新規試験法提案書

皮膚刺激性試験代替法 LabCyte EPI-MODEL 24

平成25年11月

国立医薬品食品衛生研究所

新規試験法提案書

平成 25 年 11 月 20 日

No.　2013-01

皮膚刺激性試験代替法 LabCyte EPI-MODEL24 に関する提案

平成 25 年 4 月 19 日に東京、国立医薬品食品衛生研究所にて開催された新規試験法評価会議（通称：JaCVAM 評価会議）において以下の提案がなされた。

提案内容：皮膚刺激性試験代替法 LabCyte EPI-MODEL24 は倫理的に優れた試験法であり、適切な利用条件下で適用するならば、化学物質の皮膚に対する 4 時間適用の一次刺激性を評価することが可能である。

この提案書は、European Center for the Validation of Alternative Methods(ECVAM)による第三者評価資料およびOECD Test Guideline（TG）439の成立時により準備された資料をもとに、皮膚刺激性試験代替法評価委員会によりまとめられた文書を用いてJaCVAM評価会議が評価および検討した結果、その有用性が確認されたことから作成された。

以上の理由により、行政当局の安全性評価方法として「皮膚刺激性試験代替法LabCyte EPI-MODEL24」の使用を提案するものである。

吉田武美

JaCVAM 評価会議　議長

西川秋佳

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

吉田武美　（日本毒性学会）：座長
浅野哲秀　（日本環境変異原学会）
五十嵐良明　（国立医薬品食品衛生研究所　生活衛生化学部）
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黒澤　努　（日本動物実験代替法学会）
杉山真理子　（日本化粧品工業連合会）
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牧　栄二　（日本免疫毒性学会）
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横関博雄　（日本皮膚アレルギー・接触皮膚炎学会）
吉田　緑　（国立医薬品食品衛生研究所　安全性生物試験研究センター　病理部）
吉村　功　（座長推薦）
渡部一人　（日本製薬工業協会）

任期：平成 24 年 4 月 1 日～平成 26 年 3 月 31 日
*：平成 25 年 4 月 1 日～平成 26 年 3 月 31 日

JaCVAM 運営委員会

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　究室）
本間正充　（国立医薬品食品衛生研究所　安全性生物試験研究センター　変異遺伝部）
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小島　肇　（国立医薬品食品衛生研究所　安全性生物試験研究センター　薬理部
　新規試験表評価室）：事務局

*：平成 25 年 8 月 1 日より

JaCVAM statement
on *in vitro* assays using the reconstructed human epidermis models, LabCyte EPI-MODEL24
for skin irritation testing

At the meeting concerning the above method, held on 19 April 2013 at the National Institute of Health Sciences (NIHS), Tokyo, Japan, the members of the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

The *in vitro* assay using the reconstructed human epidermis models such as LabCyte EPI-MODEL24 is considered to have sufficient accuracy and reliability for prediction of skin irritating test substances applied for 4 hours under proper conditions.

Following the review of the results of the ECVAM(European Center for the Validation of Alternative Methods) statements and OECD (Organisation for Economic Co-operation and Development) Test Guideline revised No. 439, it is concluded that two *in vitro* assays using the reconstructed human epidermis models such as EpiDerm and SkinEthics for skin irritation testing are clearly beneficial.

The JaCVAM Regulatory Acceptance Board has been regularly kept informed of the progress of the study, and this endorsement is based on an assessment of various documents, including, in particular, the evaluation report prepared by the JaCVAM ad hoc peer review panel for skin irritation testing.

Takemi Yoshida
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

20 November, 2013

The JaCVAM Regulatory Acceptance Board was established by the JaCVAM Steering Committee, and is composed of nominees from the industry and academia.

This statement was endorsed by the following members of the JaCVAM Regulatory Acceptance Board:

Mr. Takemi Yoshida (Japanese Society of Toxicology): Chairperson
Mr. Norihide Asano (Japanese Environmental Mutagen Society)
Mr. Tsutomu Ichiki (Japan Chemical Industry Association)*
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Tsutomu Miki Kurosawa (Japanese Society for Animal Experimentation)
Mr. Eiji Maki (Japanese Society of Immunotoxicology)
Mr. Mitsuteru Masuda (nominee by Chairperson)
Mr. Akiyoshi Nishikawa (NIHS)
Mr. Yasuo Ohno (nominee by Chairperson)*
Mr. Hiroshi Onodera (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Ms. Tomoko Tanita (Pharmaceuticals and Medical Devices Agency)*
Mr. Takashi Yamada (National Institute of Technology and Evaluation)*
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)
Ms. Midori Yoshida (NIHS)
Mr. Isao Yoshimura (nominee by Chairperson)
Mr. Kazuto Watanabe (Japan Pharmaceutical Manufacturers Association)

Term: From 1st April 2012 to 31st March 2014
*: From 1st April 2013 to 31st March 2014

This statement was endorsed by the following members of the JaCVAM steering Committee after receiving the report from JaCVAM Regulatory Acceptance Board:

Mr. Akiyoshi Nishikawa (BSRC, NIHS): Chairperson
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)
Mr. Jun Kanno (Division of Cellular and Molecular Toxicology, BSRC, NIHS)
Mr. Toru Kawanishi (NIHS)
Mr. Kenji Kuramochi (Ministry of Health, Labour and Welfare)*
Mr. Toshinari Mitsuoka (Ministry of Health, Labour and Welfare)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Mr. Kazuyuki Saito (Pharmaceutical & Medical Devices Agency)
Mr. Masahiro Sasaki (Ministry of Health, Labour and Welfare)
Ms. Yuko Sekino (Division of Pharmacology, BSRC, NIHS)
Mr. Atsuya Takagi (Animal Management Section of the Division of Cellular and Molecular Toxicology, BSRC, NIHS)
Mr. Junji Yamamoto (Ministry of Health, Labour and Welfare)*
Mr. Hajime Kojima (Section for the Evaluation of Novel Methods, Division of Pharmacology, BSRC, NIHS): Secretary

* Arrival at post day: 1st August 2013

皮膚刺激性試験代替法 LabCyte EPI-MODEL24

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LabCyte EPI-MODEL24 を用いた皮膚刺激性試験代替法の評価会議報告書

JaCVAM 評価会議

平成 25 年 4 月 19 日

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JaCVAM 評価会議

吉田武美（日本毒性学会）：座長
浅野哲秀（日本環境変異原学会）
五十嵐良明（国立医薬品食品衛生研究所　生活衛生化学部）
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小笠原弘道（独立行政法人　医薬品医療機器総合機構）
小野寺博志（独立行政法人　医薬品医療機器総合機構）
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吉村　功（座長推薦）
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任期：平成 24 年 4 月 1 日～平成 26 年 3 月 31 日

以上

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本報告での評価対象は、LabCyte EPI-MODEL24 を用いた皮膚刺激性試験（以下、当該試験法と略す）である。当該試験法は、株式会社ジャパン・ティッシュ・エンジニアリング（以下、JTC）によって開発された日本製の培養表皮モデルを用いたものである。培養表皮モデルを用いる皮膚刺激性試験は、被験物質が表皮モデルに与える損傷を細胞生存率を測定することで、代替的に評価するもので、すでに OECD Testing Guideline（TG）439 として認証されている [1)]。当該試験法も、OECD TG439 と同様に、ECVAM で改変された UN GHS CLP 規制に基づいて刺激性（Category2）と非刺激性（Category3 および No Category)を識別することを目的としている [1)]。今回、皮膚刺激性試験代替法の第三者評価委員会の報告 [2)]を受け、以下の 10 項目について評価したので報告する。

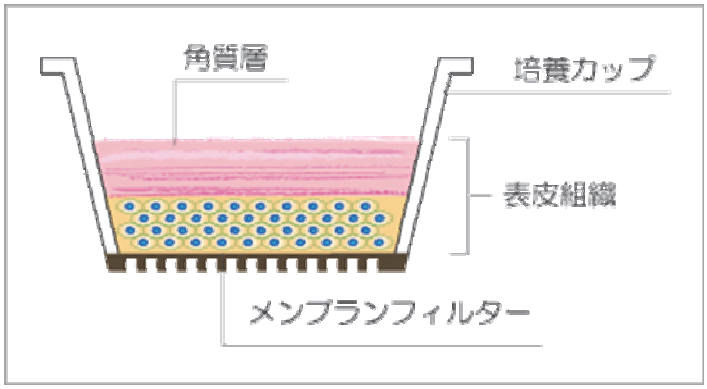
1.　当該試験法は、どのような従来試験法を代替するものか。または、どのような毒性を評価あるいは予測するものか。

当該試験法は、急性皮膚刺激性試験（OECD TG 404）の代替法である。OECD TG 404 は、ウサギの皮膚に被験物質を 4 時間適用することにより皮膚刺激性の評価を行うことを目的としている。
皮膚刺激性試験として、培養表皮モデルを用いた代替法 OECD TG439(2010)は、EpiSkinTM、EpiDermTMSIT(EPI-200)および SkinEthicTM(RHE)としてすでに認証を受けていた [1)]が、類似の培養表皮モデルとして、当該試験法も 2013 年に追認された [3)]。

2. 当該試験法と従来試験法の間にどのような科学的なつながりがあるか？

OECD TG404 では、皮膚表面に直接適用された被験物質によって引き起こされる可逆的な皮膚炎症反応を、肉眼的に紅斑および浮腫として検出し、皮膚刺激性としてとらえている。

LabCyte EPI-MODEL24 は、メンブランフィルター上に培養した角質層を含む再構築ヒト表皮モデルである。当該試験法は、この表皮モデルの上に被験物質を適用し、角質層を拡散・透過した被験物質が、角質層下の表皮細胞に与えた損傷を表皮細胞の生存率として皮膚刺激性を評価する方法である。さらに、当該試験法は OECD TG439 に性能標準 [4)]として掲載された化学物質リストをもとに、me-too（小規模）バリデーションを行い、すでに収載済みの他の表皮モデルと同等の皮膚刺激性検出能が確認されたため、科学的に妥当であるとして OECD TG 439 に追記された [3)]。



3. 当該試験法とそのデータは、透明で独立な科学的評価を受けているか。

日本動物実験代替法学会により、OECD TG 439 に性能標準[4]として掲載された化学物質リストをもとにバリデーションが行われ、さらに性能標準の変更により追加バリデーションが実施された[5]。この結果をまとめた報告書は、Independent International Scientific Peer Review Panel により評価された。

その後、Peer Review Panel の指摘[6,7]に対し、改訂プロトコル[8]を基にバリデーションが実施され、OECD 皮膚刺激専門家グループによる確認を経て OECD TG439 に追記された[3]。

以上の経緯のように、透明で独立な科学的評価を受けている。

4. 当該試験法は、従来試験法の代替法として、どのような物質又は製品を評価することを目的としているか。

当該試験法は、一般化学物質の皮膚刺激性を OECD TG439 の分類にしたがって、評価することを目的としている。

5. 当該試験法は、ハザード評価あるいはリスク評価のどちらに有用であるか。

当該試験法は、化学物質の皮膚刺激性について、UN GHS CLP 規制の分類にしたがって、刺激性と非刺激性を識別できる。

したがって、当該試験法は曝露された化学物質のハザードを評価するのに有用であるが、リスク評価への有用性は確認されていない。

6. 当該試験法は、目的とする物質又は製品の毒性を評価できるか。その場合、当該試験法の適用条件が明確になっているか。

当該試験法は、UN GHS CLP 規制に基づく分類を目的としているため、4 で示した物質を評価することは可能である。

当該試験法の適用条件は明確になっている。 MTT 還元法により細胞生存率を求めて評価に用いることから、着色による影響や還元物質による影響を受ける。また、開放適用により評価を行うため、エアロゾルやガスは適用外となる。

７．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あるか。

改訂プロトコル[9]にしたがって実施される限り、頑健である。

8. 当該試験法の技術習得は、適切な訓練と経験を経ている担当者にとって容易なものであるか。試験法の実施に特殊な設備が必要か。

当該試験法に必要な設備は、培養設備およびフォルマザンの生成量を測定するためのマイクロプレートリーダーのみであり、特殊な設備は不要である。

LabCyte EPI-MODEL24 は JTC から購入可能であるため、培養に関する適切な訓練と経験を経ている担当者であれば技術習得が容易である。

9. 当該試験法は、従来試験法と比べて時間的経費的に優れているか。

当該試験法にかかる時間は、5 日間である。試験期間は OECD TG404 と同程度であるが、馴化期間の必要な動物実験より優れている。

当該試験法では、動物実験に伴う経費が削減でき、経費的に優れている(1 キット/24 well： 5.4 万円）。

10. 当該試験法は、動物福祉の観点及び科学的見地から、目的とする物質又は製品の毒性を評価する代替法として、行政上利用することは可能か。

当該試験法は、動物を用いずに皮膚刺激性を評価できるので、動物福祉の観点から有用である。また、当該試験法は化学物質の 4 時間適用による皮膚刺激性を評価する方法である。その範囲（薬事法、農薬取締法、労働安全衛生法）において行政的な利用は可能であるが、我が国の医薬部外品、化粧品に必要とされる 24 時間適用による皮膚刺激性への応用の可能性については評価されていない。

4 時間適用にて認められる皮膚刺激性は、24 時間適用した場合、増強することが知られている[10]。したがって、当該試験法で刺激性なし（GHS 刺激性区分にて無刺激性）と評価されても、24 時間適用では刺激性ありと評価される場合がある。しかしながら、4 時間適用で検出可能な強い皮膚刺激性（GHS 刺激性区分にて刺激性）を評価可能であることから、スクリーニング法として利用できる可能性がある。

参考文献

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以上

LabCyte EPI-MODEL24 皮膚刺激性試験代替法の評価報告書

平成 24 年 10 月 31 日

皮膚刺激性試験評価委員会
委員名：
赤松浩彦（委員長：藤田保健衛生大学医学部）
寒水孝司（京都大学）

用語集

Accuracy：正確度
Concordance: 一致率
European Centre for Validation of Alternative Methods（ECVAM）：欧州代替法検証センター
False negative rate：偽陰性率
False positive rate：偽陽性率
Globally Harmonized System of Classification and Labelling of Chemicals (GHS): 化学品の分類および表示に関する世界調和システム
Independent International Scientific Peer Review Panel：第三者専門技術評価国際委員会
Inter-laboratory validation study:施設間バリデーション
Intra-laboratory validation study:施設内バリデーション
Japanese Centre for Validation of Alternative Methods（JaCVAM）：日本動物実験代替法評価センター
Performance standard：性能標準
Reliability：信頼性
Organisation for Economic Co-operation and Development（OECD）：経済協力開発機構
Test Guideline (TG):テストガイドライン
Validation study：バリデーション
Validation study management team：バリデーション運営委員会

1. 本試験法の科学的妥当性と規制試験法としての妥当性

LabCyte　EPI-MODEL24 を用いた皮膚刺激性試験代替法は、株式会社ジャパン・ティシュ・エンジニアリング（J-TEC）によって開発された日本製の培養表皮モデルである。本モデルと類似した培養表皮モデルのバリデーションが ECVAM（European　Center　for the Validation of Alternative Methods）によりバリデートされ、EU で認証された [1)]。これを受け、日本でも日本動物実験代替法学会にてプロトコル確定のためのプレバリデーション(phase Ⅰ)を経て、ECVAM で作成された GHS 分類 [1)]による皮膚刺激性試験代替法としての performance　standard（性能標準）[2)]に掲載された化学物質リストをもとに、me-too（小規模）バリデーション(phase Ⅱ)が実施された。バリデーションの実施中、ECVAM 性能標準のリストが改訂されたこともあり、追加バリデーション(phaseⅢ)を実施した [3)]。これらをまとめた報告書が Independent International Scientific Peer Review Panel（Peer Review）により評価され、その結果として、1-bromohexane の偽陰性を改善する指示があった [4)]。プロトコルの改良が J-TEC により実施され [5)]、改訂プロトコルを用い、新たに 3 施設の協力による JaCVAM（Japanese Centre for the Validation of Alternative Methods）によるバリデーション(phaseⅣ)が実施された。さらに、phaseⅣで明らかになったばらつきの大きさを改善するため、プロトコルが統一され、それを用いた追加バリデーション(phase Ⅴ)が実施されている。

本評価書は、phaseⅣからⅤにいたるバリデーション結果をもとに、LabCyte　EPI-MODEL24 を用いた皮膚刺激性試験の評価を行ったものである。なお、これらの結果は、OECD 皮膚刺激性専門家グループにおいても確認され、OECD　培養表皮モデルを用いた in vitro 皮膚刺激性試験法として、LabCyte　EPI-MODEL24 が OECD test guideline （TG439）に追記されることになった [6)]。

図 1 に、バリデーション研究過程を示すフローチャートを示す。

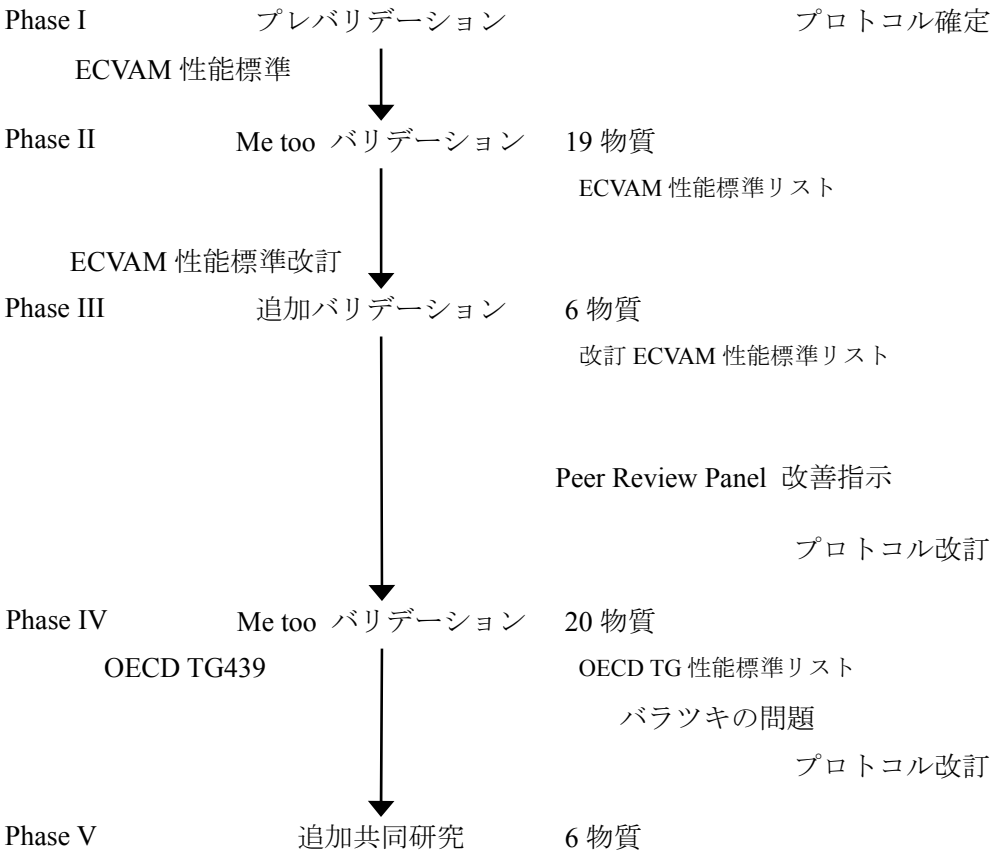

図 1　LabCyte EPI-MODEL24 皮膚刺激性試験法バリデーション研究の経過

２．試験法の妥当性

試験プロトコルは、TG 439 に示される参照試験法（EpiSkin^{TM} 試験法）とほぼ同様である。試験プロトコルは、バリデーション（phaseⅢ）からバリデーション（phaseⅣ）において、Peer Review からの指示を受けて ver.8.2 として改良された。また、バリデーション（phaseⅣ）では、参加 3 施設のうち、1 施設が特異度において TG439 に示す基準を満たさなかった。その原因として、ばらつきが大きいことが原因と判断されたため、ばらつきを減らす目的で、プロトコルを大きく変更しない範囲内での洗浄方法の詳細な統一が図られた（SOPver.8.3）[7]。変更された点を表 1 に示す。バリデーション（phase Ⅴ）では、SOPver.8.3 に従って実施された結果、試験におけるばらつきは減少した。

表 1　SOPver.8.3 における被験物質洗浄方法の明確化

洗浄操作	SOP ver.8.2	SOPver.8.3
1.洗浄前の被験物質の除去	明記されていない。	洗浄直前にインサートを傾けて廃棄する。
2.洗浄液量	明記されていない。	インサート容量以上の大量の PBS を用いて洗浄する。
3.各洗浄操作後の PBS 除去	明記されていない。	廃液ビンの上で軽くたたいて廃液する操作（タッピング）を一回行う。
4.最終洗浄後の PBS 除去	明記されていない。	インサートを傾けて除去する操作のみで上述のタッピング操作は行わない。

試験法（SOPver.8.3）の概要を以下に示す。24 ウェルプレートの各ウェルに、キットに備えつけの培養液 0.5mL を加えて LabCyte のインサートを設置し、一晩前培養して、試験に供した。被験物質が液体の場合は 25μL を LabCyte 表面に適用した。被験物質が固体の場合には 25mg をマイクロチューブに秤量しておき、予め蒸留水 25μL を添加しておいた培養表皮表面に適用した。1 被験物質につき、3 インサートを使用した。15 分間室温で静置して被験物質を暴露した。適用時間終了後、インサートを傾けてインサート内の被験物質を除去し、ポリ洗浄ビンにいれた PBS（リン酸緩衝液）をインサート内に吹き付けることにより洗浄し、インサートを傾けて PBS を除去した。この操作を 10 回以上繰り返し、組織表面の被験物質を取り除いた。その後、新しいウェルに培地 1mL を加え、インサートを移動し、42 時間培養を行った。その後、MTT を添加した培地 0.5mL を加えた新しいウェルにインサートを移動し、培養した。3 時間後、各インサートの培養表皮組織をマイクロチューブに各々入れ、イソプロパノールを 300μL 加えて一晩以上静置して MTT フォルマザンを抽出した。96 ウェルプレートに抽出液を 200μL ずつ移し、マイクロプレートリーダーを用いて 570nm、および 650nm の吸光度を測定した。570nm を測定波長とした吸光度から、650nm を参照波長とした吸光度を差し引いた値を測定値とした。イソプロパノールのみを加えたウェルをブランクとして、実測値とブランク値の差を求めた。陰性対照の吸光度を 100%とした時の各物質の細胞生存率(%)を計算した。使用した 3 組織の平均細胞生存率が 50%以下の結果を示す物質を皮膚刺激性（GHS 分類 1、あるいは 2）と判定した。陰性対照は蒸留水を用いた。陽性対照は 5% SDS（ラウリル硫酸ナトリウム）水溶液を用いた。これらの実験を、繰り返し 3 回実施し、その平均値から陽性か陰性かを判定した。

