

356 Immediately prior to the ELISA procedure, dilute or mix any assay components according to
357 the manufacturer's instructions.

358 **Step 1.** After pipetting up and down three times to mix the supernatant, transfer 50
359 μL from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

360 **Step 2.** Add 50 μL of each IL-6 standard (0 to 4000 pg/mL) into the respective
361 wells on the ELISA plate.

362 **Step 3.** Add 200 μL of the enzyme-labeled detection antibody (neat as supplied, or
363 diluted, if necessary) to each of the wells.

364 **Step 4.** Cover the microtiter plate(s) with adhesive film and incubate for 2 to 3 hr
365 at 20 to 25°C.

366 **Step 5.** Decant and wash each well five to six times with 300 mL Buffered Wash
367 Solution and then rinse three times with deionized water. Place the plates upside
368 down and tap to remove water.

369 **Step 6.** Add 200 μL of TMB/Substrate Solution to each well and incubate at RT in
370 the dark for 15 min. If necessary, decrease the incubation time.

371 **Step 7.** Add 50 μL of Stop Solution to each well.

372 **Step 8.** Tap the plate gently after the addition of Stop Solution to aid in mixing.

373 **Step 9.** Read the OD_{450} within 15 min of adding the Stop Solution. Measurement
374 with a reference wavelength of 540 to 590 nm is recommended.

375

375 **Table 6-3 ELISA Plate - Sample and Control Template**

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC ¹ 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6 ³ 0	IL-6 0
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	IL-6 500	IL-6 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-6 1000	IL-6 1000
G	TS1 ²	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-6 2000	IL-6 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-6 4000	IL-6 4000

376 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

377 ¹ EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.378 ² TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.379 ³ IL-6 values in columns 11 and 12 are in pg/mL.

380

381

382 **Table 6-4 Overview of ELISA Procedure**

Material transfer from Incubation Plate (µL)	Enzyme-labeled Antibody (µL)	Cover the Incubation Plate and incubate for 2 to 3 hr at 20 to 25°C.	TMB/Substrate Solution (µL)	Incubate 15 min at RT in the dark.	Stop Solution (µL)	Read optical density at 450 nm with a 540-590 nm wavelength reference filter.
50	200		200		50	

383 Abbreviations: RT = Room Temperature

384

385

386 **7.0 EVALUATION OF TEST METHODS**387 **7.1 OD Measurements**

388 The OD of each well is obtained by reading the samples in a standard microplate

389 spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD₄₅₀) with

390 a 540 to 590 nm reference filter (recommended). OD₄₅₀ values are used to determine assay
391 acceptability and in the decision criteria for detection of endotoxin in a test sample (see
392 **Sections 8.0** and **9.0**).

393 **8.0 CRITERIA FOR AN ACCEPTABLE TEST**

394 Obtain the PPC and the corresponding NPC by interference testing of a test substance in the
395 presence and absence of a fixed quantity of endotoxin (i.e., 1 EU/mL) in quadruplicate. An
396 EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6
397 standard curve should be included in each ELISA plate as shown in the template presented in
398 **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- 399 • The quadratic function of the IL-6 standard curve produces an $r^2 \geq 0.95$ and the
400 OD₄₅₀ of the blank control is below 0.15.
- 401 • The endotoxin standard curve produces OD₄₅₀ values that ascend in a
402 sigmoidal concentration response.

403 Blood donors are considered to be high responders if their concentration of IL-6 is greater
404 than 200 pg/mL. High responders should be excluded from analysis. From a set of four
405 donors, a maximum of one donor may be excluded from the test. Furthermore, the
406 preparation being examined is required to pass the test with blood donations from at least
407 three different donors.

408 **9.0 DATA INTERPRETATION/DECEISION CRITERIA**

409 **9.1 Decision Criteria for Determination of Pyrogenicity**

410 The validity of the endotoxin standard curve should be calculated using a four-parameter
411 logistic model. If necessary to satisfy the model, endotoxin concentrations may be modified.

412 Calculate the mean OD₄₅₀ values of all of the replicates in each experimental group. Calibrate
413 the mean OD₄₅₀ value for each test substance using the endotoxin standard curve and
414 document the estimated endotoxin concentration. Multiply the estimated endotoxin
415 concentration by the dilution factor, if necessary. This value represents the pyrogenicity of
416 the sample in terms of endotoxin equivalents for that particular donor. The *t*-test is used to
417 compare the data of a test sample against the data of the EC (0.5 EU/mL) that is performed in

418 parallel. If this test results in a significant p -value (i.e., smaller than 1%), then the sample is
419 considered to be non-pyrogenic, and as pyrogenic if otherwise (Hoffmann et al., 2005), as
420 long as the assay acceptability criteria in **Section 8.0** has been met.

421 9.1.1 Decision Level 1

- 422 • If all donors show a negative reaction, then the product passes.
- 423 • If two or more donors show a positive reaction, then the product fails.
- 424 • If one donor shows a positive reaction, then an additional test with four donors
425 has to be performed (go to Decision Level 2).

426 9.1.2 Decision Level 2

- 427 • If out of 6 to 8 donors, only one donor shows a positive reaction, then the
428 product passes.
- 429 • In any other case, the product fails.