３．バリデーション試験に用いた物質の分類と妥当性

バリデーション試験では、phaseⅣで表 2-1 および phaseⅤで表 2-2 に示すそれぞれ 20 および 6 物質、合計 21 物質が供された。これらは TG 439 の性能標準に掲載されたリスト中の 20 物質をすべて含んでいる。表 2-1 の物質 No.20　1,1,1-trichloroethane は OECD　TG 案には掲載されていたが、バリデーション phaseⅣを実施中に公定化された最終段階では tetrachloroethylene（表 2-2、物質 No.21）に置き換わった。表 1-2 に記載された残りの 5 物質は、表 2-1 にも掲載されている。

選ばれている 20 物質は、刺激性強度（GHS ランク）、物性、国際市場での入手しやすさなどを考慮して選抜され、性能標準では、小規模バリデーションにおいて、これらの物質を用いて基準以上の結果が得られることが証明されれば、既存モデルと同程度と扱うとされている。なお、category 3 に属する物質 No.8~10 は TG439 に準拠して無刺激性と分類されている。

表 2-1. Minimum List of Reference Chemicals for Determination of Accuracy and Reliability Values for Similar or Modified RhE Skin Irritation Test Methods and Codes

No.	Name	CAS number	UN GHS in vivo Cat.	Supplier
1	1-bromo-4-chlorobutane	6940-78-9	No Cat.	WPCI
2	diethyl phthalate	84-66-2	No Cat.	WPCI
3	naphthalene acetic acid	86-87-3	No Cat.	WPCI
4	allyl phenoxy-acetate	7493-74-5	No Cat.	WPCI
5	Isopropanol	67-63-0	No Cat.	WPCI
6	4-methylthio-benzaldehyde	3446-89-7	No Cat.	WPCI
7	methyl stearate	112-61-8	No Cat.	KCC
8	heptyl butyrate	5870-93-9	No Cat. (Optional Cat. 3)	Sigma-Aldrich
9	hexyl salicylate	6259-76-3	No Cat. (Optional Cat. 3)	Sigma-Fluka
10	Cinnamaldehyde	104-55-2	No Cat. (Optional Cat. 3)	Sigma-Aldrich
11	1-decanol	112-30-1	Cat.2	WPCI
12	cyclamen aldehyde	103-95-7	Cat.2	WPCI
13	1-bromohexane	11-25-1	Cat.2	WPCI
14	2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	86604-75-3	Cat.2	WPCI
15	di-n-propyl disulphide	629-19-6	Cat.2	WPCI
16	potassium hydroxide 5%	1310-58-3	Cat.2	WPCI
17	benzynethiol,5-(1,1-dimethylethyl)-2-methyl	7340-90-1	Cat.2	TCI
18	1-methyl-3-phenyl-1-piperazine	5271-27-2	Cat.2	TCI
19	Heptanal	111-71-7	Cat.2	KCC
20	1,1,1-trichloroethane	71-55-6	Cat.2	WPCI

1) CAS No.: Chemical abstracts service registry number.

KCC = Kanto Chemical Co. Inc.; TCI = Tokyo Chemical Industry Co. Ltd; WPCI = Wako Pure Chemical Industries Ltd;

No cat. = 無刺激性　Cat3 = 無刺激性, .Cat.2 = Category 2 ：刺激性

表 2-2　The list of chemicals and code with the additional study

No.	Name	CAS number	Supplier
2	diethyl phthalate	84-66-2	WPCI
4	allyl phenoxy-acetate	7493-74-5	WPCI
5	Isopropanol	67-63-0	WPCI
13	1-bromohexane	11-25-1	WPCI
15	di-n-propyl disulphide	629-19-6	WPCI
21	Tetrachloroethylene	3446-89-7	WPCI

WPCI = Wako Pure Chemical Industries

４．試験法の正確性を評価するために用いられた参照化合物の *in vivo* 参照データ

in vivo 参照データは、GHS 刺激性区分を 2.3 として、それ以上（Category2）を 10 物質、それ未満を 10 物質選定している。Category3　刺激性 1.7-2.3 は無刺激性に区分されている [9]。その情報は、TG439 性能標準で確認できる。

５．試験法のデータと結果の利用性

バリデーション PhaseIV の結果を表 3 に、phaseV の結果を表 4 に示す。標準偏差（SD）が 18%を超える結果は、採用されておらず、追加実験結果が採用された。表 3 に物質 No.15 の施設 b は 1 ～3 回目までの結果が採用されなかった。OECD 性能標準には追加実験は 2 回までとされていることから [10]、4 および 5 回目の結果のみが採用された。この結果から、施設 b における物質 No.15 については繰り返し数が 3 回未満なので、判定結果は欠測になった。表 3 に示すように、バリデーション（phaseV）では、施設 b における物質 No.15 は、2 回目から 4 回目をもとに、判定された。

なお、表 3 に掲載された物質 No.20 の結果は、3 節で述べた理由から解析には利用していない。

表 3.　Mean viability of chemicals at each laboratory (Phase V)

Chem. No.	Lab a				Lab b					Lab c				
	1	2	3	4	1	2	3	4	5	1	2	3	4	5
1	12.4	11.3	19.0		16.5	10.7	10.6			9.0	9.8	9.8		
2	91.7	81.5	69.6		60.9	57.5	*65.5	69.5		90.5	*77.4	102.0	93.0	#66.7
3	108.0	113.0	105.0		96.5	96.7	90.2			89.4	*90.8	106.0	98.9	#96
4	19.1	*43.4	65.1	59.3	66.6	70.6	*48.1	66.2		90.1	93.0	93.2		
5	89.6	77.0	67.6		75.9	*57.5	74.8	77.1		*68.5	86.6	*66.4	67.2	74.4
6	16.2	15.9	17.0		17.3	13.5	11.4			15.5	16.1	12.0		
7	110.0	110.0	104.0		98.8	93.1	76.3			91.2	102.0	108.0		
8	109.0	122.0	111.0		93.1	106.0	86.6			95.5	106.0	119.0		
9	105.0	111.0	102.0		98.0	95.7	83.5			99.6	100.0	113.0		
10	15.7	20.3	16.0		11.5	15.9	11.4			17.3	14.1	14.9		
11	14.2	16.5	9.4		12.4	17.3	16.2			22.1	15.1	14.1		
12	8.9	15.9	10.0		11.0	7.8	9.0			6.0	7.4	5.7		
13	*48	16.2	16.1	15.5	*39.5	6.6	*49.6	17.2	19.0	17.5	17.0	16.2		
14	2.1	4.3	4.1		4.9	5.2	9.1			2.8	3.4	3.2		
15	19.9	95.9	83.5		*39.1	*28	*52.7	17.5	18.5	81.1	83.2	86.3		
16	0.9	1.7	1.6		4.6	2.0	3.3			0.9	3.1	11.6		
17	6.9	46.6	1.0		10.6	21.0	11.6			6.3	5.0	6.6		
18	6.7	4.5	3.6		9.8	10.9	11.0			1.3	1.8	2.2		
19	9.5	10.3	10.4		9.5	7.0	9.5			11.9	10.2	10.9		
20	8.7	12.0	7.8		9.1	7.9	37.6	#17.4		7.6	7.0	6.8		

* SD >18%, Not accepted data.
\# Additional experiments,Not accepted data.
In vitro skin no irritant
In vitro skin irritant

表 4.　Mean cell viability at each Lab in the additional validation study (Phase V)

No	Name	Lab a			Lab b				Lab c		
		1	2	3	1	2	3	4	1	2	3
2	diethyl phthalate	94.5	90.7	92.5	*49.9	48.2	53.6	67.3	94.4	77.8	92.5
4	allyl phenoxy-acetate	83.1	79.9	67.3	*64.7	62.4	57.8	64.0	91.0	84.3	79.2
5	Isopropanol	77.3	79.2	80.4	79.0	78.9	78.2		93.7	86.2	82.1
13	1-bromohexane	17.5	17.1	18.0	14.3	25.0	20.3		11.7	11.5	7.6
15	di-n-propyl disulphide	96.5	84.5	86.4	*72.1	31.1	29.6	43.8	36.9	78.6	70.7
21	Tetrachloroethylene	5.6	5.0	5.3	7.1	9.9	13.0		8.7	7.1	7.0

* SD >18%
In vitro skin no irritant
In vitro skin irritant

６．試験法の正確性

表 3 および 4 の結果をもとに、平均値から求められた判定結果を表 5 および 6 に示す。表 4 に示すように、物質 No.4 施設 a の判定結果は 3 回中 2 回が陰性であったが、3 回の平均値を用いると陽性なり、結果が食い違った。追加バリデーションでは、さらなるプロトコルの改良ですべての施設のすべての実験で陰性となった。表 5 および 6 に示すように、物質 No.13 はすべての施設のすべての実験で陽性となり、プロトコルの改良が確認できた。

TG439 性能標準に準拠し、PhaseⅣおよびⅤの結果を組み合わせて、物質 2、4、5、13、15、2 1 については、phaseV の結果を、それ以外の物質は PhaseⅣの結果を最終結果として表 8 にまとめると、感度が 90%（2 施設）、100%（1 施設）、特異度が 70%（3 施設）、正確度 80%（2 施設）、85%（1 施設）となり、性能標準の基準である感度 90%、特異度 70%、正確度 80%に全て適合した。これにより、最終的なプロトコル（SOPver.8.3）を用いれば、LabCyte を用いた皮膚刺激性試験の正確性は高いと判断した。ただし、14 物質については、旧プロトコル（SOPver.8.2）で実施された結果であり、最終プロトコル（SOPver.8.3）に適合するかは不明である。そこで、最終プロトコルで実施された 14 物質を含んだより広範な物質のデータ（表 9、図 2,3）を J-TEC より提供を受け、プロトコルの改訂に伴う結果への影響を評価した。沿

結果、偏りは小さく、精度の向上に寄与していると判断した。

表 5.　Classification using three independent viabilities (Phase IV)

No.	UN GHS *in vivo* Cat	Lab a				Lab b				Lab c			
		1	2	3	F	1	2	3	F	1	2	3	F
1	No Cat.	P	P	P	P	P	P	P	P	P	P	P	P
2	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
3	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
4	No Cat.	P	N	N	P	N	N	N	N	N	N	N	N
5	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
6	No Cat.	P	P	P	P	P	P	P	P	P	P	P	P
7	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
8	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
9	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
10	No Cat.	P	P	P	P	P	P	P	P	P	P	P	P
11	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
12	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
13	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
14	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
15	Cat.2	P	N	N	N	P	P	ND	ND	N	N	N	N
16	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
17	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
18	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
19	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
20	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P

P: Positive, N: Negative, F: Final detemination by median, ND: Not detected for invalid

表 6.　Classification using three independent viabilities (Phase V)

No	Name	Lab.a		Lab.b		Lab.c	
		Mean	Judge	Mean	Judge	Mean	Judge
2	diethyl phthalate	92.5	NI	56.4	NI	88.3	NI
4	allyl phenoxy-acetate	76.8	NI	61.4	NI	84.8	NI
5	Isopropanol	79.0	NI	78.7	NI	87.3	NI
13	1-bromohexane	17.5	I	19.9	I	10.3	I
15	di-n-propyl disulphide	89.1	NI	34.8	I	62.1	NI
21	Tetrachloroethylene	5.3	I	10.5	I	7.6	I

表 7　Classification using three independent viabilities (Phase IV and phase V)

No.	UN GHS *in vivo* Cat	Lab a				Lab b				Lab c			
		1	2	3	F	1	2	3	F	1	2	3	F
1	No Cat.	P	P	P	P	P	P	P	P	P	P	P	P
2	No Cat.	N	N	N	N	P	N	N	N	P	N	N	N
3	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
4	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
5	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
6	No Cat.	P	P	P	P	P	P	P	P	P	P	P	P
7	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
8	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
9	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
10	No Cat.	P	P	P	P	P	P	P	P	P	P	P	P
11	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
12	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
13	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
14	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
15	Cat.2	N	N	N	N	P	P	P	P	P	N	N	N
16	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
17	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
18	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
19	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
20	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P

表 8. 2x2 tables merged with additional study

Lab a		In vivo classification		
		Irritant	Non-Irritant	Total
In vitro prediction	Irritant	9	3	12
	Non-irritant	1	7	8
	Total	10	10	20

Sensitivity (%)	90.0
Specificity (%)	70.0
Accuracy (%)	80.0

Lab b		In vivo classification		
		Irritant	Non-Irritant	Total
In vitro prediction	Irritant	10	3	13
	Non-irritant	0	7	7
	Total	10	10	20

Sensitivity (%)	100.0
Specificity (%)	70.0
Accuracy (%)	85.0

Lab c		In vivo classification		
		Irritant	Non-Irritant	Total
In vitro prediction	Irritant	9	3	12
	Non-irritant	1	7	8
	Total	10	10	20

Sensitivity (%)	90.0
Specificity (%)	70.0
Accuracy (%)	80.0

表 9.　SOPver.8.2 と SOPver.8.3 を用いた LabCyte EPI-MODEL24 皮膚刺激性試験

Chemical			LabCyte EPI-MODEL24 SIT			
Name	In vivo score	In vivo class	Ver.8.2 Viability (%)	In vitro Class I/NI	Ver8.3 Viability (%)	In vitro Class I/NI
1-bromo-4-chlorobutane	0	NI	19.8 ± 3.4	I	27.8 ± 0.8	I
diethyl phthalate	0	NI	87.6 ± 27.9	NI	90.8 ± 15.7	NI
di-propylene glycol	0	NI	101.0 ± 8.5	NI	95.4 ± 3.8	NI
naphtalen acetic acid	0	NI	99.7 ± 7.6	NI	94.6 ± 6.6	NI
3-ChloronitroBenzene	0	NI	99.2 ± 4.7	NI	88.8 ± 7.1	NI
3,3-Dithiodipropionic Acid	0	NI	96.3 ± 5.5	NI	99.0 ± 2.4	NI
4,4-Methylenebis(2,6-di-tert-buthylphenol)	0	NI	101.3 ± 2.2	NI	99.1 ± 5.6	NI
4-Amino-1,2,4-Triazole	0	NI	99.1 ± 1.8	NI	94.5 ± 2.1	NI
Benzyl Benzoate	0	NI	101.9 ± 3.3	NI	96.5 ± 2.8	NI
Sodium Bicarbonate	0	NI	100.0 ± 0.3	NI	101.0 ± 4.5	NI
Erucamide	0	NI	95.0 ± 6.6	NI	97.0 ± 3.5	NI
1,5-hexadiene	0	NI	91.2 ± 5.1	NI	64.9 ± 10.1	NI
Polyethlene glycol 400	0	NI	102.7 ± 4.3	NI	96.3 ± 2.4	NI
Glycerol	0	NI	109.1 ± 14.7	NI	96.6 ± 4.6	NI
3,3-Dimethylpentane	0	NI	79.8 ± 9.8	NI	65.1 ± 7.8	NI
allyl phenoxy-acetate	0.3	NI	82.3 ± 22.9	NI	81.4 ± 9.1	NI
Isopropanol	0.3	NI	84.6 ± 2.7	NI	74.7 ± 7.4	NI
Benzyl Salicylate	0.3	NI	97.2 ± 3.3	NI	98.5 ± 7.7	NI
Lauric Acid	0.3	NI	104.4 ± 9.0	NI	93.6 ± 4.1	NI
4-methyl-thio-benzaldehyde	1	NI	22.6 ± 1.8	I	33.1 ± 2.4	I
Methyl Stearate	1	NI	104.4 ± 7.7	NI	90.8 ± 5.1	NI
Benzyl Acetate	1	NI	26.2 ± 13.4	I	25.6 ± 1.8	I
Hydroxycitronellal	1	NI	24.2 ± 7.1	I	26.2 ± 1.9	I
Isopropyl Myristate	1	NI	100.8 ± 6.2	NI	96.0 ± 8.6	NI
Isopropyl Palmitate	1	NI	107.5 ± 7.3	NI	100.0 ± 8.7	NI
n-Buthyl Propionate	1	NI	36.8 ± 12.1	I	33.8 ± 3.5	I
Sodium Bisulphite	1	NI	49.0 ± 33.5	NI	66.9 ± 1.8	NI
Benzyl Alcohol	1.3	NI	13.6 ± 7.0	I	12.6 ± 2.0	I
allyl heptanoate	1.7	NI	103.0 ± 6.8	NI	88.7 ± 9.9	NI
heptyl butyrate	1.7	NI	108.1 ± 2.0	NI	104.2 ± 5.9	NI
2-Ethoxy Ethyl Methacrylate	1.7	NI	49.0 ± 23.1	I	31.5 ± 10.7	I
hexyl salicylate	2	NI	106.7 ± 16.3	NI	97.2 ± 4.9	NI
Linalyl Acetate	2	NI	93.5 ± 7.3	NI	95.0 ± 10.0	NI
terpinyl acetate	2	NI	32.1 ± 5.3	I	28.9 ± 8.8	I
Linalol	2	NI	16.6 ± 8.8	I	26.6 ± 4.9	I
Cinnamaldehyde	2	NI	29.1 ± 7.2	I	30.2 ± 5.9	I
Eugenol	2	NI	27.0 ± 7.4	I	35.4 ± 4.4	I
cyclamen aldehyde	2.3	I	30.1 ± 5.9	I	26.8 ± 1.9	I
1-decanol	2.3	I	32.3 ± 8.8	I	27.9 ± 6.8	I
1-bromohexane	2.7	I	39.9 ± 3.3	I	32.7 ± 4.0	I
alpha-Terpineol	2.7	I	21.4 ± 10.3	I	25.1 ± 0.5	I
1-BromoPentane	2.7	I	21.9 ± 3.6	I	29.8 ± 3.5	I
2-chloromethyl-3,5-dimethyl-4-methoxypyridine HC	2.7	I	12.7 ± 3.2	I	13.1 ± 1.3	I
butyl methacrylate	3	I	28.9 ± 4.6	I	30.0 ± 6.6	I
di-n-propyl disulphide	3	I	71.7 ± 7.5	NI	69.8 ± 20.3	NI
Potassium Hydroxide 5%	3	I	3.0 ± 0.5	I	0.1 ± 0.2	I
Heptanal	3.3	I	17.3 ± 3.2	I	21.4 ± 0.2	I
benzynethiol, 5-(1,1-dimethylethyl)-2-methyl	3.3	I	27.6 ± 8.4	I	28.1 ± 4.9	I
1-methyl-3-phenyl-1-piperazine	3.3	I	15.2 ± 4.6	I	5.3 ± 6.9	I
SLS (20% aq)	4	I	12.0 ± 2.3	I	12.7 ± 1.6	I
1,1,1 Trichloroethane	4	I	11.8 ± 1.7	I	20.9 ± 2.6	I
Tetrachlotroethylene	4	I	17.0 ± 5.7	I	22.9 ± 1.4	I
Capric acid (decanoic acid)	4	I	11.2 ± 5.9	I	17.0 ± 1.3	I
SLS (5% aq)		I	13.4 ± 1.7	I	15.9 ± 0.2	I

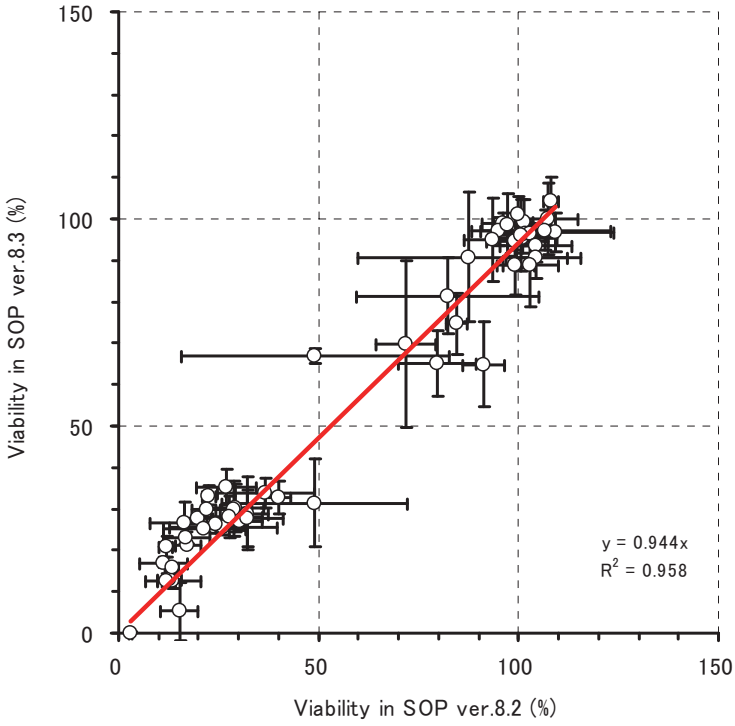

図 2　SOPver.8.2 と SOPver.8.3 を用いた LabCyte EPI-MODEL24 皮膚刺激性試験における生細胞率の相関

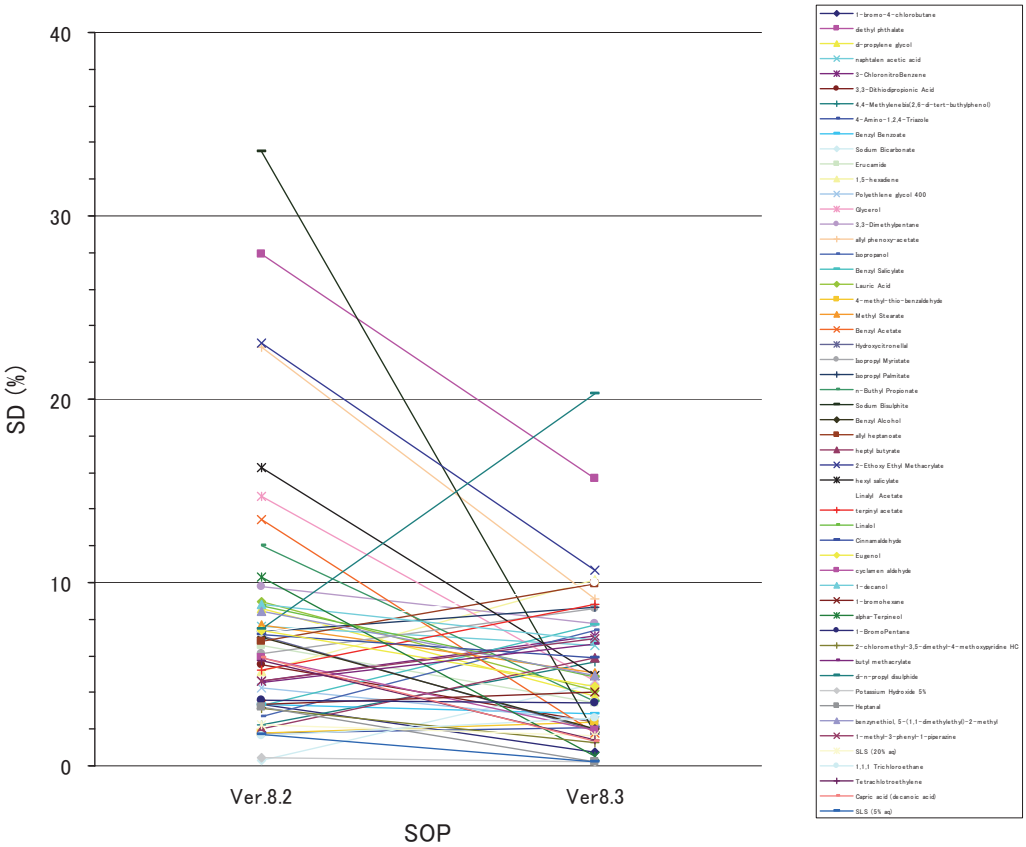

図 3　SOPver.8.2 と SOPver.8.3 を用いた LabCyte EPI-MODEL24 皮膚刺激性試験における生細胞率 SD の相関

７．試験方法の信頼性

表 10 に示すように、phaseⅣでは 5 物質において、ばらつきの大きな結果が得られた。各物質で SD が 18%を越えるデータが 2～3 みられ、合計 13 データとなった。データはしめしていないが、ばらつきの大きかった 5 物質の SD は phase Ⅱ と比べ、phaseⅣでは大きくなっている。すなわち、1-bromohexsane の偽陰性を改善するために行った被験物質の洗い方改は適切ではなかったといえる。そこで、さらなるプロトコルの改善がなされた phase Ⅴでは、SD 値は小さくなり、表 3 に示すように、SD が 18%を越える値は 3 データのみに改善された。

バリデーション PhaseIV、および V を組み合わせた施設内の再現性については、表 8 に示すように、3 回の繰り返し試験で判定結果が全て同じ結果となった物質の割合は、95%（2 施設：施設 b, c 19/20 物質）、100%（1 施設）となり、性能標準の基準である 90%以上に全施設が適合した。

バリデーション PhaseIV、および V を組み合わせた施設間の再現性については、表　に示すように、3 施設の判定結果が全て同じ結果となった物質の割合は、95%（19/20 物質、除く物質 No.15）となり、性能標準の基準である 80%以上に適合した。

以上の結果および、表 5 および 7 に示す施設内、施設間の高い再現性結果から本試験法の改訂プロトコルの信頼性は高いと判断した。

表 10. Chemicals obtained large variations at me-too validation study

		Data number of SD > 18%			
No.	Name	Lab a	Lab b	Lab c	Total
2	diethyl phthalate	0/3	1/4	1/4	2/11
4	allyl phenoxy-acetate	1/4	1/4	0/3	2/11
5	Isopropanol	0/3	1/4	2/5	3/12
13	1-bromohexane	1/4	2/5	0/3	3/12
15	di-n-propyl disulphide	0/3	3/5	0/3	3/11

８．データの質

試験に参加した施設は GLP の精神に則り、試験に関するすべての記録を残し、記録用紙を各施設の試験責任者が確認した後、バリデーション実行委員長に送付した。バリデーション実行委員長はすべての記録用紙を確認し、不明な点があれば問い合わせ、相互で確定したデータのみを解析に用いている。よって、データの質は確保されていると判断した。

９．試験法の有用性と限界

LabCyte EPI-MODEL24（24 インサート/キット）は、1 キット 5 万 4 千円 1 キットで未知検体を 6 物質（他に陰性対照、陽性対照）評価可能である。

動物実験と比較して、5 日間という短時間で結果を得ることができる。一方、LabCyte EPI-MODEL24 入手後は 4 日以内に試験を開始する必要があり、試験実施に 5 日間を要するため、火曜日到着ですぐに試験を実施しない場合、土曜日、日曜日での作業が発生する。

OECD TG404 に示されるドレイズ試験は、3 匹のウサギを用いて 4 時間閉塞貼付を行い、除去後、1,24,48,72 時間後の反応をドレイズ評価基準で評価する方法である。GHS 分類においては、区分 2（刺激性：少なくとも 2 個体で 2.3 以上）、区分 1（弱刺激性：少なくとも 2 個体で 1.7 以上 2.3 未満）、区分外（非刺激性：1.7 未満）となっている。このうち、TG439 では区分 3 を分類することはできないとされている。

適用限界として、MTT 干渉作用のある物質と着色性物質が挙げられる。プロトコルには、これらの物質の検出方法についての記載があり、その影響が明らかな物質については、その程度を定量する必要があると考えられる。また、エアロゾルやガスについては、適用外であるとされている。

10. その他の試験方法の科学的な報告

TG439 に掲載されている培養表皮モデル　EpiSkin、EpiDerm、SkinEthics を用いた皮膚一次刺激性試験に関する論文報告は多い。これらの試験法は JaCVAM 皮膚刺激性評価委員会においても、国内の専門家により評価され、OECD　TG 内容が認証されている [11]。

11. 結論

3 次元培養表皮モデル LabCyte EPI-MODEL24 を用いた皮膚刺激性試験法は、GHS 分類による皮膚刺激性の識別をと予測する方法として、TG439 性能標準に従いバリデーションが実施されており、その結果、正確性、信頼性についても妥当であった。したがって、本試験法は GHS 分類による皮膚刺激性を予測する *in vitro* 試験として有用な方法であると判断した。

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No. 81, *Summary Report of the Validation Peer Review for the Updated Test Guideline 407, and Agreement of the Working Group of National Coordinators of the Test Guidelines Programme on the follow-up of this report (2007)*

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No. 93, *Report of the Validation of an Enhancement of OECD TG 211: Daphnia Magna Reproduction Test (2008)*

No. 94, *Report of the Validation Peer Review for the 21-Day Fish Endocrine Screening Assay and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-up of this Report (2008)*

No. 95, *Detailed Review Paper on Fish Life-Cycle Tests (2008)*

No.96, *Guidance Document on Magnitude of Pesticide Residues in Processed Commodities (2008)*

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No. 100, *Report of the Second Survey on Available Omics Tools (2009)*

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No. 114, *Performance Assessment of Different Cytotoxic and Cytostatic Measures for the In Vitro Micronucleus Test (MNVIT): Summary of results in the collaborative trial (2010)*

No. 115, *Guidance Document on the Weanling Hershberger Bioassay in Rats: A Short-term Screening Assay for (Anti) Androgenic Properties (2009)*

No. 116, *Guidance Document on the Design and Conduct of Chronic Toxicity and Carcinogenicity Studies, Supporting TG 451, 452 and 453 (2010)*

No. 117, *Guidance Document 117 on the Current Implementation of Internal Triggers in Test Guideline 443 for an Extended One Generation Reproductive Toxicity Study, in the United States and Canada (2011)*

No. 118, *Workshop Report on OECD Countries Activities Regarding Testing, Assessment and Management of Endocrine Disrupters Part I and Part II (2010)*

No. 119, *Classification and Labelling of chemicals according to the UN Globally Harmonized System: Outcome of the Analysis of Classification of Selected Chemicals listed in Annex III of the Rotterdam Convention (2010)*

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No. 121, *Detailed review paper (DRP) on Molluscs life-cycle Toxicity Testing (2010)*

No. 122, *Guidance Document on the determination of the Toxicity of a Test Chemical to the Dung Beetle Aphodius Constans (2010)*

No. 123, *Guidance Document on the Diagnosis of Endocrine-related Histopathology in Fish Gonads (2010)*

No. 124, *Guidance for the Derivation of an Acute Reference Dose (2010)*

No. 125, *Guidance Document on Histopathology for Inhalation Toxicity Studies, Supporting TG 412 (Subacute Inhalation Toxicity: 28-Day) and TG 413 (Subchronic Inhalation Toxicity: 90-Day) (2010)*

No. 126, S*hort Guidance on the Threshold approach for Acute Fish Toxicity (2010)*

No. 127, *Peer review report of the validation of the 21-day androgenised female stickleback screening assay (2010)*

No. 128, *Validation Report of the 21-day Androgenised Female Stickleback Screening Assay (2010)*

No. 129, *Guidance Document on using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests (2010)*

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No. 132, *Report of the Multi-Laboratory Validation of the H295R Steroidogenesis Assay to Identify Modulators (2010)*

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No.135, *Detailed Review Paper on Environmental Endocrine Disruptor Screening: The use of Estrogen and Androgen Receptor Binding and Transactivation Assays in Fish (2010)*

No. 136, *Validation Report of t he Chironomid Full Life-Cycle Toxicity Test (2010)*

No. 137, *Explanatory Background Document to the OECD Test Guideline On In Vitro Skin Irritation Testing (2010)*

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No. 139, *Report of the Expert Consultation on Scientific and Regulatory Evaluation of Organic Chemistry Mechanism Based Structural Alerts for the Identification of Protein-binding Chemicals (2011)*

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No. 142, *Report of the Phase 2 of the Validation of the Fish Sexual Development Test for the Detection of Endocrine Active Substances (2011)*

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No. 145, *Transgenic Rodent Somatic and Germ Cell Gene Mutation Assay: Retrospective Performance Assessment (2011)*

No. 148, *Guidance Document on the Androngenised Female Stickleback Screen (2011)*

No. 152, *Case Study: Assessment of an Extended Chemical Category, the Short-chain Methacrylates, Targeted on Bioaccumulation (2011)*

No. 153, *Guidance Document for the Derivation of an Acute Reference Concentration (Arfc) (2011)*

No. 156, *Guidance Notes on Dermal Absorption* (2011)

No. 157, *Validation Report Phase 1 for the Zebrafish Embryo Toxicity Test (2011)*

No. 158, *Report of Progress on the Interlaboratory Validation of the OECD Harpacticoid Copepod Development and Reproduction Test (2011)*

No. 159, *Validation Report for the Skin Irritation Test Method using Labcyte Epi-Model24(2011)*

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or contact:

OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France

Fax: (33-1) 44 30 61 80 E-mail: ehscont@oecd.org

E-mail: ehscont@oecd.org

FOREWORD

This document presents the validation report for the "*Skin Irritation Test Using the LabCyte EPI-MODEL24*". The project for developing a Test Guideline for an *in vitro* epidermal model to assess skin irritation using the LabCyte EPI-MODEL24, led by Japan, was included in the work plan of the Test Guidelines Programme in 2009. The Working of National Coordinators of the Test Guidelines Programme endorsed this validation at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (Joint Meeting) agreed to its declassification on 5 August 2011.

A validation peer review report, accompanied by a report on additional validation work, is also expected to be published in the Series on Testing and Assessment.

This document is published under the responsibility of the Joint Meeting.

MEMBERS OF LABCYTE VALIDATION MANAGEMENT TEAM

Mr. Hajime Kojima, JaCVAM: Japanese Centre for the Validation of Alternative Methods
Mr. Takashi Omori; Kyoto Univ.
Mr. Kenji Idehara; Daicel Chemical Co.
Mr. Isao Yoshimura; Tokyo University of Science
Mr.Etsuyoshi Miyaoka; Tokyo University of Science
Mr. Masakazu Kato; Japan Tissue Engineering Co. Ltd.
Mr. Toshiro Yokouchi; Japan Tissue Engineering Co. Ltd.

Participant laboratories

- Aiken Co., Ltd. (Ms Yoko Ando and Ms Yui Asako)
- KOBAYASHI Pharmaceutical Co., Ltd. (Mr. Yoshihiro Yamaguchi and Ms Maki Nakamura)
- The Institute of Environmental Toxicology (Mr Tadashi Kosaka and Mr Koichi hayashi)
- Fancl Corp. (Ms. Tamie Suzuki and Ms Runa Izumi)
- FUJIFILM Corporation (Ms. Atsuko Yuasa, and Mr Shinichi Akimoto)
- Maruishi Pharmaceutical Co., Ltd. (Mr Yukihiko Watanabe and Mr Osamu Mitani)
- Drug Safety Testing Center Co., Ltd. (Mr Shinsuke Shinoda and Ms Saori Hagiwara)

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1. Goal statement

- The aim of this study was to validate *in vitro* skin irritation tests in a formal inter-laboratory study, the ultimate goal of the test strategy will be to replace the regulatory Draize skin irritation test according OECD TG 404 (OECD, 2002).
- The primary goal of this validation study was an evaluation of the ability of the *in vitro* tests to reliably discriminate skin irritant (I) from non-irritant (NI) chemicals, as defined according to the OECD and United Nations proposal for Globally Harmonised System (GHS) for the classification and labelling of skin irritation (category 1/category 2; no category; Anon., 2003) .

2. Objective

1. The *in vitro* test system, employing reconstructed human epidermis model (RhE: LabCyte EPI-MODEL24), has progressed through protocol optimisation as in vitro skin irritation test. The multi-laboratory assessment of this system performed based on the a few ECVAM performance standards (ESAC statement, 2007, 2008, 2009). This report shows the results of 3rd phase validation study in accordance with the revised reference chemicals described by the new ESAC statement 2009.

2. The present objective was to conduct a validation study to assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of this test system with a challenging set of coded 25 test chemicals for which high quality *in vivo* data were available. The validation study was undertaken in accordance with the principles and criteria documented in the OECD *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* (No. 34, OECD, 2005) and according to the Modular Approach to validation (Hartung *et al.* 2004).

3. Test Method

3-1. Reconstructed human cultured epidermal model

3. LabCyte EPI-MODEL24 is a new, commercially available RhE model produced by Japan Tissue Engineering Co. Ltd. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand human keratinocytes while maintaining their phenotype, they were cultured with 3T3-J2 cells as a feeder layer (Rheinwald and Green, 1975; Green, 1978). Reconstruction of human cultured epidermis is achieved by cultivating and proliferating keratinocytes on an inert filter substrate (surface 0.3 cm^2) at the air-liquid interface for 13 days with an optimized medium containing 5% fetal bovine serum. It constructs a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum. LabCyte EPI-MODEL24 is embedded in an agarose gel containing nutrient solution and shipped in 24-well plates at around 18°C (Kato, 2009).

3-2. MODEL SUPPLIER

4. According to OECD GLP Consensus Document No.5 "*Compliance of Laboratory Suppliers with GLP Principles*" the responsibility for the quality and fitness for use of equipment and materials rests entirely with the management of the test facility (OECD, 1999).

5. The acceptability of equipment and materials in laboratories complying with GLP-like principles should therefore be guaranteed to any regulatory authority to which studies were submitted. In some countries where GLP has been implemented, suppliers belong to national regulatory or voluntary accreditation schemes (for laboratory animals) which can provide users with additional documentary evidence that they are using a test system of a defined quality.

6. The audits focused on the procedures established to guarantee a defined quality of the tissue models.

4. Validation Management structure

7. This validation study was performed by the Japanese Society for the Alternative to Animal Experiments (JSAAE).

The management structure of the study is shown in Figure 1.

4-1. Validation Management Group

8. The Validation Management Team (VMT), which plays a central role overseeing the conduct of the validation study, includes:

1) Goal statement
2) Project plan including objective
3) Study protocol / amendments
4) Outcome of QC audits
5) Test chemicals
6) Data management procedures
7) Timeline/ study progression
8) Study interpretation and conclusions
9) Reports and publication

9. The final decision on which laboratories participate in the validation study is the responsibility of the VMT.

Members:

A chair (Hajime Kojima, JaCVAM: Japanese Centre for the Validation of Alternative Methods)
The sponsor representative: representatives of JSAAE (Takashi Omori; Kyoto Univ., Kenji Idehara; Daicel Chemical Co. and Isao Yoshimura; Tokyo University of Science)
The sponsor representative, LabCyte EPI-MODEL24suppliers and lead lab (Masakazu Kato : Japan Tissue Engineering Co., Ltd, J-TEC)

4-2. Chemical selection, acquisition, coding and distribution

1) Definition of selection criteria
2) Chemical selection
3) Liaise with suppliers
4) Final check of chemicals provided
5) Acquisition
6) Coding
7) Distribution

Member

Hajime Kojima, JaCVAM

4-3 . Independent biostatisticians

1) Approve spreadsheets
2) Collect data
3) Analyse data

Members:
Takashi Omori: Kyoto Univ., Etsuyoshi MIyaoka and Kenya Ishiyama: Tokyo University of Science

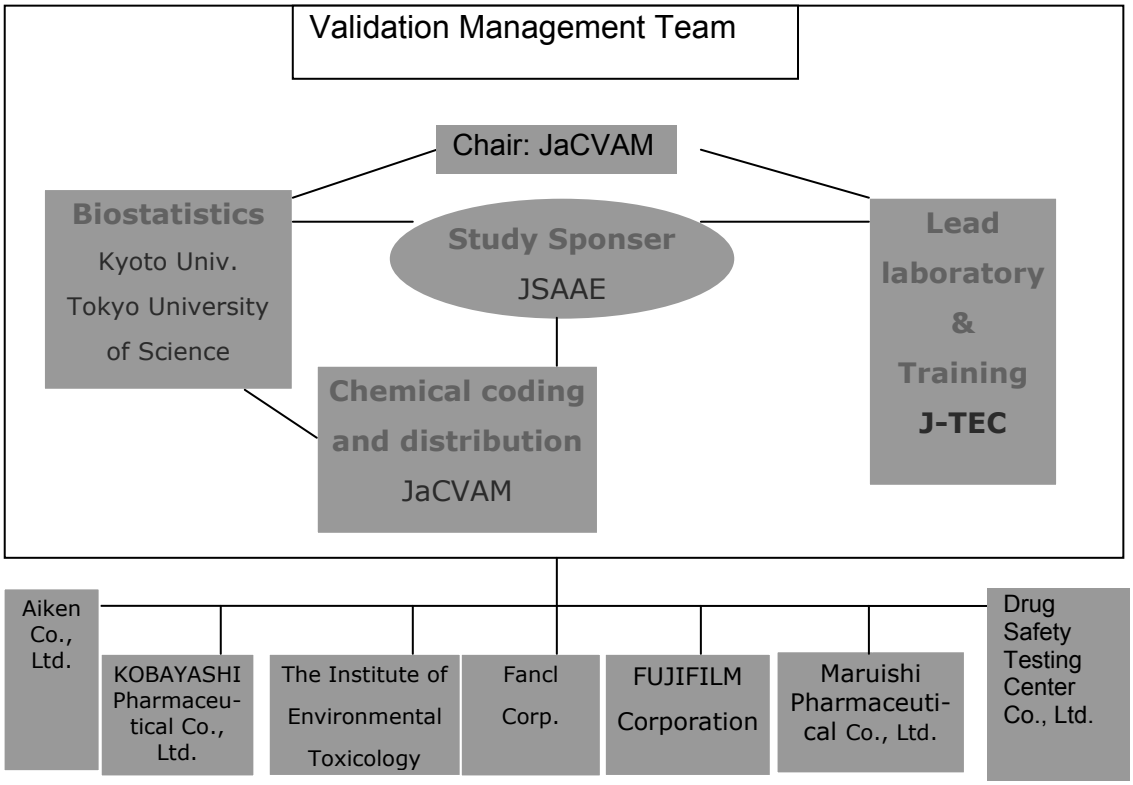


Figure 1. Management structure of the JSAAE skin irritation validation study

4-4. Participating laboratories

The laboratories participating in the study are to be defined as shown in **Fig. 1**.

The following 6 laboratories participated in the validation study for the evaluation of the LabCyte EPI-MODEL24 assays:

- Laboratory 1 – Aiken Co., Ltd. (Yoko Ando and Yui Asako)
- Laboratory 2 – KOBAYASHI Pharmaceutical Co., Ltd. (Yoshihiro Yamaguchi and Maki Nakamura)
- Laboratory 3 – The Institute of Environmental Toxicology (Tadashi Kosaka and Koichi hayashi)
- Laboratory 4 – Fancl Corp. (Tamie Suzuki and Runa Izumi)
- Laboratory 5 – FUJIFILM Corporation (Atsuko Yuasa, and Shinichi Akimoto)

  This laboratory was not participated at the 3rd Phase study.
- Laboratory 6 – Maruishi Pharmaceutical Co., Ltd. (Yukihiko Watanabe and Osamu Mitani)
- Laboratory 7 – Drug Safety Testing Center Co., Ltd. (Shinsuke Shinoda and Saori Hagiwara)

A lead laboratory is also identified as J-TEC (Mr. Masakazu Kato and Mr Toshihiro Yokouchi). This laboratory was not participated in the validation study.

Each laboratory also was responsible for complying with GLP-like principles and specifying QA aspects.

4-5. Sponsorship

The study was managed and finance by JSAAE and J-TEC .

1) JSAAE finance:

- the management of the study (VMT meetings)
- the independent statistical support (biostatistician)
- the responsible for the chemicals purchase, coding and distribution to the laboratories
- the independent QC audit of the data
- the publication of the study

2) *J-TEC finance:*

- the lead laboratories for the test method
- training for the participating laboratories
- the independent QC audit on the LabCyte EPI-MODEL24
- the financial assistance for the participated laboratories

5. Study design

10. Before this validation study, the training course using LabCyte EPI-MODEL24 was performed by J-TEC on April, 2008. All technicians from each laboratory participated at this training course.

11. Three phases of validation studies were performed. In the 1st phase, we confirmed the transferability of the test protocol and assessed its reproducibility, by testing three coded chemicals (ethanol, glycerol and napthalen acetic acid) and a positive control (5% sodium lauryl sulfate solution) in seven laboratories between June and July of 2008.