430 **10.0 STUDY REPORT**

431 The test report should include the following information:

432 *Test Substances and Control Substances*

- 433 • Name of test substance
- 434 • Purity and composition of the substance or preparation
- 435 • Physicochemical properties (e.g., physical state, water solubility)
- 436 • Treatment of the test/control substances prior to testing (e.g., vortexing,
437 sonication, warming, resuspension solvent)

438 *Justification of the Test Method and the Protocol Used*

439 *Test Method Integrity*

- 440 • The procedure used to ensure the integrity (i.e., accuracy and reliability) of the
441 test method over time
- 442 • If the test method employs proprietary components, documentation on the
443 procedure used to ensure their integrity from “lot-to-lot” and over time

- 444 • The procedures that the user may employ to verify the integrity of the
445 proprietary components

446 *Criteria for an Acceptable Test*

- 447 • Acceptable concurrent positive control ranges based on historical data
448 • Acceptable negative control data

449 *Test Conditions*

- 450 • Cell system used
451 • Calibration information for the spectrophotometer used to read the ELISA
452 • Details of test procedure used
453 • Description of any modification to the test procedure
454 • Reference to historical data of the model
455 • Description of the evaluation criteria used

456 *Results*

- 457 • Tabulation of data from individual test samples

458 *Description of Other Effects Observed*

459 *Discussion of the Results*

460 *Conclusion*

461 *A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies*

- 462 • This statement should indicate all inspections made during the study and the
463 dates any results were reported to the Study Director. This statement should
464 also confirm that the final report reflects the raw data.

465 If GLP-compliant studies are performed, then additional reporting requirements provided in
466 the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be
467 followed.

468

468 **11.0 REFERENCES**

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491

492

492 12.0 TERMINOLOGY AND FORMULA

493 12.1 Assay Sensitivity (λ)

494 For an *in vitro* cell-based assay, the variable λ is defined as the lowest statistically significant
495 point on the standard endotoxin concentration-response curve and represents the relative
496 sensitivity of the test method for the detection of endotoxin (i.e., level of detection).

497 12.2 Endotoxin Control

498 The EC is incubated with WB and serves as the positive control for the experiment. The
499 results should be compared to historical values to insure that it provides a known level of
500 cytokine release relative to the NSC.

501 12.3 Endotoxin Limit Concentration (ELC)

502 The ELC is the maximum allowable concentration of endotoxin for a particular product and
503 is expressed in EU per volume (mL) or weight (mg). The ELC is defined by the FDA or
504 specified in the USP². It is calculated as the product of K/M, where:

505 K is the threshold pyrogen dose for parenteral use in rabbits or humans (5.0 EU/kg). At an
506 injection volume of 10 mL/kg, K is equal to 0.5 EU/mL.

507 M is the larger of the rabbit dose or maximum human dose administered in one hour as
508 defined below and varies with test substance³

509 12.4 Maximum Valid Dilution (MVD)

510 The MVD is the maximum dilution of a test substance that can be tolerated in a test system
511 without exceeding the ELC, if the test substance must be diluted as a result of assay
512 interference. Dilutions beyond the MVD would not be valid for endotoxin detection in the

² ELC values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, Pharmacopoeial guidelines, FDA publications).

³ Values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, Pharmacopoeial guidelines, FDA publications).

513 test system. Calculation of the MVD is dependent on whether or not the ELC for a test
514 substance is published. When the ELC is known, the MVD is:

515
$$\text{MVD} = (\text{ELC} \times \text{Product Potency [PP]})/\lambda$$

516 As an example, for "Cyclophosphamide Injection," the ELC is 0.17 EU/mg, PP is 20
517 mg/mL, and the assay sensitivity is 0.1 EU/mL. The calculated MVD would be 34. The test
518 substance can be diluted no more than 1:34 prior to testing.

519 If the ELC is not known, the MVD is:

520
$$\text{MVD} = \text{PP}/\text{Minimum Valid Concentration (MVC)}$$

521 where, $\text{MVC} = (\lambda \times \text{M})/\text{K}$

522 where, M is the maximum human dose

523 As an example, for "Cylophosphamide Injection," the PP is 20 mg/mL, M is 30
524 mg/kg, and assay sensitivity is 0.1 EU/mL. The calculated MVC is 0.6 mg/mL and the MVD
525 is 33.3. The test substance can be diluted no more than 1:33 prior to testing.

526 **12.5 Negative Product Control (PPC)**

527 The NPC is a test sample to which PFS is added. The NPC is the baseline for determination
528 of cytokine release relative to the endotoxin-spiked PPC.

529 **12.6 Negative Saline Control (NSC)**

530 The NSC is WB incubated with PFS (used for dilution of test substance) and is used as the
531 blank.

532 **12.7 Non-intrathecal Threshold Pyrogen Dose (K)**

533 The value K represents the threshold pyrogen dose for parenteral products for rabbits and
534 humans. Based on experimental data, K is fixed at 5.0 EU/kg. For intrathecal products, K is
535 0.2 EU/kg.

536 **12.8 Positive Product Control (PPC)**

537 The PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an
538 amount of endotoxin equal to that which produces $\frac{1}{2}$ the maximal increase in OD from the

539 endotoxin standard curve) to insure that the test system is capable of endotoxin detection in
540 the product as diluted in the assay.

541 **12.9 Product Potency (PP)**

542 The concentration for a test substance is the PP typically expressed as $\mu\text{g/mL}$ or mg/mL .

543 **12.10 Rabbit Pyrogen Test Dose of Maximum Human Dose (M)**

544 The variable M represents the rabbit test dose or the maximum human dose in 1 hr. The
545 variable M is expressed in mg/kg and varies with the test substance. For
546 radiopharmaceuticals, M should be adjusted to account for product activity (radioactive
547 decay) at time administration. An average human standard weight of 70 kg is used for the
548 calculation. If a pediatric dose should be used and it is higher than the adult dose, then it
549 should be used in the formula.

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