12. In the 2nd phase study, we confirmed the intra- and inter-laboratory reproducibility robustness, and the correlation of test using 19 new chemicals tested in reference to the original EPISKIN performance standards (ECVAM, 2007) . These tests were conducted by 7 laboratories between September 2008 and January of 2009.

13. In the 3rd phase study, we confirmed the intra- and inter-laboratory reproducibility robustness, and the correlation of test using 6 chemicals tested in reference to the new EPISKIN performance standards (ESAC statement, 2009). This study was conducted by 6 laboratories, which attend the 1st and 2nd phase validation study between April to May, 2009.

6. Test Chemical

6-1. Chemicals Selection and list

14. In 1st phase study, JaCVAM selected three coded chemicals (ethanol, glycerol and napthalen acetic acid) to test.

15. According to the original ESAC Performance Standard (ESAC statement,2007) in 2nd Phase, the VMT selected 19 new chemicals to test in Table 1. One chemical, tri-isobuthyl phosphate (No. 13) on the chemical list reference for the original ECVAM Performance Standard cannot be purchased on the Japanese market. The VMT is responsible for the final approval of the chemicals proposed by JaCVAM. To avoid any potential bias in the final selection, the laboratory representatives on the VMT were not party to these discussions, nor were they informed of the final list of test chemicals for either phase of the validation study.

16. According to the new ECVAM performance standard (ESAC statement, 2009) in 3rd phase, the VMT selected 6 new chemicals tested in Table 2. The final approval of the chemicals proposed by JaCVAM is the responsibility of the VMT. To avoid any potential for bias in the final selection, the laboratory representatives on the VMT did not be party to these discussions, nor were they made aware of the chemicals finally approved for testing in either phase of the validation study.

Table 1. Reference test chemicals and codes

No.	Chemical	CAS number	GHS label	In vivo score (PII)	Laboratory						
					a	b	c	d	e	f	g
01	1-bromo-4-chlorobutane	6940-78-9	no	0	A-01	B-099	C-077	D-115	E-133	F-031	G-049
02	diethyl phthalate	84-66-2	no	0	A-02	B-100	C-078	D-116	E-134	F-032	G-050
03	di-propylene glycol	25265-71-8	no	0	A-03	B-081	C-079	D-117	E-135	F-033	G-051
04	naphtalen acetic acid	86-87-3	no	0	A-04	B-082	C-080	D-118	E-136	F-034	G-052
05	allyl phenoxy-acetate	7493-74-5	no	0.3	A-05	B-083	C-061	D-119	E-137	F-035	G-053
06	isopropanol	67-63-0	no	0.3	A-06	B-084	C-062	D-120	E-138	F-036	G-054
07	4-methyl-thio-benzaldehyde	3446-89-7	no	1	A-07	B-085	C-063	D-101	E-139	F-037	G-055
08	methyl stearate	112-61-8	no	1	A-08	B-086	C-064	D-102	E-140	F-038	G-056
09	allyl heptanoate	142-19-8	no	1.7	A-09	B-087	C-065	D-103	E-121	F-039	G-057
10	heptyl butyrate	5870-93-9	no	1.7	A-10	B-088	C-066	D-104	E-122	F-040	G-058
11	hexyl salicylate	6259-76-3	no	2	A-11	B-089	C-067	D-105	E-123	F-021	G-059
12	terpinyl acetate	80-26-2	no	2	A-12	B-090	C-068	D-106	E-124	F-022	G-060
13	5(W/V %) SLS				A-13	B-091	C-069	D-107	E-125	F-023	G-041
14	1-decanol	112-30-1	Category 2	2.3	A-14	B-092	C-070	D-108	E-126	F-024	G-042
15	cyclamen aldehyde	103-95-7	Category 2	2.3	A-15	B-093	C-071	D-109	E-127	F-025	G-043
16	1-bromohexane	111-25-1	Category 2	2.7	A-16	B-094	C-072	D-110	E-128	F-026	G-044
17	α-terpineol	98-55-5	Category 2	2.7	A-17	B-095	C-073	D-111	E-129	F-027	G-045
18	di-n-propyl disulphide	629-19-6	Category 2	3	A-18	B-096	C-074	D-112	E-130	F-028	G-046
19	butyl methacrylate	97-88-1	Category 2	3	A-19	B-097	C-075	D-113	E-131	F-029	G-047
20	heptanal	111-71-7	Category 2	4	A-20	B-098	C-076	D-114	E-132	F-030	G-048

1) CAS No.: Chemical abstracts service registry number.
2) PII: Primary irritation index.

Table 2. Test chemicals and code.

No.	Chemical	CAS number	GHS label	In vivo Score	Laboratory					
					a	b	c	d	f	g
A	Cinnamaldehyde	104-55-2	no	2	A-151	B-176	C-196	D-216	F-236	G-256
B	2-Chroromethyl-3,5-dimethyl-4-methoxypyridine HCl	322-76821	Category 2	2.7	A-154	B-173	C-192	D-211	F-233	G-253
C	Potassium hydroxide (5%aq)	168-21815	Category 2	3	A-156	B-175	C-194	D-213	F-232	G-251
D	Benzenethiol, 5-(1,1-dimethyethyl)-2-methyl	7340-90-1	Category 2	3.3	A-153	B-172	C-191	D-214	F-234	G-254
E	1-Methyl-3-phenyl-1-piperazine	5271-27-2	Category 2	3.3	A-152	B-171	C-195	D-215	F-235	G-255
F	1,1,1-Torichloroethane	200-02463	Category 2	4	A-155	B-174	C-193	D-212	F-231	G-252

1)CAS No.: Chemical abstracts service registry number.

6-2. Deficit chemical

17. In Table1, tri-isobuthyl phosphate (No. 13) could not be used in the examination because it was not available in Japan. Therefore, a 5% SLS solution was used instead of tri-isobuthyl phosphate. The data obtained with the 5% SLS solution were not used for calculating the predictivity of the test.

6-3. Chemical Coding and distribution

18. Independent coding and distribution of chemicals were contracted out by JaCVAM to an independent laboratory. The (company's name) is certified according to ISO 9001, EN 4500 and GLP, and has proven experience of reliable services. The codes were provided by JaCVAM.

7. Protocol

7-1. Protocol of the skin irritation test with LabCyte EPI-MODEL

19. In 2nd phase study, we used the SOP (ver. 5.0) and we used the SOP (ver. 6.1) in 3rd phase study. The revised points, which make the deletion measurement of IL-1α, revise calculating formula of viability, classification used median of 3trails and how to treat of volatile substances were shown in change tracking of the SOP (ver. 6.1). The VMT made judgments that these revise points were minor and difference with the SOP (ver.5.0) used by 2nd phase study and this version was little in the VMT meeting on July 17, 2009.

20. LabCyte EPI-MODEL tissues were shipped from the supplier on Mondays and delivered to recipients on Tuesdays. Upon receipt, the tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 mL), and incubated overnight (37°C, 5% CO_2 humidified atmosphere). On the following day, the tissues were topically exposed to the test chemicals. Liquids (25 µL) were applied with a micropipette, and solids (25 mg) were applied from microtubes and moistened with 25 µL sterile water. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied using a cell-saver-type tip with a micropipette. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were treated with 25 µL distilled water, and three tissues serving as

positive controls were exposed to 5% SLS. After a 15-minute exposure, each tissue was carefully washed with PBS (Invitrogen, CA, USA) 10 times using a washing bottle to remove any remaining test chemical from the surface. The blotted tissues were then transferred to new 24-well plates containing 1 mL of fresh assay medium.

21. The treated and control tissues were incubated for 42 hours (37°C, 5% CO_2 humidified atmosphere). When the 42-hour post-incubation period was complete, blotted tissues were transferred to new 24-well plates containing 0.5 mL of freshly prepared MTT medium (1 mg/mL; Dojindo Co., Kumamoto, Japan) for the MTT assay and conditioned medium was collected to determine the interleukin-1 alpha (IL-1α) levels. Tissues were incubated for three hours (37°C, 5% CO_2 humidified atmosphere) and then transferred to microtubes containing 0.3 mL isopropanol, which completely immersed the tissue. Formazan extraction was performed at room temperature, and the tissues were allowed to stand overnight. Subsequently, 200-µL extracts were transferred to a 96-well plate. The optical density was measured at 570 nm and 650 nm as a reference absorbance, with isopropanol as a blank.

22. The tissue viability was calculated as a percentage relative to the viability of the negative controls. The median of three values from identically treated tissues was used to classify a chemical according to the prediction model.

23. The amount of IL-1α released in the conditioned medium after 42 hours was determined using an IL-1α ELISA kit (Invitrogen, CA, USA), following the manufacturer's detailed instructions.

7-2. Prediction model of skin irritation

24. In this study, the prediction model of skin irritation potential with LabCyte EPI-MODEL was set to refer to the conditions for EPISKIN described in the ECVAM Performance Standards. This prediction model is described in Table 3. In the event that the three independent results within an individual batch were not consistent, the result that occurred twice was used.

Acceptance criteria

1) OD_{NC} of the negative control is greater than 0.7.
2) The viability of the positive control is less than 40%.

Table 3. Positive Criteria.

Tissue Viability (primary)	IL-1α ELISA (secondary)	Classification
Mean tissue viability ≤ 50%		Irritant
Mean tissue viability > 50%	Mean IL-1α release ≥ 120 pg/tissue	
Mean tissue viability > 50%	Mean IL-1α release < 120 pg/tissue	Non-irritant

7-3. Difference between LabCyte EPI-MODEL 24 protocol and EPISKIN protocol

25. The differences between the LabCyte EPI-MODEL 24 protocol and EPISKIN protocol are summarized in Table 3. Although the amount of medium (Table 4(A)), amount of test chemicals (Table 4(B)), and threshold of IL-1α content (Table 4(C)) for the LabCyte EPI-MODEL 24 protocol are different from the EPISKIN protocol, their conditions meet the descriptions of the Performance Standards.

Table 4. Differences between the LabCyte EPI-MODEL 24 protocol and EPISKIN protocol.
(A) Amount of medium.

	LabCyte EPI-MODEL 24 SOP	EPISKIN SOP	Reason
Pre-incubation	0.5 mL	2 mL	LabCyte EPI-MODEL 24 cultures are performed in 24-well culture plates. A medium volume of 0.5 mL to 1 mL is appropriate to add to the 24-well culture plate. A medium volume of 1 mL is necessary for a 42-hour culture.
Post-incubation	1 mL	2 mL	
MTT assay	0.5 mL	2 mL	

These conditions meet the descriptions of the Performance Standards.

(B) Amount of test chemicals.

Test chemical	LabCyte EPI-MODEL 24 SOP	EPISKIN SOP	Reason
Liquid	25 μL (75 μL/cm^2)	10 μL (25 μL/cm^2)	The lowest amount of the test chemical that spread uniformly was applied to the test model.
Solid	25 mg+25 μL DW (75 μL/cm^2)	10 mg+10 μL DW (25 μL/cm^2)	

These conditions meet the descriptions of the Performance Standards.

(C) Amount of test chemicals.

LabCyte EPI-MODEL 24 SOP	EPISKIN SOP	Performance Standards (EPISKIN)
IL-1α content ≥ 120 pg/tissue (IL-1α content ≥ 120 pg/mL)	IL-1α content ≥ 100 pg/tissue (IL-1αcontent ≥ 50 pg/mL)	IL-1α content ≥ 120 pg/tissue (IL-1α ≥ 60 pg/mL)

The threshold of IL-1α released in LabCyte EPI-MODEL was set based on the conditions for EPISKIN described in the Performance Standards.

7-4. Data collection, handling, and analysis

26. The independent biostatisticians for the study collected and organised the data using specific data collection software (Datasheet4.0:20080910.xls in 2nd phase study and Datasheet5.0:20090430.xls in 3rd phase study). They will work in close collaboration with the biostatisticians, (Takashi Omori, Etsuyoshi Miyaoka, and Kenya Ishiyama). After decoding the data, they will perform statistical analyses. The data management procedures and statistical tools applied will be approved by the VMT.

7-5. Quality assurance, GLP

LABORATORIES

27. All participating laboratories worked in the spirit of OECD GLP-like principles.

QA aspects

28. Takashi Omori, Kenya Ishiyama and Hajime Kojima assured the quality of all the data and records.

8. Results

8-1 1st Phase

8-1-1 Negative control

29. In 1st phase data, Table 5 shows the absorbance values for the negative control. All data for the negative control met the acceptance criteria.

Table 5. Absorbance of negative control by 1st phase study.

	Exp.				
	1	2	3		
Lab.	Value	Value	Value	Mean	SD
a	1.073	0.928	1.007	1.003	0.073
b	0.93	1.245	1.042	1.072	0.16
c	0.96	0.869	0.761	0.863	0.1
d	0.987	0.928	0.939	0.951	0.031
e	0.84	0.884	0.973	0.899	0.068
f	1.049	0.934	0.968	0.984	0.059
g	1.147	1.159	1.074	1.127	0.046

8-1-2 Positive control and test chemicals

30. Table 6 shows the testing chemicals did not show any great score when the scores on tests were repeated in each laboratory. Furthermore, there was no significant inter-laboratory variation. These experiments suggested the feasibility of the LabCyte EPI-MODEL24 through the experiment. All laboratories were judged to participate at the Phase II by the validation management team.

Table 6. Viability of the positive control and three coded chemicals by 1st phase study

		1	2	3		
Chem.	**Lab.**	**Viability**	**Viability**	**Viability**	**Mean**	**SD**
PC	a	6.35	27.55	15.67	16.52	10.63
	b	3.94	3.51	3.97	3.81	0.26
	c	5.45	4.81	3.49	4.58	1
	d	11.74	7.22	14.08	11.02	3.49
	e	31.6	9.76	38.61	26.66	15.05
	f	3.1	2.89	2.93	2.97	0.11
	g	4.46	7.17	2.62	4.75	2.29
P01	a	62.67	39.12	46.61	49.46	12.03
Ethanol	b	41.08	50.86	86.58	59.51	23.95
	c	68.13	34.13	67.31	56.53	19.4
	d	68.57	40.52	33.03	47.37	18.73
	e	54.19	72.08	60.55	62.27	9.07
	f	.	64.16	47.98	56.07	11.44
	g	4.68	5.23	6.67	5.53	1.03
P02	a	103.63	104.17	98.48	102.09	3.14
Glycerol	b	85.5	100.58	67.97	84.68	16.32
	c	101.24	99.41	104.84	101.83	2.76
	d	103.3	101.35	89.73	98.13	7.34
	e	101.75	98.06	99.04	99.62	1.91
	f	.	97.23	96	96.62	0.87
	g	94	98.16	103.6	98.59	4.82
P03	a	109.13	90.73	97.78	99.22	9.28
naphtalen acetic acid	b	93.96	103.91	103.96	100.61	5.76
	c	103.66	102.11	117.3	107.69	8.36
	d	102.28	98.15	94.56	98.33	3.86
	e	107.11	104.39	97.36	102.95	5.03
	f	.	101.34	102.07	101.7	0.52
	g	92.2	101.04	105.52	99.59	6.78

8-2. 2nd phase & 3rd phase

8-2-1. Comments at the Datasheet

31. All tests were sufficient with acceptance criteria. There were a few comments from each laboratory in Tables 7 -9. By an application of Potassium hydroxide (5%aq) (B175, D213 and F232), the model's layers were desquamated. By an application of cinnamicaldehyde (D216 and G256), the cups were discoloured and crystallized.

Table 7. Comments on the datasheets (Viability) by 2nd phase

Lab ID	Exp.No.	Lot	Date	Comments
a	Main-2	LCE24-081013-B	2008/10/20	This test was recorded as the Main-1.
a	Main-3	LEC24-081117-B	2008/11/1	This test was recorded as the Main-2.
a	Main-4	LCE24-081117-B	2008/11/22	This test was recorded as the Main-3.
b	Main-1	LCE24-081013-B	2008/10/20	
b	Main-2	LCE24-081027-B	2008.11.04	
b	Main-3	LCE24-081117-B	2008/11/25	
c	1	LCE24-080929-B	2008.10.6	
c	2	LCE24-081020-B	2008/10/27	
c	3	LCE24-081027-B	2008.11.3	
d	81021	LCE24-081020-B	2008/10/27	
d	81028	LCE24-081027-B	2008/11/4	
d	81118	LCE24-081117-B	2008/11/25	
e	Main-1	LCE24-081006-B	2008/10/14	
e	Main-2	LCE24-081013-B	2008/10/20	
e	Main-3	LCE24-081020-B	2008/10/27	
f	LAB-08VAL	LCE24-080929-B	2008/10/6	
f	Maruishi	LCE24-081013-B	2008/10/20	
f	LAB-08VAL	LCE24-081103-B	2008/11/10	
g	Main-1	LCE24-080929-B	2008.10.06	By an application of G49,G53,G55, the model's cap was discolored.
g	Main-2	LCE24-081013-B	2008.10.20	By an application of G49,G53,G55, the model's cap was discolored.
g	Main-3	LCE24-081027-B	2008.11.03	By an application of G49,G53,G55, the model's cap was discolored.

Table 8. Comments on the datasheets (ELISA) by 2nd phase

Lab_ID	Exp.No.	Lot	Date	Comments
a	Main-2	LCE24-081013-B	2008/10/20	This test was recorded as the Main-1.
a	Main-3	LEC24-081117-B	2008/11/1	This test was recorded as the Main-2.
a	Main-4	LCE24-081117-B	2008/11/22	This test was recorded as the Main-3.
b	Main-1	LCE24-081013-B	2008/12/12	
b	Main-2	LCE24-081027-B	2008/12/12	
b	Main-3	LCE24-081117-B	2008.12.26	
c	1	LCE24-080929-B	2008/10/7	
c	2	LCE24-081020-B	2008/10/30	
c	3	LCE24-081027-B	2008.11.3	
d	81021	LCE24-081020-B	2008/11/11	
d	81028	LCE24-081027-B	2008/11/26	
d	81118	LCE24-081117-B	2009/1/7	
e	Main-1	LCE24-081006-B	2008/12/2	
e	Main-2	LCE24-081013-B	2008/12/2	
e	Main-3	LCE24-081020-B	2008/12/19	
f	Maruishi	LCE24-081013-B	2008/11/25	
f	Maruishi	LCE24-081013-B	2008/11/27	
f	LAB-08VAL	LCE24-081103-B	2008/12/25	
g	Main-1	LCE24-080929-B	2008.10.09	
g	Main-2	LCE24-081013-B	2008.10.22	
g	Main-3	LCE24-081027-B	2008.11.05	

Table 9. Comments on the datasheets (Viability) by 3rd phase study

Lab ID	Exp.No.	Lot	Date	Comments
a	No.1	LCE24-090420-A	2009/4/27	
a	No.2	LEC24-090511-A	2009/5/18	
a	No.3	LEC24-090518-A	2009/5/25	
b	20090421-1	LCE24-090420-A	2009/4/27	By an application of B175, the model's layers were desquamated.
b	20090421-2	LEC24-090511-A	2009/5/20	By an application of B175, the model's layers were desquamated.
b	20090421-3	LEC24-090518-A	2009/5/25	By an application of B175, the model's layers were desquamated.
c	1	LCE24-090420-A	2009/4/27	
c	2	LEC24-090511-A	2009/5/18	
c	3	LEC24-090518-A	2009/5/25	
d	90512	LEC24-090511-A	2009/5/18	By an application of D213, the model's layers were desquamated. By an application of D216, white crystallizations in the cup were detected.
d	90519	LEC24-090518-A	2009/5/25	By an application of D213, the model's layers were desquamated. By an application of D216, white crystallizations in the cup were detected.
d	90526	LEC24-090525-A	2009/6/1	By an application of D213, the model's layers were desquamated. By an application of D216, white crystallizations in the cup were detected.
f	LAB-09VAL	LCE24-090420-A	2009/4/27	By an application of F232, the model's layers were desquamated.
f	LAB-09VAL	LEC24-090511-A	2009/5/18	
f	LAB-09VAL	LEC24-090518-A	2009/5/25	By an application of F232, the model's layers were desquamated.
g	①	LCE24-090420-A	2009/4/27	By an application of G256, the model's caps were discolored.
g	②	LCE24-090427-A	2009/5/4	By an application of G256, the model's caps were discolored.
g	③	LEC24-090511-A	2009/5/18	By an application of G256, the model's caps were discolored.

8-2-2. Negative control

32. In Table 10 and Fig.2, absorbances of negative control are shown. All data of negative control were sufficient with acceptance criteria excluding Lab a, test1. The mean OD of lab a, test 1 is 0.59 (0.61, 0.58, 0.57). We were not accepted at this result, and accepted the results of test 2-4 re-tested at Lab a.

Table 10 Absorbance of negative control

		Lab.					
Study	Run	a	b	c	d	f	g
2	1	0.75 (0.02)	0.93 (0.01)	0.91 (0.01)	0.82 (0.02)	0.84 (0.01)	1.13 (0.01)
	2	0.86 (0.02)	0.85 (0.04)	1.01 (0.02)	0.90 (0.04)	0.79 (0.02)	1.18 (0.02)
	3	0.82 (0.04)	0.84 (0.03)	0.93 (0.02)	0.96 (0.03)	0.83 (0.00)	1.05 (0.05)
3	1	0.90 (0.02)	0.96 (0.02)	1.04 (0.02)	1.11 (0.05)	0.90 (0.02)	0.91 (0.04)
	2	0.72 (0.02)	1.01 (0.02)	1.06 (0.01)	1.11 (0.04)	0.94 (0.02)	1.08 (0.01)
	3	0.80 (0.02)	0.97 (0.04)	1.01 (0.02)	1.13 (0.03)	0.92 (0.03)	0.88 (0.03)
Mean		0.81	0.93	0.99	1.01	0.87	1.04
Median		0.81	0.94	1.01	1.03	0.87	1.06
Min		0.72	0.84	0.91	0.82	0.79	0.88
Max		0.9	1.01	1.06	1.13	0.94	1.18
SD		0.07	0.07	0.06	0.13	0.06	0.12
Range		0.17	0.17	0.15	0.31	0.15	0.3

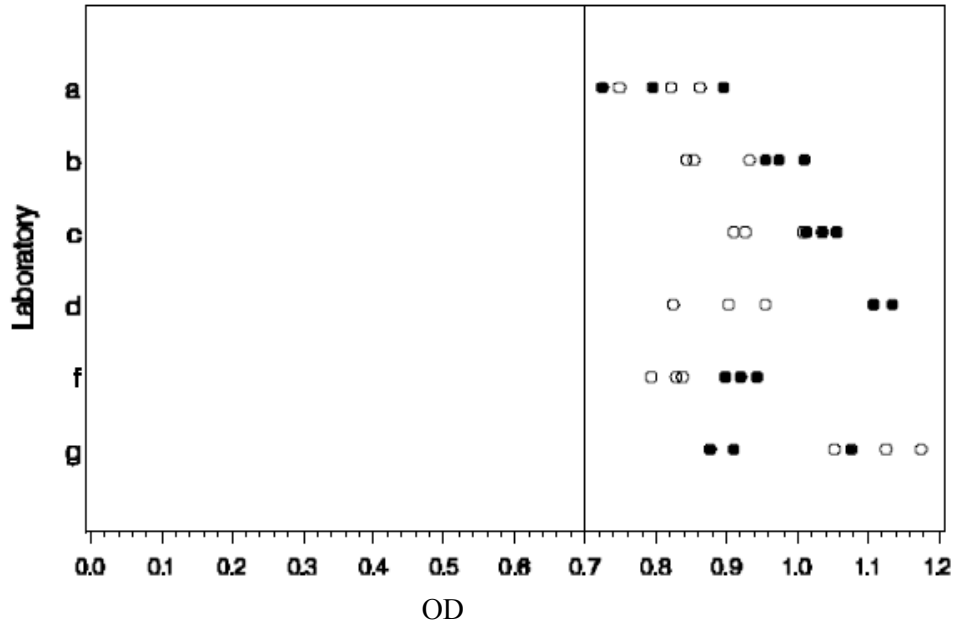


Fig.2 Distribution of Absorbance on negative control

8-2-3. Positive control

33. Table 11 and Fig.3 show three independent viabilities and statistical analysis of positive control at each laboratory. All data were sufficient with acceptance criteria of positive control.

Table 11. Viability of positive control

		Lab.					
Study	Run	a	b	c	d	f	g
2	1	5.9 (1.3)	5.2 (2.3)	4.1 (0.5)	5.7 (2.3)	3.5 (0.4)	3.1 (0.2)
	2	8.8 (4.8)	12.3 (6.9)	5.4 (3.0)	2.6 (0.3)	2.9 (0.3)	10.7 (5.3)
	3	2.5 (0.4)	7.8 (2.4)	3.8 (0.0)	3.3 (0.3)	3.2 (0.3)	4.2 (1.3)
3	1	6.4 (1.8)	9.3 (6.8)	8.2 (3.4)	3.5 (0.9)	8.5 (1.9)	11.7 (2.5)
	2	2.2 (0.4)	2.2 (0.1)	7.3 (2.2)	2.5 (0.3)	4.1 (1.3)	2.5 (0.1)
	3	1.8 (0.2)	1.6 (0.3)	2.4 (0.2)	2.1 (0.4)	2.7 (0.0)	3.3 (0.3)
Mean		4.6	6.4	5.2	3.3	4.1	5.9
Median		4.2	6.5	4.7	2.9	3.3	3.7
Min		1.8	1.6	2.4	2.1	2.7	2.5
Max		8.8	12.3	8.2	5.7	8.5	11.7
SD		2.9	4.2	2.2	1.3	2.2	4.1
Range		7	10.7	5.7	3.6	5.8	9.2

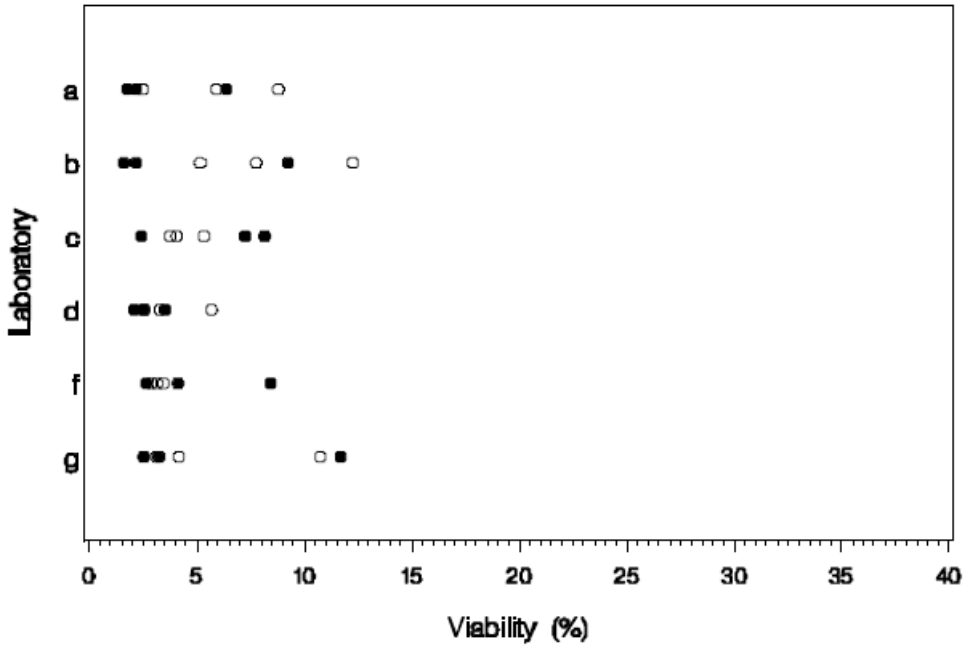


Fig.3 Distribution of viability on positive control

8-2-4. Skin irritation test by cell viability

34. The results of the skin irritation test with LabCyte EPI-MODEL 24 when it was only evaluated cell viabilities as indicator are shown in Table 12 in 2nd phase study and Table 14 in 3rd phase study. Summary statistical analysis of viability each chemical are shown in Table 13 and Fig.4 in 2nd phase study and Table 15 and Fig.5 in 3rd phase study.

35. Invalid data obtained only Lab a, run 1. This lab performed at retesting. Therefore, the data of lab a were accepted among run 2-4.

Table 12. Viability of chemicals at each laboratory by 2nd phase study.

				Lab.						
Chem.	Vivo	Score	Exp.	a	b	c	d	e	f	g
01	no	0	1	31.0	47.1	10.6	14.3	38.1	14.3	10.6
			2	11.2	10.4	20.3	9.1	25.2	11.2	10.6
			3	11.6	16.1	12.4	9.6	32.3	10.4	14.0
02	no	0	1	79.8	66.9	88.1	102.3	101.8	75.3	96.0
			2	76.5	61.7	89.7	89.8	76.4	67.2	94.8
			3	65.2	88.7	85.8	67.6	85.8	75.7	103.3
03	no	0	1	109.1	93.3	94.6	105.1	129.6	94.2	100.5
			2	103.9	99.8	93.1	112.8	106.6	97.9	93.4
			3	100.9	102.3	95.7	101.4	103.9	92.5	111.1
04	no	0	1	106.3	94.4	97.1	106.1	127.1	100.1	104.8
			2	95.2	100.2	99.9	100.9	113.6	92.8	103.3
			3	96.5	98.6	97.8	98.4	105.2	92.7	109.8
05	no	0.3	1	78.5	61.7	91.4	79.4	103.0	71.9	96.8
			2	78.5	71.9	95.2	70.5	90.3	39.3	89.9
			3	74.1	84.5	89.2	66.1	89.6	55.1	88.4
06	no	0.3	1	92.5	77.9	81.0	91.3	97.0	87.8	87.2
			2	79.4	83.5	79.1	102.4	81.5	94.4	81.2
			3	82.4	80.5	83.6	82.7	90.7	81.1	54.1
07	no	1	1	24.1	10.8	20.8	21.7	17.5	15.8	31.5
			2	12.6	12.6	16.2	13.8	22.2	31.1	22.5
			3	17.8	13.2	15.2	19.8	21.3	15.6	19.9
08	no	1	1	111.9	86.7	75.3	109.4	114.9	89.7	101.1
			2	90.2	100.6	82.3	107.5	100.9	97.8	100.9
			3	95.3	104.8	77.2	103.0	100.9	96.5	109.0
09	no	1.7	1	112.8	96.7	106.6	105.0	115.8	98.8	102.3
			2	97.1	110.1	96.8	103.4	108.6	86.5	103.4
			3	101.1	109.5	93.5	98.1	103.9	97.7	112.1
10	no	1.7	1	115.9	115.4	107.5	114.3	132.0	104.0	107.9
			2	104.1	110.1	103.6	108.2	117.0	101.2	108.4
			3	86.5	111.3	103.7	105.5	107.5	101.2	113.1

Table 12. continued

				Lab.						
Chem.	Vivo	Score	Exp.	a	b	c	d	e	f	g
11	no	2	1	113.7	105.0	101.0	102.4	123.1	103.1	102.8
			2	98.1	106.6	94.6	105.8	110.4	98.0	100.5
			3	112.6	103.7	94.1	102.7	105.5	94.6	109.0
12	no	2	1	28.2	24.6	24.9	54.3	55.6	27.2	87.7
			2	18.4	24.6	44.8	76.2	57.8	65.2	98.0
			3	15.3	15.9	28.1	27.4	57.2	66.0	112.6
14	Category 2	2.3	1	11.1	12.1	14.7	10.7	14.2	13.1	13.5
			2	6.6	8.3	9.5	11.7	12.0	16.7	12.0
			3	6.8	8.8	9.1	10.2	10.4	17.0	10.6
15	Category 2	2.3	1	11.1	9.3	13.1	8.0	11.0	8.6	9.2
			2	7.1	10.2	19.3	8.6	11.3	5.9	24.7
			3	8.2	9.9	8.1	9.2	8.7	7.1	9.2
16	Category 2	2.7	1	67.9	92.0	51.5	18.1	98.2	59.6	64.9
			2	32.2	54.1	86.3	79.2	90.6	50.4	79.6
			3	59.8	98.3	81.7	37.7	78.7	67.5	86.5
17	Category 2	2.7	1	6.1	4.5	5.3	6.6	8.9	6.9	6.2
			2	4.8	4.7	6.0	5.3	6.3	5.5	5.3
			3	5.6	5.7	5.9	3.9	5.4	4.5	5.3
18	Category 2	3	1	82.1	46.5	91.2	83.7	98.9	69.2	92.4
			2	78.3	50.6	87.3	69.9	87.2	80.6	85.9
			3	25.3	100.0	87.5	59.0	69.1	71.9	94.4
19	Category 2	3	1	15.0	74.6	10.0	30.4	83.1	40.1	35.8
			2	19.9	10.9	22.4	28.3	26.1	87.0	44.7
			3	51.1	32.0	35.0	18.2	69.4	71.8	38.7
20	Category 2	4	1	31.1	24.8	10.4	9.6	10.7	8.1	8.8
			2	9.3	8.0	7.6	16.9	8.2	7.8	6.7
			3	29.5	9.3	7.6	30.9	6.2	8.2	8.6

Table 13. Summary of the statistical analysis of the viability for each chemical by 2nd phase study.

		Lab.						
Chem.	Stat.	a	b	c	d	e	f	g
01	Mean	17.9	24.5	14.4	11.0	31.9	12.0	11.7
	Median	11.6	16.1	12.4	9.6	32.3	11.2	10.6
	Min	11.2	10.4	10.6	9.1	25.2	10.4	10.6
	Max	31.0	47.1	20.3	14.3	38.1	14.3	14.0
02	Mean	73.8	72.4	87.8	86.6	88.0	72.7	98.0
	Median	76.5	66.9	88.1	89.8	85.8	75.3	96.0
	Min	65.2	61.7	85.8	67.6	76.4	67.2	94.8
	Max	79.8	88.7	89.7	102.3	101.8	75.7	103.3
03	Mean	104.7	98.5	94.5	106.4	113.3	94.8	101.7
	Median	103.9	99.8	94.6	105.1	106.6	94.2	100.5
	Min	100.9	93.3	93.1	101.4	103.9	92.5	93.4
	Max	109.1	102.3	95.7	112.8	129.6	97.9	111.1
04	Mean	99.3	97.8	98.2	101.8	115.3	95.2	105.9
	Median	96.5	98.6	97.8	100.9	113.6	92.8	104.8
	Min	95.2	94.4	97.1	98.4	105.2	92.7	103.3
	Max	106.3	100.2	99.9	106.1	127.1	100.1	109.8
05	Mean	77.0	72.7	91.9	72.0	94.3	55.4	91.7
	Median	78.5	71.9	91.4	70.5	90.3	55.1	89.9
	Min	74.1	61.7	89.2	66.1	89.6	39.3	88.4
	Max	78.5	84.5	95.2	79.4	103.0	71.9	96.8
06	Mean	84.8	80.7	81.2	92.1	89.7	87.8	74.2
	Median	82.4	80.5	81.0	91.3	90.7	87.8	81.2
	Min	79.4	77.9	79.1	82.7	81.5	81.1	54.1
	Max	92.5	83.5	83.6	102.4	97.0	94.4	87.2
07	Mean	18.2	12.2	17.4	18.4	20.3	20.8	24.6
	Median	17.8	12.6	16.2	19.8	21.3	15.8	22.5
	Min	12.6	10.8	15.2	13.8	17.5	15.6	19.9
	Max	24.1	13.2	20.8	21.7	22.2	31.1	31.5
08	Mean	99.1	97.4	78.3	106.6	105.6	94.7	103.7
	Median	95.3	100.6	77.2	107.5	100.9	96.5	101.1
	Min	90.2	86.7	75.3	103.0	100.9	89.7	100.9
	Max	111.9	104.8	82.3	109.4	114.9	97.8	109.0
09	Mean	103.7	105.4	98.9	102.2	109.4	94.3	105.9
	Median	101.1	109.5	96.8	103.4	108.6	97.7	103.4
	Min	97.1	96.7	93.5	98.1	103.9	86.5	102.3
	Max	112.8	110.1	106.6	105.0	115.8	98.8	112.1
10	Mean	102.1	112.2	104.9	109.3	118.8	102.1	109.8
	Median	104.1	111.3	103.7	108.2	117.0	101.2	108.4
	Min	86.5	110.1	103.6	105.5	107.5	101.2	107.9
	Max	115.9	115.4	107.5	114.3	132.0	104.0	113.1

Table 13. continued.

		Lab.						
Chem.	Stat.	a	b	c	d	e	f	g
11	Mean	108.1	105.1	96.6	103.6	113.0	98.6	104.1
	Median	112.6	105.0	94.6	102.7	110.4	98.0	102.8
	Min	98.1	103.7	94.1	102.4	105.5	94.6	100.5
	Max	113.7	106.6	101.0	105.8	123.1	103.1	109.0
12	Mean	20.7	21.7	32.6	52.6	56.9	52.8	99.5
	Median	18.4	24.6	28.1	54.3	57.2	65.2	98.0
	Min	15.3	15.9	24.9	27.4	55.6	27.2	87.7
	Max	28.2	24.6	44.8	76.2	57.8	66.0	112.6
14	Mean	8.2	9.7	11.1	10.9	12.2	15.6	12.0
	Median	6.8	8.8	9.5	10.7	12.0	16.7	12.0
	Min	6.6	8.3	9.1	10.2	10.4	13.1	10.6
	Max	11.1	12.1	14.7	11.7	14.2	17.0	13.5
15	Mean	8.8	9.8	13.5	8.6	10.3	7.2	14.4
	Median	8.2	9.9	13.1	8.6	11.0	7.1	9.2
	Min	7.1	9.3	8.1	8.0	8.7	5.9	9.2
	Max	11.1	10.2	19.3	9.2	11.3	8.6	24.7
16	Mean	53.3	81.4	73.1	45.0	89.1	59.1	77.0
	Median	59.8	92.0	81.7	37.7	90.6	59.6	79.6
	Min	32.2	54.1	51.5	18.1	78.7	50.4	64.9
	Max	67.9	98.3	86.3	79.2	98.2	67.5	86.5
17	Mean	5.5	4.9	5.8	5.3	6.9	5.6	5.6
	Median	5.6	4.7	5.9	5.3	6.3	5.5	5.3
	Min	4.8	4.5	5.3	3.9	5.4	4.5	5.3
	Max	6.1	5.7	6.0	6.6	8.9	6.9	6.2
18	Mean	61.9	65.7	88.7	70.9	85.1	73.9	90.9
	Median	78.3	50.6	87.5	69.9	87.2	71.9	92.4
	Min	25.3	46.5	87.3	59.0	69.1	69.2	85.9
	Max	82.1	100.0	91.2	83.7	98.9	80.6	94.4
19	Mean	28.7	39.2	22.5	25.6	59.5	66.3	39.8
	Median	19.9	32.0	22.4	28.3	69.4	71.8	44.7
	Min	15.0	10.9	10.0	18.2	26.1	40.1	35.8
	Max	51.1	74.6	35.0	30.4	83.1	87.0	44.7
20	Mean	23.3	14.0	8.6	19.2	8.4	8.0	8.1
	Median	29.5	9.3	7.6	16.9	8.2	8.1	8.6
	Min	9.3	8.0	7.6	9.6	6.2	7.8	6.7
	Max	31.1	24.8	10.4	30.9	10.7	8.2	8.8

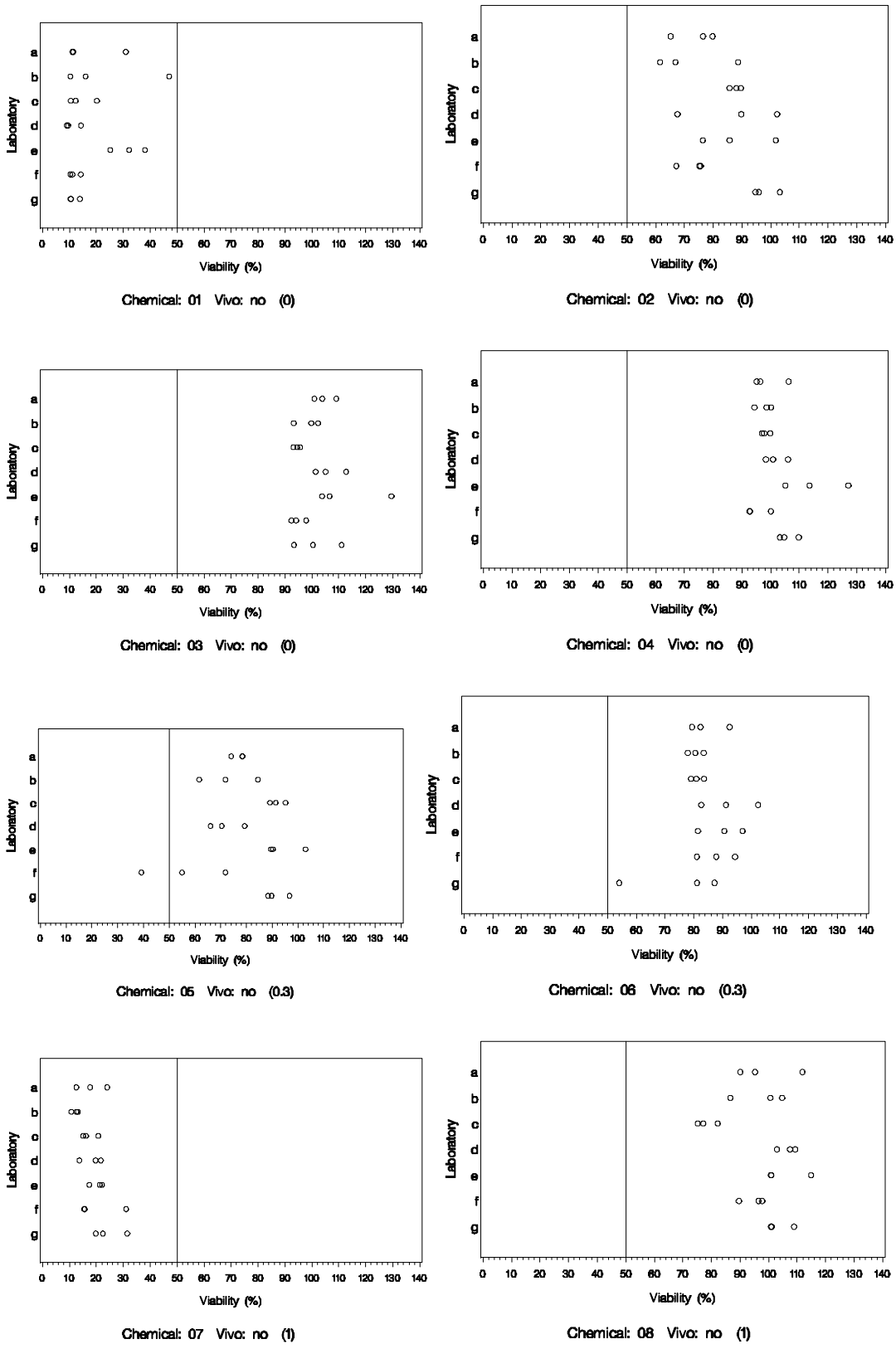


Fig. 4. Distribution of the viability for each chemical.

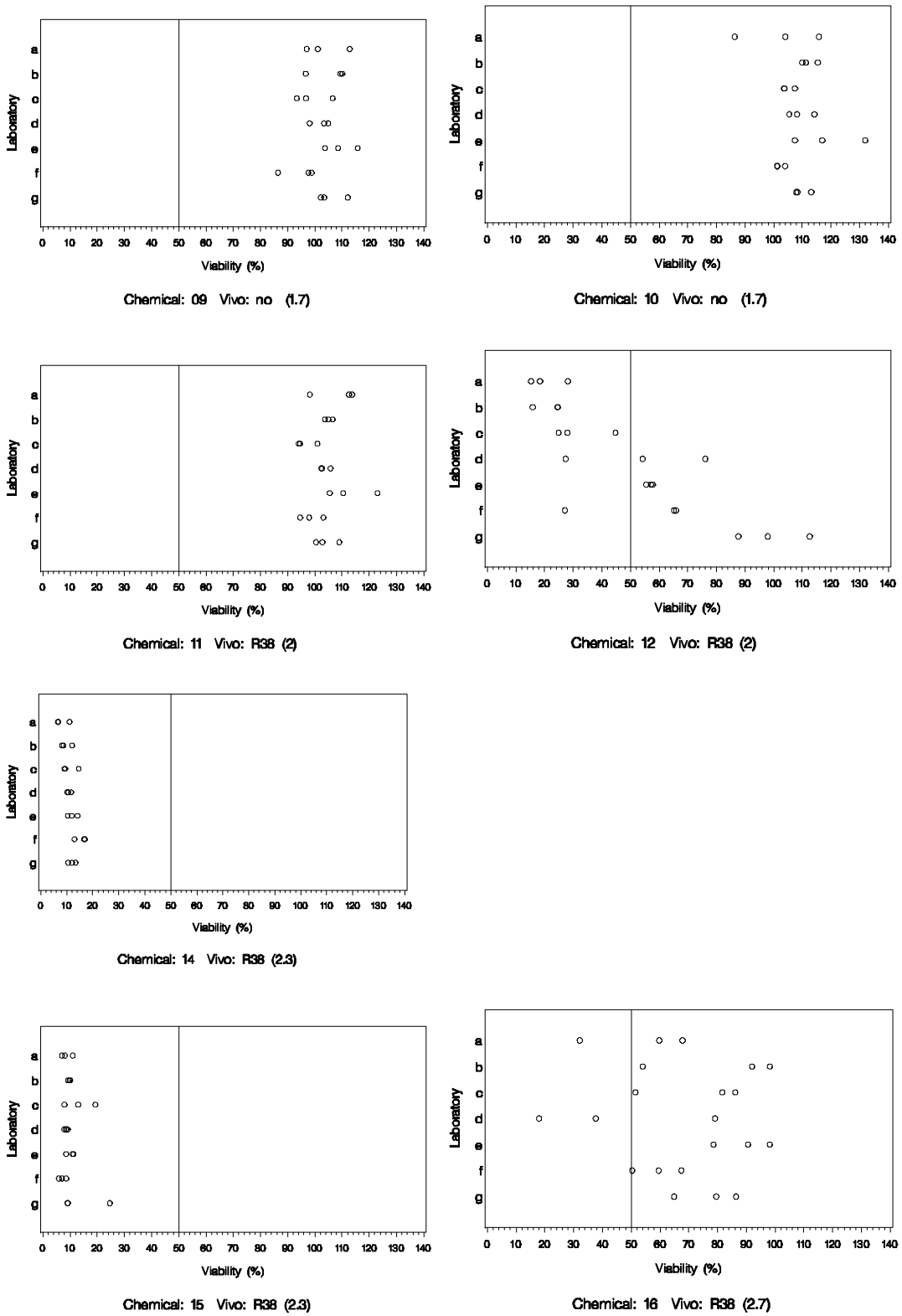


Fig. 4. continued

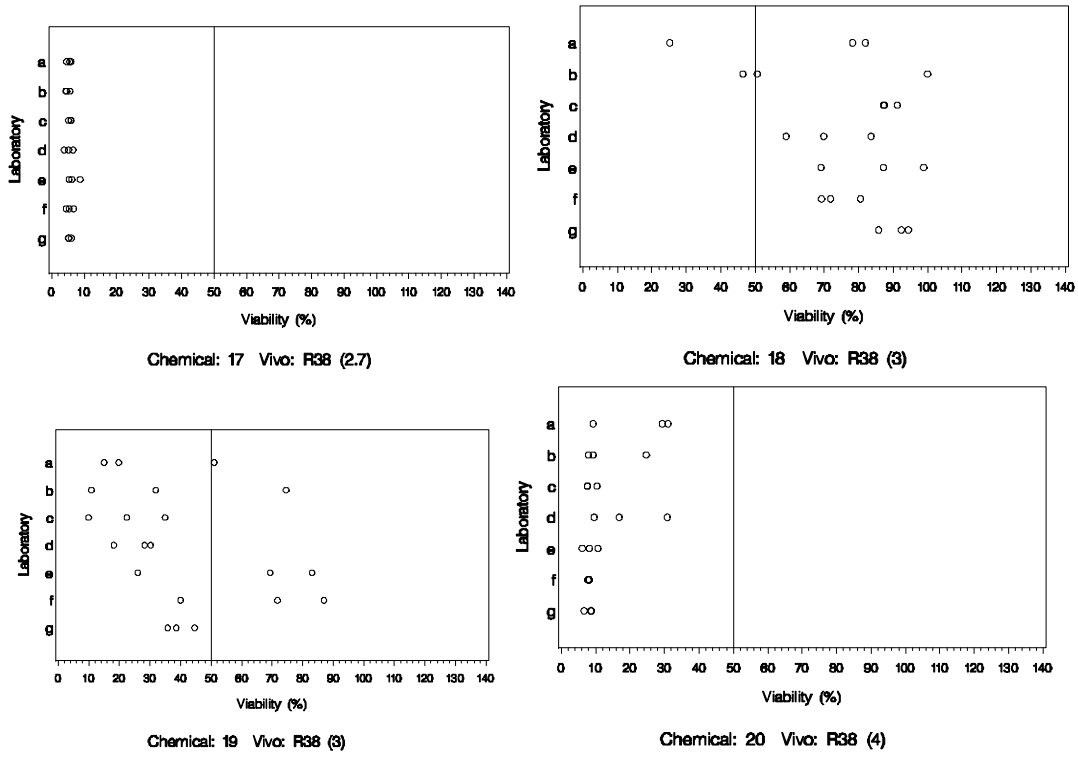


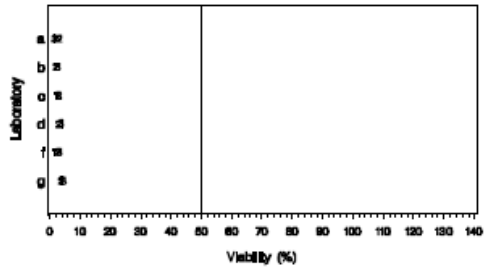
Fig. 4. continued.

Table 14. Viability of chemicals each laboratory by 3rd phase study

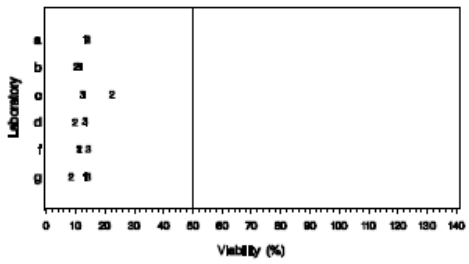
				Lab.					
Chem.	Vivo	Score	Exp.	a	b	c	d	f	g
A	no	2	1	13.3	11.8	13.2	13.8	11.4	13.7
			2	14.2	10.2	22.5	9.9	11.3	8.7
			3	14.0	11.1	12.3	13.2	14.3	14.3
B	Category 2	2.7	1	1.5	2.2	2.5	4.0	1.7	3.9
			2	3.1	2.2	2.9	3.0	2.6	3.7
			3	1.5	2.5	3.0	3.9	3.2	4.7
C	Category 2	3	1	0.7	0.7	0.7	6.9	0.8	1.0
			2	1.3	1.1	1.4	2.0	4.8	0.4
			3	0.5	0.8	1.0	0.8	1.0	0.3
D	Category 2	3.3	1	14.5	24.0	12.7	10.3	13.8	19.3
			2	13.6	16.0	12.5	18.3	8.8	15.2
			3	18.6	15.5	12.6	23.0	19.2	14.1
E	Category 2	3.3	1	3.9	3.4	3.4	8.2	3.2	4.1
			2	4.5	2.7	3.3	3.9	4.2	3.1
			3	1.8	3.5	3.5	3.7	5.0	5.1
F	Category 2	4	1	5.6	7.2	6.5	6.4	5.2	7.2
			2	5.7	6.1	6.8	5.4	7.4	6.8
			3	5.4	4.2	6.5	5.4	5.0	7.6

Table 15 Summary statistical analysis of viability each chemical by 3rd phase study

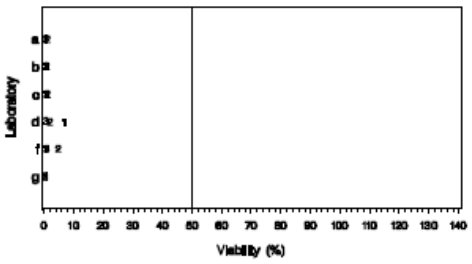
		Lab.					
Chem.	Stat.	a	b	c	d	f	g
A	Mean	13.8	11.0	16.0	12.3	12.3	12.2
	Median	14.0	11.1	13.2	13.2	11.4	13.7
	Min	13.3	10.2	12.3	9.9	11.3	8.7
	Max	14.2	11.8	22.5	13.8	14.3	14.3
B	Mean	2.0	2.3	2.8	3.6	2.5	4.1
	Median	1.5	2.2	2.9	3.9	2.6	3.9
	Min	1.5	2.2	2.5	3.0	1.7	3.7
	Max	3.1	2.5	3.0	4.0	3.2	4.7
C	Mean	0.8	0.8	1.0	3.2	2.2	0.6
	Median	0.7	0.8	1.0	2.0	1.0	0.4
	Min	0.5	0.7	0.7	0.8	0.8	0.3
	Max	1.3	1.1	1.4	6.9	4.8	1.0
D	Mean	15.6	18.5	12.6	17.2	13.9	16.2
	Median	14.5	16.0	12.6	18.3	13.8	15.2
	Min	13.6	15.5	12.5	10.3	8.8	14.1
	Max	18.6	24.0	12.7	23.0	19.2	19.3
E	Mean	3.4	3.2	3.4	5.3	4.2	4.1
	Median	3.9	3.4	3.4	3.9	4.2	4.1
	Min	1.8	2.7	3.3	3.7	3.2	3.4
	Max	4.5	3.5	3.5	8.2	5.0	5.1
F	Mean	5.5	5.8	6.6	5.7	5.9	7.2
	Median	5.6	6.1	6.5	5.4	5.2	7.2
	Min	5.4	4.2	6.5	5.4	5.0	6.8
	Max	5.7	7.2	6.8	6.4	7.4	7.6



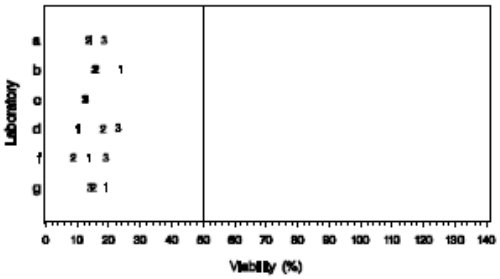
2−chloromethyl−3,5−dimethyl−4−methoxypyridine HCl



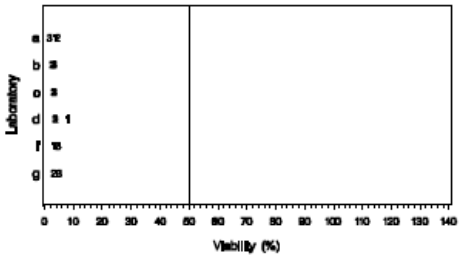
cinnamaldehyde



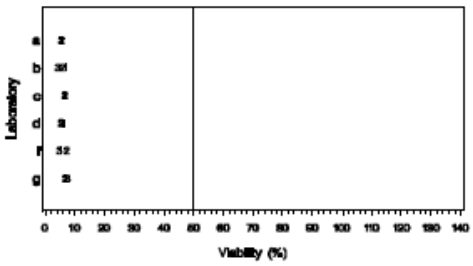
potassium hydroxide (5% aq.)



benzenethiol, 5−(1,1−dimethylethyl)−2−methyl



1−methyl−3−phenyl−1−piperazine



1,1,1−trichloroethane

Fig.5 Distribution of viability each chemical

8-2-5. IL-1α

36. The results of the LabCyte EPI-MODEL 24 skin irritation test when IL-1α was evaluated as an indicator are summarized in Table 16.

Table 16. IL-1α levels from each laboratory.

				Lab.						
Chem.	GHS	Score	Exp.	a	b	c	d	e	f	g
01	no	0	1	.	.	.	.	.	.	.
			2	.	.	.	.	.	.	.
			3	.	.	.	.	.	.	.
02	no	0	1	132.8	52.9	59.3	41.2	60.7	61.3	9.4
			2	68.1	56.5	37	89.1	68.4	99.3	9.6
			3	97.6	41.1	76	72.4	46	70.1	12.6
03	no	0	1	12	9.5	15.5	8.6	23.2	12.7	8.1
			2	7.1	8.6	11.7	19.9	10.5	9.2	11.9
			3	10.7	10.3	12.9	9.4	11.3	6.7	15.7
04	no	0	1	10	6	8	11.7	9.5	2.5	6.3
			2	5.3	8	5.5	13.2	15.1	2.6	8.6
			3	6.3	4.7	7.2	7.9	9.7	3.4	6.8
05	no	0.3	1	122	97.6	24.3	81.2	57.7	183.5	15.4
			2	35.7	63.5	35.1	115.3	36.6	.	28.5
			3	44.4	26	31.2	49.4	33	191.6	33.2
06	no	0.3	1	59	85.7	114	85.6	94.4	60.8	112.5
			2	62.9	93.6	104.9	139.5	81.4	48.1	62.1
			3	68.8	85.1	82.9	64.5	52.9	54.8	147.1
07	no	1	1	.	.	.	.	.	.	.
			2	.	.	.	.	.	.	.
			3	.	.	.	.	.	.	.
08	no	1	1	8.2	9.4	84.1	4.1	6.9	21.4	5.3
			2	3.6	6.4	31.6	10.4	8.5	4.9	5.8
			3	6	4.1	33.1	5.2	6.7	2.1	7.2
09	no	1.7	1	10.9	17.1	11.2	42.6	29.5	33	7.4
			2	19.8	8.8	8.8	32.2	6.5	25.3	9.7
			3	31.3	6.8	20.1	21.3	11.2	24.7	10.6
10	no	1.7	1	27.9	7.4	31.3	41.2	46.5	39.3	9.8
			2	17.1	12.7	15	50.4	26.7	26.7	14.5
			3	66.2	12.2	30	42.1	26.3	24.2	13.2

Table 16. continued.

				Lab.						
Chem.	GHS	Score	Exp.	a	b	c	d	e	f	g
11	no	2	1	5	31.1	18	15.3	10.4	16.2	6.4
			2	3.3	11.9	15.8	19	9.7	8.1	7.5
			3	18.2	5	8.9	8.7	8.6	12.6	11.9
12	no	2	1	.	.	.	157.2	120.4	.	34.5
			2	.	.	.	113	118.6	90.2	27.3
			3	.	.	.	.	58.3	66.2	13.6
14	Category 2	2.3	1	.	.	.	.	.	.	.
			2	.	.	.	.	.	.	.
			3	.	.	.	.	.	.	.
15	Category 2	2.3	1	.	.	.	.	.	.	.
			2	.	.	.	.	.	.	.
			3	.	.	.	.	.	.	.
16	Category 2	2.7	1	86.9	68.1	129.4	.	126.8	116.5	90.8
			2	.	100.2	74.4	169.7	76.1	107.5	70.9
			3	121.2	42.5	83.6	.	73.1	87.3	79.2
17	Category 2	2.7	1	.	.	.	.	.	.	.
			2	.	.	.	.	.	.	.
			3	.	.	.	.	.	.	.
18	Category 2	3	1	61.5	.	60.6	90.3	86.9	114.5	18
			2	57.7	104.9	45.8	221.3	98.7	76.4	45.1
			3	.	17.2	51.4	138.1	63.9	102.2	22.1
19	Category 2	3	1	.	57.3	.	.	109.2	.	.
			2	.	.	.	.	.	69.2	.
			3	102.3	.	.	.	68	59.5	.
20	Category 2	4	1	.	.	.	.	.	.	.
			2	.	.	.	.	.	.	.
			3	.	.	.	.	.	.	.

Cells highlighted in yellow indicate that the classification changed based on the IL-1α data.

8-2-6. Classification of three independent viabilities at each laboratory

37. The classifications from mean of three independent viabilities only evaluated MTT assay were shown in Table 17 in 2nd phase study and Table 19 in 3rd phase study. Refer to Table 18, the IL-1α results changed the classification for only 3 data points. The classification of **Allyl phenoxy-acetate** by Lab f was changed the misunderstood classification. The other two chemicals were changed the correct classification. Regarding the ILα only a few chemicals showed different results but the overall call was that ILα did not significantly contribute to the performance of the assay.

Table 17.Classification using three independent viabilities by 2nd phase study
「P」:Positive、「N」: Negative

			Lab.						
Chem.	**GHS**	**Score**	**a**	**b**	**c**	**d**	**e**	**f**	**g**
01	no	0	P	P	P	P	P	P	P
02	no	0	N	N	N	N	N	N	N
03	no	0	N	N	N	N	N	N	N
04	no	0	N	N	N	N	N	N	N
05	no	0.3	N	N	N	N	N	N	N
06	no	0.3	N	N	N	N	N	N	N
07	no	1	P	P	P	P	P	P	P
08	no	1	N	N	N	N	N	N	N
09	no	1.7	N	N	N	N	N	N	N
10	no	1.7	N	N	N	N	N	N	N
11	no	2	N	N	N	N	N	N	N
12	no	2	P	P	P	N	N	N	N
14	Category 2	2.3	P	P	P	P	P	P	P
15	Category 2	2.3	P	P	P	P	P	P	P
16	Category 2	2.7	**N**	**N**	**N**	P	**N**	**N**	**N**
17	Category 2	2.7	P	P	P	P	P	P	P
18	Category 2	3	**N**	**N**	**N**	**N**	**N**	**N**	**N**
19	Category 2	3	P	P	P	P	**N**	**N**	P
20	Category 2	4	P	P	P	P	P	P	P

Table.18. Classification of chemicals by MTT assay demolished by additional IL-1α measurement

No.	**Chemical**	**CAS number**	**GHS label**	**In vivo score (PII)**	**Lab.**	**Classification by MTT assay**	**Classification by** MTT+IL-1α
05	**Allyl phenoxy-acetate**	**7493-74-5**	**no**	**0.3**	f	N	P
16	**1-bromohexane**	**111-25-1**	**Category 2**	**2.7**	**a**	**N**	**P**
18	**di-n-propyl disulphide**	**629-19-6**	**Category 2**	**3**	**d**	**N**	**P**

Table 19 Classification using three independent viabilities by 3rd phase study
「P」:Positive、「N」: Negative

			Lab.					
Chem.	**in vivo**	**Score**	**a**	**b**	**c**	**d**	**f**	**g**
A	no	2	P	P	P	P	P	P
B	Category 2	2.7	P	P	P	P	P	P
C	Category 2	2.7	P	P	P	P	P	P
D	Category 2	3.3	P	P	P	P	P	P
E	Category 2	3.3	P	P	P	P	P	P
F	Category 2	4	P	P	P	P	P	P

Table 20. Sensitivity, specificity and accuracy on MTT assay vs GHS-EU classification in the 2nd + 3rd Phase validation study (25 substances)

Index	Lab.						
	a	b	c	d	f	g	
Sensitivity	10/12	10/12	10/12	11/12	9/12	10/12	
	83.3	83.3	83.3	91.6	75	83.3	
Specificity	9/13	9/13	9/13	10/13	10/13	10/13	
	69.2	69.2	69.2	76.9	76.9	76.9	
Accuracy	19/25	19/25	19/25	21/25	19/25	20/25	
	76	76	76	84	76	80	

Table 21. Sensitivity, specificity and accuracy on MTT assay vs GHS-EU classification in 2nd phase study (19 substances).

Index	Lab.						
	a	b	c	d	e	f	g
Sensitivity	5/7	5/7	5/7	6/7	4/7	4/7	5/7
	71.4	71.4	71.4	85.7	57.1	57.1	71.4
Specificity	9/12	9/12	9/12	10/12	10/12	10/12	10/12
	75	75	75	83.3	83.3	83.3	83.3
Accuracy	14/19	14/19	14/19	16/19	14/19	14/19	15/19
	73.7	73.7	73.7	84.2	73.7	73.7	78.9

Table 22. Sensitivity, specificity and accuracy of the MTT assay and IL-1α vs. the GHS-EU classification in 2nd phase study (19 substances).

Index	Lab.						
	a	b	c	d	e	f	g
Sensitivity	6/7	5/7	5/7	7/7	4/7	4/7	5/7
	85.7	71.4	71.4	100	57.1	57.1	71.4
Specificity	9/12	9/12	9/12	10/12	10/12	9/12	10/12
	75	75	75	83.3	83.3	75	83.3
Accuracy	15/19	14/19	14/19	17/19	14/19	13/19	15/19
	78.9	73.7	73.7	89.5	73.7	68.4	78.9

Table 23(A). Mean and range of Sensitivity, specificity and accuracy on the MTT assay using LabCyte EPI-MODEL vs. GHS-EU classification in the 2nd + 3rd Phase validation study (25 substances)

	N	Mean	Min.	Max.	ECVAM criteria
Sensivitity (%)	6	83.3	75.0	91.6	80.0
Specificity (%)	6	73.1	69.2	76.9	70.0
Accuracy (%)	6	78.0	76.0	84.0	75.0

Table 23(B). Mean and range of sensitivity, specificity and accuracy of the MTT assay vs. the GHS-EU classification in 2nd phase study (19 substances).

	N	Mean	Min.	Max.	ECVAM criteria
Sensivitity (%)	7	69.4	57.1	85.7	80.0
Specificity (%)	7	79.7	75.0	83.3	70.0
Accuracy (%)	7	75.9	73.7	84.2	75.0

Table 23(C). Mean and range of sensitivity, specificity and accuracy of the MTT assay and IL-1α vs. the GHS-EU classification in 2nd phase study (19 substances).

	N	Mean	Min.	Max.	ECVAM criteria
Sensivitity (%)	7	73.4	57.1	100.0	80.0
Specificity (%)	7	78.6	69.2	76.9	70.0
Accuracy (%)	7	76.7	68.4	89.5	75.0

9. Discussion

9-1. Reliability

38. All data of negative control and positive control each laboratory in 2nd and 3rd phase study was sufficient with the acceptance criteria as shown in Tables 10 and 11. There were high respectabilities within and between laboratories in this model.

39. In all data, Invalid data obtained only one data (Lab a, run 1). This lab performed at retesting and we accepted data of run 2-4. Therefore, the rate of invalid at this assay is 0.2% (total 1/508, 400 data: 3runs X 7 labs X 19 chemicals+1 run in 2nd phase study & 108 data; 3 runs X 6 labs X 6 chemicals in 3rd phase study). Based on a comparison of the results from the seven laboratories, the classification of 3 chemicals (No. 12, 16 and 19) should be potentially changed. However, the classifications of the remaining chemicals were not changed. The variations of these chemicals and No.18 are larger than those of others. The IL-1α data changed the classification for No. 5, 16 and 18 at Lab. f (No. 5), Lab. a (No. 16), and Lab. d (No. 18). The effect of IL-1α on the reliability of these results is small.

9-2. Predictivity

40. In December 2008, the EU adopted the UN Globally Harmonised System for Classification and Labelling and will implement this by means of the so-called CLP regulation (Regulation EC 1272/2008). The new EU classification system based on UN GHS (abbreviated here as "GHS-EU") continues to use two categories to distinguish non-irritant (no-category) from irritant (category 2) substances. However, according to the new rules for skin irritation classification and labelling, the cut-off score to distinguish between no-category and category 2 substances was shifted to 2.3 from a value of 2.0 (EU classification system). Consequently substances with an in vivo score between 2.0 and 2.3 that are considered irritant under the existing EU classification system will be considered non-irritants under the future GHS-EU classification system, which does not use the optional UN GHS category 3.

41. The prediction values of the LabCyte EPI-MODEL 24 skin irritation test when it was evaluated by cell viabilities (MTT) as an indicator, and the GHS-EU classifications are shown in Table 20. The sensitivity, specificity and accuracy of this prediction model at each laboratory were 75-91.6 %, 69.2-76.9 %, and 76-84 %, respectively. These predictivities were similar with each laboratory. The mean and range of prediction values of the skin irritation test with LabCyte EPI-MODEL 24 when it was only evaluated by MTT as an indicator and the GHS-EU classification are shown in Table 23(A). The mean sensitivity, specificity and accuracy of this prediction model are 83.3%, 73.1%, and 78.0%, respectively. Some deviations from the ESAC Performance standard (sensitivity of 80%, specificity of 70% and an accuracy of 75%) that were specific adaptations for the Japanese model.The effect of IL-1α on the predictivity was small compared with results in Tables 21,22, 23 (B) and 23(c).

10. Conclusions

42. Based on the GHS-EU classification, 12 irritants and 13 non-irritants in the ECVAM Performance Standards(2007,2009) were tested by the 7 labs using **LabCyte** EPI-MODEL. The assay demonstrated high reliability within and between laboratories, and acceptable reliability of the positive control (100%) and accuracy (77.5% overall accuracy, 82.3% overall sensitivity, 72.6% overall specificity) on the MTT assay for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

This report summarized at JSAAE 1st report and 2nd report on this validation study.

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OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France

Fax: (33-1) 44 30 61 80 E-mail: ehscont@oecd.org

FOREWORD

This document presents the Peer Review Report of the validation of the "*Skin Irritation Test using LabCyte EPI-MODEL24*", as a "*me-too*" development following the Performance Standards of Test Guideline 439: *In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method,* including four annexes. Additional information was generated after the peer review because the first validation study was on-going when OECD experts were discussing draft Performance Standards (PS) for TG 439.

The LabCyte test method has been considered as similar to the Reconstructed Human Epidermis Test Method that is the basis of TG 439, and has therefore been subjected to an additional validation study: a "*me-too*" validation study based on the PS of TG 439. This document also presents:

- Appendix 1: JaCVAM Re-analysis of the initial validation data according to the Performance Standards of TG 439
- Appendix 2: Background Document for a modified skin irritation test using LabCyte EPI-MODEL24
- Appendix 3: Standard Operating Procedure for the Skin irritation test using the LabCyte EPI-MODEL24 for the additional validation study
- Appendix 4: additional validation study: "me-too" validation study according to peer review report.

Finally, the report includes the agreement of the Working Group of National Coordinators of the Test Guidelines Programme (WNT) on the follow-up to the peer review.

The project for developing a Test Guideline for the in vitro epidermal model LabCyte EPIMODEL24 to assess skin irritation was proposed by Japan and included in the work plan of the Test Guidelines Programme in 2009. A validation report was published as No. 147 in the Series on Testing and Assessment. An OECD Peer Review was performed in December 2009 and the final Peer Review Panel (PRP) Report was available on 17th of March 2010.

This document was endorsed by the WNT at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (Joint Meeting) agreed to its declassification on 5 October 2011.

This document is published under the responsibility of the Joint Meeting.

Agreement of the Working Group of National Coordinators of the Test Guidelines Programme on the follow-up to the Peer Review Report

The peer review report of the validation of the modified skin irritation test method using the LabCyte EPI-Model24 as a TG 439 "me-too" test was submitted to the Working Group of National Coordinators of the Test Guidelines Programme (WNT) at its meeting held on 12-14 April 2011, for endorsement.

Considering

- the issues raised in the peer review report regarding the performance of the assay including within and between laboratory reproducibility, variability between replicates and most notably, the misclassification of the strong irritant reference chemical, 1-bromohexane,

- Additional information provided by Japan to address the issues raised in the peer review report,

The WNT agreed that before adding the "me too test" to TG 439, the Expert Group on Skin Irritation and Corrosion should review all existing data of the LabCyte test method, and submit a recommendation for WNT approval of the test method as a "me-too" test method to TG 439, or for further work if necessary. In case the Expert Group agrees on that the LabCyte test method is validated and should be added to TG 439, this approval would be requested from the WNT by a written procedure.

SUMMARY REPORT OF THE PEER REVIEW PANEL ON LABCYTE EPI-MODEL 24 IN VITRO TEST METHOD FOR THE ASSESSMENT OF SKIN IRRITATION POTENTIAL OF CHEMICALS

prepared for

Environment Directorate
Organisation for Economic Co-operation and Development Paris

March 17, 2010

PREAMBLE

This document presents the summary report of the assessment by an independent Peer Review Panel on the LabCyte EPI-MODEL 24 in vitro test method for the assessment of skin irritation potential of chemicals.

Until today, for regulatory purposes the skin irritation potential of chemicals has been investigated by the Draize skin test. In this in vivo experiment, the chemical is applied for usually four hours to the skin of rabbits. Based on a scoring of the two endpoints erythema and oedema formation and the reversibility of such effects, the skin irritation potential is assessed.

However, the advancement of in vitro methods has brought forward reconstructed human epidermis models. These models are based on non-transformed human-derived epidermal keratinocytes. The keratinocytes are cultured to form a multilayered, highly differentiated model of the human *epidermis.* After topical exposure to a neat test chemical, cell viability is measured by the amount MTT [(3-4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazoliumbromide] being reduced by dehydrogenase. The relative reduction of viability is then related to the skin irritation potential of the chemical.

After several years of evaluation and optimisation, two models underwent formal validation by the European Centre for the Validation of Alternative Methods (ECVAM) resulting in a statement on the scientific validity of the tests by ECVAM Scientific Advisory Board (ESAC). As a consequence, the Organisation for Economic Co-operation and Development (OECD) is currently in the process of drafting the respective OECD test guideline 'In Vitro Skin Irritation: Reconstructed Human Epidermis (RhE) Test Method'.

Anticipating that in future further reconstructed human epidermis models will be developed, the draft test guideline includes the annex 'Performance standards for assessment of proposed similar or modified in vitro reconstructed human epidermis (RhE) test methods for skin irritation'. These performance standards can be used to assess other analogous test methods (colloquially referred to as "me-too" tests) that are based on similar scientific principles and measure or predict the same biological or toxic effect.

The LabCyte EPI-MODEL 24 in vitro test method is considered to potentially be such a test method, to which the performance standards apply. The method was validated in a study employing several Japanese laboratories. The documentation of this study together with some additional information has been provided to the OECD and constitutes the basis for this peer review.

Summary Report of the Peer Review Panel
on the LabCyte EPI-MODEL 24 in vitro test method for the assessment of skin irritation potential of chemicals

The peer review process

1. The Peer Review Panel (Panel) was constituted in December 2009, to provide an independent review of the validation of the LabCyte EPI-MODEL 24 in vitro test method for the assessment of skin irritation potential of chemicals. The test method has been considered as a similar Reconstructed Human Epidermis (RhE) Test Method and underwent therefore a validation study referring to the respective performance standards. In this respect it has to be noted that the validation of the LabCyte EPI-MODEL 24 took place while the OECD test guideline 'In Vitro Skin Irritation: Reconstructed Human Epidermis (RhE) Test Method' including the annex on performance standards was still in the drafting process. The work of the Panel was coordinated by a panel chair contracted by the OECD. In addition to experts invited by the Secretariat, potential Panel members were nominated by the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) and then approached by the chair. From the originally five assigned panel members, one resigned from the assignment in January 2010, but was immediately replaced. The members of the Panel are listed in Annex 1.

Furthermore, an independent contact person who is familiar with the LabCyte EPI-MODEL 24 was nominated to support the panel in case of open issues needing clarification.

2. The Panel was asked to evaluate the data collected on the test method, and to answer specific charge questions. These questions were proposed by the panel chair and agreed by the (OECD). Panel members were asked to base their review on nine documents, which have been provided by the OECD. These documents contained information relevant to the validation of the LabCyte EPI-MODEL 24.

As background information, they were also provided with the three following documents:

- OECD Guidance Document on the Validation and International Acceptance of New or Updated Methods for Hazard Assessment, Series on Testing and Assessment, Number 34, 2005
  (last access on March 08, 2010, under http://www.olis.oecd.org/olis/2005doc.nsf/LinkTo/NT00002EAE/$FILE/JT00188291.pdf)
- OECD GUIDELINE FOR THE TESTING OF CHEMICALS (DRAFT PROPOSAL FOR A NEW GUIDELINE) - *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* (RhE) Test Method
  (last access on March 08, 2010, under http://www.oecd.org/dataoecd/1/59/43664841.pdf)
- European Commission: Explanatory Background Document to the OECD Draft Test Guideline on in vitro Skin Irritation Testing
  (last access on March 08, 2010, under http://ecvam.jrc.ec.europa.eu/ft_doc/ECVAM-BfR-OECDv3.0_OECD_post_ECM2c.pdf)

Two further documents, one describing the content of the review documents, and the other providing a complete version of Table 16 of the file 'D3 LabCyte_skin_irritation_testprotocol_ver_7.2 NC', were added by the PRP chair to facilitate the review. All documents, identified by their file names as provided to the PRP, are listed in Annex 2.

The charge to the Panel was to assess to what extent the eight OECD validation criteria set out in the OECD Guidance Document had been met and to evaluate whether the test method complies with the performance standards of the draft OECD Test Guideline on Skin Irritation. It has to be noted that these

two sets of charge questions are not to be considered independent as they address similar aspects. All eleven charge questions are listed in Annex 3. A summary of the Panel's responses to the individual questions is presented in paragraphs 4 to 40 below. For transparency, the individual comments from the Panel members are provided in Annex 4.

3. During the evaluation process, the Panel held two teleconferences which were organised and coordinated by the chair. Subsequently, each Panel member provided written responses on the charge questions to the PRP chair by February 15, 2010. Based on these responses, a draft report was compiled by the chair and provided to the PRP for review and comments (February 22, 2010). In this draft all aspects raised by at least one reviewer were included. Furthermore, it contained some clarifications on specific issues added by the chair. The PRP commented on the draft report until March 02, 2010. Accounting for this feedback and resolving remaining open issues, the final report was drafted by the chair and send to the PRP for approval on March 09, 2010. This report presents the resulting approved responses of the Panel to each of the charge questions.

General Panel responses

4. The Panel stated that the presentation of the review information could have been more structured and focused, e.g. by referencing to the performance standards and/or to the charge questions. Therefore, it proposes to facilitate future reviews by aligning the presentation of information with the review charge questions.
All information in the review documents on IL-1α has not been considered by the Panel because this end-point has not been previously declared as formally validated and there are no performance standards available

Panel responses to the charge questions - part I: The eight OECD principles and criteria for test method validation

5. The PRP reached consensus regarding the charge questions relating to the eight OECD principles and criteria for test method validation.

Charge question 1: A rationale for the test method should be available, including a clear statement of scientific need and regulatory purpose.

6. The Panel agreed that this criterion had been fully met. The rationale for the test method is clearly stated with regard to the scientific basis and regulatory purpose. In addition, as the LabCyte EPI-MODEL 24 refers to performance standards of presumably similar methods, which are in the process of OECD adoption, the general need and regulatory purpose as defined in the draft guideline applies as well.

Charge question 2: The relationship between the test method endpoint(s) and the biological effect and to the toxicity of interest should be addressed, describing limitations of the test methods.

7. The Panel agreed that this criterion had been partly met.

8. In particular, it has not been met with regard to the description of limitations. In the provided SOP this topic is only touched, but not sufficiently covered. For example, substances sticking to the tissue might create problems. Respective information might be deductible from the literature of the validated test methods, e.g. from paragraph 7 of the respective OECD draft test guideline.

9. Although, in general acceptable, the relationship of the method endpoint and the biological effect could be described in more detail. As a potential me-too method this relationship has been described earlier for the already validated methods and does thus apply also to the LabCyte EPI-MODEL 24. However, addition of information as given in paragraph 11 of the OECD Draft guideline (Version 7.6) would be helpful.

Charge question 3: A detailed protocol for the test method should be available

10. The Panel agreed that this criterion had been partly met.

11. In particular, a measure of variability between tissue replicates has not been defined.

12. Furthermore, as already addressed under paragraph 8, limitations are not sufficiently covered.

13. Particular protocol changes have been proposed by the panel. The most important suggestion was to explain the prediction model in more detail. Minor suggestions were
- to better define the storage temperature of the MTT solution and the positive control substance
- to better and more objectively define changes considered significant for chemicals that stain the tissue
- to change the instruction '...must be repeated three times...' to '...must be performed three times in total...'
- to include instructions for the case, when only two tissues provide measurements, e.g. when one tissue is damaged

Charge question 4: Within- and between-laboratory reproducibility of the test method should be demonstrated

14. The Panel agreed that this criterion had been partly met.

15. Most importantly, within- and between-laboratory reproducibility have not been calculated. All required data for the calculation have been provided, but appropriate statistical analysis and documentation is missing and should be included. In particular, these should be addressed by an appropriate measure of variability between runs viabilities, e.g. the standard deviation, and the concordance of classifications based on run. Results should be discussed, especially with regard to less reproducible chemicals

16. Regarding intra-assay reproducibility, a measure of variability between tissues has not been defined, nor calculated (see also paragraph 11.)

Charge question 5: Demonstration of the test method's performance should be based on testing of representative, preferably coded reference chemicals

17. The Panel agreed that this criterion had been met.

18. All chemicals currently proposed by the OECD draft test guideline have been tested, with one acceptable exception, in a coded manner.

Charge question 6: The performance of test methods should have been evaluated in relation to existing relevant toxicity data as well as information from the relevant target species.

19. The Panel agreed that this criterion had been met.

20. As only chemicals which have been included in other validation activities leading to the OECD draft test guideline, were tested, the relation to existing toxicity data from the relevant target species was established.

Charge question 7: All data supporting the assessment of the validity of the test method should be available for expert review

21. The Panel agreed that this criterion had been partly met.

22. As mentioned before, within- and between-laboratory reproducibility have not been calculated and documented (see paragraph 15).

23. Regarding intra-assay reproducibility, a measure of variability between tissues has not been defined, nor calculated (see also paragraph 11).

Charge question 8: Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of Good Laboratory Practice (GLP)

24. The Panel agreed that this criterion had been partly met. It has to be noted that this optional criterion initially led to different individual interpretation by the PRP members.

25. In general, the conduct of the study in the spirit of GLP is acceptable. For example, also in the original validation study, which led to the draft OECD test guideline, not all laboratories were GLP compliant. However, the panel agreed that the adherence to GLP principles could have been described in more detail. If available, documentation on this issue generated in the frame of the validation study of the LabCyte-EPIMODEL 24 should be provided.

Panel responses to the charge questions - part II: Performance Standards of the draft Test Guideline on Skin Irritation

26. The PRP reached consensus regarding the charge questions relating to the Performance Standards of the draft Test Guideline on Skin Irritation.

Charge question 9: Adherence to the essential test method components should be demonstrated

27. The Panel agreed that this criterion had been partly met. This charge question refers to the test method components as specified in the draft OECD test guideline. The respective components will be addressed individually.

28. Functional conditions – Viability: An upper acceptance limit for the negative control tissues, e.g. defined by a maximum optical density (OD), is missing. Although the data base for defining this might be small, it should nevertheless be defined if possible. Furthermore, it has not been defined what is considered as an acceptable variation of viabilities from replicate tissues.

29. Functional conditions – Barrier function: Further data on lipid composition could be added if available.

30. Functional conditions – Morphology: Acceptance criteria required for batch acceptance should be defined.

31. Functional conditions – Reproducibility: A measure of variability between tissues has not been defined, nor calculated (see also paragraph 11).

32. Functional conditions – Quality control (QC): It is not entirely evident how the model producer intends to ensure and demonstrate that each batch of the RhE model used meets defined production release criteria.

Charge question 10: Reliability and accuracy should be demonstrated by using at least the recommended reference chemicals

33. The Panel agreed that this criterion had been met.

Charge question 11: The test should have been assessed based on the defined reliability and accuracy values

34. The Panel agreed that this criterion had not been met. When addressing this question, the panel agreed to exclude data of 'Lab e' from analysis according to data 'Rule 2' of paragraph 14 of annex 2 of the OECD draft test guideline, which addresses the performance standards. Therefore, the data of 'Lab e' have not been considered.

35. Most importantly, the specific restriction of the performance standards that only two *in vivo* Category 2 substances, 1-decanol and di-n-propyl disulphide, may be misclassified as No Category by more than one participating laboratory, has not been met. In particular, 1-bromohexane, Category 2, has been misclassified as No Category by five of six participating laboratories (excluding 'Lab e' data). In addition, but without consequence, butyl methacrylate, Category 2, has been misclassified as No Category in one of six laboratories (excluding 'Lab e' data, which also misclassified butyl methacrylate). As a potential solution optimisation of the protocol either regarding the application time (prolongation beyond 30 minutes, with the risk of a decrease in specificity and overall accuracy) or regarding the test substance application (re-spreading of the material during application) has been proposed.

36. The panel agreed that the within- and between laboratory reproducibility has not been assessed with regard to the requirements of the performance standards. A detailed extended evaluation of these reproducibility values addressing the performance standards requirements should be presented (see also paragraph 15).

37. The panel anticipated that one result of a detailed analysis of the within- and between laboratory reproducibility would be that two laboratories might have a within-laboratory reproducibility of lower than 90%. This would not comply with the requirement of paragraph 14 of annex 2 of the OECD draft test guideline that '...within-laboratory variability should show a concordance of classifications (GHS Category 2/No Category) obtained in different, independent test runs of the 20 Reference Chemicals within one single laboratory equal or higher (≥) than 90%'.

38. In contrast to the performance standards, the median viability instead of the mean viability, as required in 'Rule 2' of paragraph 9 of annex 2 of the OECD draft test guideline, which addresses the performance standards, has been used for deriving a final classification for a complete run sequence of a given laboratory. The panel expects that this will result in only minor differences in results, but suggests to re-analyse the data and document the results accordingly.

39. Regarding the predictive values, the panel agreed that the requirements, i.e. sensitivity ≥ 80%, specificity ≥ 70% and accuracy ≥ 75%, were met, when considering all 25 tested chemicals. It was noted,

however, that the overall sensitivity would be ≤ 80% when considering only the 19 chemicals tested in the second phase of the study.

Recommendations

40. The Panel agrees that this report provides a summary of their views on the status of the validation of the LabCyte EPI-MODEL 24 in vitro test method for the assessment of skin irritation potential of chemicals, as detailed in the responses to the questions posed to the Panel and based on the information related to the test method validation provided to the Panel.

41. The report of the Panel, along with the provided review documents on the LabCyte EPI-MODEL 24 should form the basis for decisions on whether the validation meets the OECD principles for validation and the performance standards of the draft OECD Test Guideline '*In Vitro* Skin Irritation: Reconstructed Human *Epidermis* (RhE) Test Method'. The Panel recommends that the OECD considers the Panel report as guidance for recommending additional work required to fully meet all OECD principles and performance standards.

42. Future work should focus especially on the following aspects. Most importantly, the issue of misclassifying 1-bromohexane should be resolved. Furthermore, an extensive analysis of the within- and between reproducibility referring to the performance standards of the draft OECD Test Guideline should be carried out and appropriately documented. It is also recommended to assess variability between replicate tissues and to define a respective acceptance criterion. In order to comply better with the performance standards, analyses using the mean instead of the median for deriving a final classification for a complete run sequence of a given laboratory should be carried out. Finally, appropriate documentation describing and demonstrating the adherence to GLP principles should be provided.

ANNEX 1

MEMBERS OF THE PEER REVIEW PANEL

Panel member	Affiliation
Craig Blackstock	Charles River, Edinburgh, UK
Penny Jones	Unilever, Bedford, UK
Kristina Kejlová	National Institute of Public Health, Prague, Czech Republic
Albrecht Poth	Harlan Cytotest Cell Research GmbH, Rossdorf, Germany
Klaus Rudolf Schröder	BioMed- zet Life Science GmbH, Linz, Austria (until 31.12.2009 Henkel AG & Co. KGaA, Germany)

ANNEX 2

List of all documents provided to the PRP
(identified by file name)

- A1 _J_ToxicolSci(2009).pdf
- A4 LabCyte_skin_irritation_testprotocol_ver_6.01 NC.pdf
- A6 Draft 2nd report LabCyte0907____.pdf
- Appendix7.pdf
- D1 A suitable exposure time for SIT with LabCyte EPI-MODEL24.pdf
- D2 LabCyte EPI-MODEL background data document.pdf
- D3 LabCyte_skin_irritation_testprotocol_ver_7.2 NC.pdf
- LabCyte report 091008.pdf
- D3 Table 16.pdf
- List of J-TEC reference document.pdf
- Content of review documents.docx
- 3rd TG SKIN IRR_V.7.6_9 Sept_09_Clean.pdf
- GD No.34.pdf
- SKIN IRR BACKGROUND DOC (V.3 0) 14 August 2009.pdf

ANNEX 3

Charge questions

PRP Charge questions - part I:
The eight OECD principles and criteria for test method validation

1. A rationale for the test method should be available, including a clear statement of scientific need and regulatory purpose.

2. The relationship between the test method endpoint(s) and the biological effect and to the toxicity of interest should be addressed, describing limitations of the test methods.

3. A detailed protocol for the test method should be available.

4. Within- and between-laboratory reproducibility of the test method should be demonstrated.

5. Demonstration of the test method's performance should be based on testing of representative, preferably coded reference chemicals.

6. The performance of test methods should have been evaluated in relation to existing relevant toxicity data as well as information from the relevant target species.

7. All data supporting the assessment of the validity of the test method should be available for expert review.

8. Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of Good Laboratory Practice (GLP)

PRP Charge questions - part II:
Performance Standards of the draft Test Guideline on Skin Irritation

9. Adherence to the essential test method components should be demonstrated *(see also question 3)*.

10. Reliability and accuracy should be demonstrated by using at least the recommended reference chemicals *(see also question 5)*.

11. The test should have been assessed based on the defined reliability and accuracy values *(see also questions 4 and 6*

ANNEX 4

COLLATED COMMENTS FROM THE PEER REVIEW PANEL ON THE LABCYTE EPI-MODEL24 IN VITRO TEST METHOD FOR THE ASSESSMENT OF SKIN IRRITATION POTENTIAL OF CHEMICALS

ISSUES	COMMENTS AND RECOMMENDATIONS
General comments	The overall impression of the Panel is that the LabCyte EPI-MODEL 24 has been properly evaluated in validation activities. Consensus has been reached for all charge questions. Regarding the eight OECD principles and criteria for test method validation, several issues still need to be addressed more thoroughly. The major criticism from all Panel members was that the within-and between-laboratory reproducibility has not been properly analysed and documented. Furthermore, a measurement for variability between identically treated tissues needs still to be defined together with a respective acceptance criterion. Regarding the additional three charge questions referring to the performance standards of the draft Test Guideline on Skin Irritation, the Panel members agreed that the specific restriction that only two *in vivo* Category 2 substances, 1-decanol and di-n-propyl disulphide, may be misclassified as 'No Category' by more than one participating laboratory, has not been met. In particular, 1-bromohexane, Category 2, has been misclassified as 'No Category' by five of six participating laboratories.
VALIDATION CRITERIA	
1. Rationale for the test method	The Panel agreed that the rationale for the test method is clearly stated, while the scientific basis and the regulatory purpose are well described. The test is intended to replace the Draize skin irritation test for regulatory purposes as currently defined in OECD Test Guideline 404. **The Panel agreed that this criterion has been met.**

2. Relationship between the test method endpoint(s) and the biological effect and to the toxicity of interest	The panel agreed that the relevance of using chemically induced cytotoxicity measured by MTT to a human skin model to assess skin irritation potential has been well established already in the evaluation of the models leading to draft OECD Test Guideline. However, it is recommended to describe this more explicitly, e.g. as done in the draft guideline (Version 7.6) in paragraph 11. Furthermore, the panel agreed that the limitations of the model, e.g. regarding certain properties of test chemicals, should be described in more detail as for example done in paragraph 7 the draft guideline (Version 7.6). **The Panel agreed that this criterion had been partly met.**
3. Availability of a detailed test method protocol	The panel evaluated the provided protocol (version 7.2). Although largely appropriate, the panel recommends to: - elaborate on the test method limitations - include a measurement of variability between replicate tissues including a respective acceptance criterion - explain the prediction model in more detail - better define the storage temperature of the MTT solution and the positive control substance - better and more objectively define changes considered significant for chemicals that stain the tissue - change the instruction '...must be repeated three times...' to '...must be performed three times in total...' - include instructions for the case, when only two tissues provide measurements, e.g. when one tissue is damaged. **The Panel agreed that this criterion had been partly met.**
4. Demonstration of within- and between-laboratory reproducibility	The Panel agreed that the presented information on within- and between-laboratory reproducibility is not sufficient. As the generated data would allow a comprehensive evaluation of this aspect, the panel recommends to conduct and document a detailed statistical analysis of within- and between-laboratory reproducibility, including an evaluation of variability between replicate tissues. **The Panel agreed that this criterion had been partly met.**

5. Demonstration of the test method's performance based on testing of representative reference chemicals	The Panel agreed that the test chemicals, which were tested coded, was representative and appropriate for the demonstration of the test method's performance. In particular, all reference chemicals currently proposed in the draft OECD Test Guideline have been tested with the acceptable exception of tetrachloroethylene. **The Panel agreed that this criterion has been met.**
6. Test methods evaluation related to existing relevant toxicity data	The panel agreed that the data from the test method have been compared with relevant toxicity data, namely with Draize skin irritation test data which it is designed to replace. Furthermore, all chemicals tested have been used previously in validation studies of similar models. **The Panel agreed that this criterion has been met.**
7. Availability of all relevant data for expert review	The panel assumed that all relevant data has been provided. Furthermore, it noted that the standard protocol is available to the public. **The Panel agreed that this criterion has been met.**
8. GLP (ideally)	Initially, the optional nature of this criterion led to diverging interpretations. While some reviewers considered the statement that the validation study was carried out in the spirit of GLP as sufficient to fulfil this criterion, other reviewers requested evidence substantiating this statement and considered the criterion not fulfilled. After a discussion of this issue during the review process of this report, the understanding and interpretation of this criterion could be harmonised. As a result, the Panel agreed that the carrying out the validation in the spirit of GLP is acceptable. Nevertheless, the Panel recommended to better document how this was established and to provide respective documentation, if available. **The Panel agreed that this criterion had been partly met.**
PERFORMANCE STANDARDS	
9. Test method components	The panel agreed that some detailed information to comprehensively address the test method components was missing. Regarding 'Functional conditions – Viability', an upper acceptance limit for the negative control tissues, e.g.

	defined by a maximum optical density (OD), is missing. Although the data base for defining this might be small, it should nevertheless be defined if possible. Furthermore, it has not been defined what is considered as an acceptable variation of viabilities from replicate tissues. Regarding 'Functional conditions – Morphology', an acceptance criteria required for batch acceptance should be defined. Regarding 'Functional conditions – Reproducibility': A measure of variability between tissues should be defined. Regarding 'Functional conditions – Quality control (QC)', it was not entirely evident to the Panel how the model producer intends to ensure and demonstrate that each batch of the RhE model used meets defined production release criteria. **The Panel agreed that this criterion had been partly met.**
10. Demonstration of reliability and relevance using reference chemicals	After clarification that a lot of review comments to this charge question would be equally well or better suited for charge question 11, the panel agreed that, with one acceptable exception, the currently recommended reference chemicals have been used to demonstrate reliability and relevance of the test method. In addition, six further chemicals, which have been used in the validation of the models leading to the draft OECD Test Guideline, have been tested. **The Panel agreed that this criterion has been met.**
11. Test assessment based on defined reliability and accuracy values	The panel agreed on several crucial issues. Most importantly and the main reason for failing this criterion, the specific requirement of paragraph 12, Annex 2, of the draft OECD Test guideline ('...further specific restriction applies to the sensitivity of the proposed *in vitro* test method inasmuch as only two *in vivo* Category 2 substances, 1-decanol and di-n-propyl disulphide, may be misclassified as No Category by more than one participating laboratory.') was not met. The chemical 1-bromohexane, which is an in vivo Category 2 substance, has been classified as 'negative' by five out of six laboratories in the validation exercise (excluding laboratory e). Furthermore, within-and between laboratory reproducibility have not been appropriately evaluated. In this regard, the Panel anticipated that, while the between-laboratory reproducibility might fulfil the respective requirements, two laboratories (a and f) might not meet the requirement that the within-laboratory variability should show a concordance of classifications (GHS Category 2/No Category) obtained in different, independent test runs of the 20 Reference Chemicals within one single laboratory equal or higher (≥) than 90%. Related to this, the Panel recommends additional analyses based on final classifications for a complete run sequence of a given laboratory derived from the mean viabilities (instead of median viabilities) as specified

	in the performance standards. Regarding the predictive values, the panel agreed that the requirements, i.e. sensitivity ≥ 80%, specificity ≥ 70% and accuracy ≥ 75%, were met, when considering all 25 tested chemicals. It was noted, however, that the overall sensitivity would be ≤ 80% when considering only the 19 chemicals tested in the 2nd phase of the study. **The Panel agreed that this criterion has not been met.**

Appendix 1

JaCVAM Re-Analysis of initial validation data according the Performance Standards of TG 439 August 23, 2010

During our validation studies, the draft OECD performance standards (PS) that is based on the ECVAM performance standards had been on the table by OECD international experts. We performed our studies using the reference chemical list in the original or revised ECVAM performance standards (ECVAM 2007), in accordance with the validation plan. On the other hand, we did not follow other rules of the performance standard that were not included in the plan. Therefore, we could not calculate within- and between-laboratory reproducibility or analyze our data according to the rules of the performance standard (annex2) in the OECD draft test guideline.
With instructions related to our reanalyzing the data to OECD peer review panel, in this report I have analyzed our data according to the OECD draft performance standards.
With reference to definitions of the rules for reliability and accuracy values, the validation data were set in order as shown below.

1) Invalid data obtained only from Lab a, during run 1 (data not shown). This is because the negative OD detected during run 1 was lower than the acceptable limit. This lab carried out retesting. Therefore, the data Lab a, runs 2-4, were accepted as complete run sequences. All run sequences were completed in all other laboratories.

2) Twenty chemicals from the reference chemical (RC) list in the OECD performance standard (table 1) are used in this report.

3) With the exclusion of the data from Lab e, fully complete data from six laboratories were analyzed with three complete run sequences using all 20 RCs.

Below are our answers to questions regarding data analysis.
1. To questions 3, 4, 7 and 9 in the summary report of the LabCyte peer review panel:
A measure of variability between tissue triplicates was defined in the SOP and calculated in Table 2, and detailed data is presented in the attached files.
2. To questions 4, 7 and 11 in the summary report of the LabCyte peer review panel:
Within- and between-laboratory reproducibility were calculated according to annex 2.
As shown in Tables 3-1 and 3-2, there was no difference in median and mean variability among tissue triplicates.
Within-laboratory reproducibility: equal or higher than 90% in all laboratories as shown in Table 4. 2

Between-laboratory reproducibility: equal or higher than 80% in all laboratories as shown in Table 4.
As shown in Tables 5 and 6, accuracy of the required predictive values was sufficient relative to the ECVAM criteria.

Table 1: Reference test chemicals according to OECD performance standards (PS)

No.	Validation code	Chemical	CAS number	GHS label	In vivo score (PII)
1	01	1-bromo-4-chlorobutane	6940-78-9	no	0
2	02	diethyl phthalate	84-66-2	no	0
3	04	naphtalen acetic acid	86-87-3	no	0
4	05	allyl phenoxy-acetate	7493-74-5	no	0.3
5	06	isopropanol	67-63-0	no	0.3
6	07	4-methyl-thio-benzaldehyde	3446-89-7	no	1
7	08	methyl stearate	112-61-8	no	1
8	10	heptyl butyrate	5870-93-9	no	1.7
9	11	hexyl salicylate	6259-76-3	no	2
10	A	Cinnamaldehyde	104-55-2	no	2
11	14	1-decanol	112-30-1	Category 2	2.3
12	15	cyclamen aldehyde	103-95-7	Category 2	2.3
13	16	1-bromohexane	111-25-1	Category 2	2.7
14	B	2-Chroromethyl-3,5-dimethy l-4-methoxypyridine HCl	322-76821	Category 2	2.7
15	C	Potassium hydroxide (5%aq)	168-21815	Category 2	3
16	18	di-n-propyl disulphide	629-19-6	Category 2	3
17	D	Benzenethiol, 5-(1,1-dimethyethyl)-2-methyl	7340-90-1	Category 2	3.3
18	E	1-Methyl-3-phenyl-1-piperazine	5271-27-2	Category 2	3.3
19	20	heptanal	111-71-7	Category 2	4
20	F	1,1,1-Torichloroethane	200-02463	Category 2	4

Table 2: Mean viability at each test for each chemical according to OECD PS

					Lab					
No	Code	Vivo	Score	Exp	a	b	c	d	f	g
01	01	no	0	1	31.0	47.1	10.6	14.3	14.3	10.6
				2	11.2	10.4	20.3	9.1	11.2	10.6
				3	11.6	16.1	12.4	9.6	10.4	14.0
02	02	no	0	1	79.8	66.9	88.1	102.3	75.3	96.0
				2	76.5	61.7	89.7	89.8	67.2	94.8
				3	65.2	88.7	85.8	67.6	75.7	103.3
3	04	no	0	1	106.3	94.4	97.1	106.1	100.1	104.8
				2	95.2	100.2	99.9	100.9	92.8	103.3
				3	96.5	98.6	97.8	98.4	92.7	109.8
4	05	no	0.3	1	78.5	61.7	91.4	79.4	71.9	96.8
				2	78.5	71.9	95.2	70.5	39.3	89.9
				3	74.1	84.5	89.2	66.1	55.1	88.4
5	06	no	0.3	1	92.5	77.9	81.0	91.3	87.8	87.2
				2	79.4	83.5	79.1	102.4	94.4	81.2
				3	82.4	80.5	83.6	82.7	81.1	54.1
6	07	no	1	1	24.1	10.8	20.8	21.7	15.8	31.5
				2	12.6	12.6	16.2	13.8	31.1	22.5
				3	17.8	13.2	15.2	19.8	15.6	19.9
7	08	no	1	1	111.9	86.7	75.3	109.4	89.7	101.1
				2	90.2	100.6	82.3	107.5	97.8	100.9
				3	95.3	104.8	77.2	103.0	96.5	109.0
8	10	no	1.7	1	115.9	115.4	107.5	114.3	104.0	107.9
				2	104.1	110.1	103.6	108.2	101.2	108.4
				3	86.5	111.3	103.7	105.5	101.2	113.1
9	11	no	2	1	113.7	105.0	101.0	102.4	103.1	102.8
				2	98.1	106.6	94.6	105.8	98.0	100.5
				3	112.6	103.7	94.1	102.7	94.6	109.0
10	A	No	2	1	13.3	11.8	13.2	13.8	11.4	13.7
				2	14.2	10.2	22.5	9.9	11.3	8.7
				3	14	11.1	12.3	13.2	14.3	14.3
11	14	Category 2	2.3	1	11.1	12.1	14.7	10.7	13.1	13.5
				2	6.6	8.3	9.5	11.7	16.7	12.0
				3	6.8	8.8	9.1	10.2	17.0	10.6
12	15	Category 2	2.3	1	11.1	9.3	13.1	8.0	8.6	9.2
				2	7.1	10.2	19.3	8.6	5.9	24.7
				3	8.2	9.9	8.1	9.2	7.1	9.2
13	16	Category 2	2.7	1	67.9	92.0	51.5	18.1	59.6	64.9
				2	32.2	54.1	86.3	79.2	50.4	79.6
				3	59.8	98.3	81.7	37.7	67.5	86.5
14	B	Category 2	2.7	1	1.5	2.2	2.5	4	1.7	3.9
				2	3.1	2.2	2.9	3	2.6	3.7
				3	1.5	2.5	3	3.9	3.2	4.7
15	C	Category 2	3	1	0.7	0.7	0.7	6.9	0.8	1
				2	1.3	1.1	1.4	2	4.8	0.4
				3	0.5	0.8	1	0.8	1	0.3
16	18	Category 3	3	1	82.1	46.5	91.2	83.7	69.2	92.4
				2	78.3	50.6	87.3	69.9	80.6	85.9
				3	25.3	100.0	87.5	59.0	71.9	94.4
17	D	Category 2	3.3	1	14.5	24	12.7	10.3	13.8	19.3
				2	13.6	16	12.5	18.3	8.8	15.2
				3	18.6	15.5	12.6	23	19.2	14.1
18	E	Category 2	3.3	1	3.9	3.4	3.4	8.2	3.2	4.1
				2	4.5	2.7	3.3	3.9	4.2	3.1
				3	1.8	3.5	3.5	3.7	5	5.1
19	20	Category 3	4	1	31.1	24.8	10.4	9.6	8.1	8.8
				2	9.3	8.0	7.6	16.9	7.8	6.7
				3	29.5	9.3	7.6	30.9	8.2	8.6
20	F	Category 2	4	1	5.6	7.2	6.5	6.4	5.2	7.2
				2	5.7	6.1	6.8	5.4	7.4	6.8
				3	5.4	4.2	6.5	5.4	5	7.6

Table 3-1: Mean viability at three tests for each chemical according to OECD PS

NO.	**Code**	GHS label	**a**	**b**	**c**	**d**	**f**	**g**
1	01	no	17.9	24.5	14.4	11.0	12.0	11.7
2	02	no	73.8	72.4	87.8	86.6	72.7	98.0
3	04	no	99.3	97.8	98.2	101.8	95.2	105.9
4	05	no	77.0	72.7	91.9	72.0	55.4	91.7
5	06	no	84.8	80.7	81.2	92.1	87.8	74.2
6	07	no	18.2	12.2	17.4	18.4	20.8	24.6
7	08	no	99.1	97.4	78.3	106.6	94.7	103.7
8	10	no	102.1	112.2	104.9	109.3	102.1	109.8
9	11	no	108.1	105.1	96.6	103.6	98.6	104.1
10	A	no	13.8	11.0	16.0	12.3	12.3	12.2
11	14	Category 2	8.2	9.7	11.1	10.9	15.6	12.0
12	15	Category 2	8.8	9.8	13.5	8.6	7.2	14.4
13	16	Category 2	53.3	81.4	73.1	45.0	59.1	77.0
14	B	Category 2	2.0	2.3	2.8	3.6	2.5	4.1
15	C	Category 2	0.8	0.8	1.0	3.2	2.2	0.6
16	18	Category 2	61.9	65.7	88.7	70.9	73.9	90.9
17	D	Category 2	15.6	18.5	12.6	17.2	13.9	16.2
18	E	Category 2	3.4	3.2	3.4	5.3	4.2	4.1
19	20	Category 2	23.3	14.0	8.6	19.2	8.0	8.1
20	F	Category 2	5.5	5.8	6.6	5.7	5.9	7.2

Table 3-2: Median viability at three tests for each chemical according to OECD PS

NO.	**Code**	GHS label	**a**	**b**	**c**	**d**	**f**	**g**
1	01	no	11.6	16.1	12.4	9.6	11.2	10.6
2	02	no	76.5	66.9	88.1	89.8	75.3	96.0
3	04	no	96.5	98.6	97.8	100.9	92.8	104.8
4	05	no	78.5	71.9	91.4	70.5	55.1	89.9
5	06	no	82.4	80.5	81.0	91.3	90.7	81.2
6	07	no	17.8	12.6	16.2	19.8	21.3	22.5
7	08	no	95.3	100.6	77.2	107.5	100.9	101.1
8	10	no	104.1	111.3	103.7	108.2	101.2	108.4
9	11	no	112.6	105.0	94.6	102.7	98.0	102.8
10	A	no	14.0	11.1	13.2	13.2	11.4	13.7
11	14	Category 2	6.8	8.8	9.5	10.7	16.7	12.0
12	15	Category 2	8.2	9.9	13.1	8.6	7.1	9.2

13	16	Category 2	59.8	92.0	81.7	37.7	59.6	79.6
14	B	Category 2	1.5	2.2	2.9	3.9	2.6	3.9
15	C	Category 2	0.7	0.8	1.0	2.0	1.0	0.4
16	18	Category 2	78.3	50.6	87.5	69.9	71.9	92.4
17	D	Category 2	14.5	16.0	12.6	18.3	13.8	15.2
18	E	Category 2	3.9	3.4	3.4	3.9	4.2	4.1
19	20	Category 2	23.3	14.0	8.6	19.2	8.0	8.1
20	F	Category 2	5.6	6.1	6.5	5.4	5.2	7.2

Table 4: Reproducibility according to OECD PS using mean viabilities

Index	Lab.					
	a	b	c	d	f	g
Within-laboratory	18/20	19/20	20/20	19/20	20/20	20/20
	90	95	100	95	100	100
Between-laboratory	19/20					
	95.0					

Table 5: Sensitivity, specificity and accuracy on MTT assay vs GHS-EU classification according to OECD PS

Index	Lab.					
	a	b	c	d	f	g
Sensitivity	8/10	8/10	8/10	9/10	8/10	8/10
	80	80	80	90	80	80
Spescificity	7/10	7/10	7/10	7/10	7/10	7/10
	70	70	70	70	70	70
Accuracy	15/20	15/20	15/20	16/20	15/20	15/20
	75	75	75	80	75	75

Table 6: Mean and range of sensitivity, specificity and accuracy on the MTT assay using LabCyte EPI-MODEL vs. GHS-EU classification according to OECD PS

	N	Mean	Min.	Max.	ECVAM criteria
Sensivitity (%)	6	81.7	80.0	90.0	80.0
Specificity (%)	6	70.0	70.0	70.0	70.0
Accuracy (%)	6	76.7	75.0	80.0	75.0

Appendix 2

Background document for the modified skin irritation test using LabCyte EPI-MODEL24 (LabCyte EPI-MODEL24 SIT)

Japan Tissue Engineering Co., Ltd. R&D

TABLE OF CONTENTS

1. Abstract

A validation study of an *in vitro* skin irritation test method using a reconstructed human epidermal model for replacement of the Draize test was conducted by the European Centre for the Validation of Alternative Methods (ECVAM), and a protocol using EpiSkin™ (SkinEthic, France) was approved as a validated reference method (VRM) in April, 2007. Structural and performance test criteria for skin models are defined in the ECVAM Performance Standards. We have performed several evaluations of our reconstructed human epidermal model LabCyte EPI-MODEL24 (Japan Tissue Engineering Co., Ltd, Japan), and have confirmed that it is suitable for skin irritation tests defined by the ECVAM Performance Standards.

The original study for the development of a test method showed that test chemicals could be classified into non-irritants and irritants using the LabCyte EPI-MODEL24 (ORIGINAL STAGE). The VRM consists of a 15 minute exposure period, followed by a rinsing step and a 42 hour post-incubation period before quantitative measurement of cell viability using an MTT reaction. First, we examined the exposure period when the test chemicals are applied and found that the optimal exposure period is 15 minutes. After that, the performance of the original LabCyte EPI-MODEL24 SIT, which included an optimization study, was confirmed in intra-laboratory study and then evaluated on relevance and ability to meet minimum criteria described in the ECVAM performance standards for phase 1, 2 and 3 validation studies. The results of the validation studies for the original LabCyte EPI-MODEL24 SIT were summarized in JSAAE REPORTS and then these reports were assessed by a peer review panel in OECD, according to the OECD draft test guideline for skin irritation testing, which was presented in 2009.

However, the OECD peer review panel pointed out that the performance of the original LabCyte EPI-MODEL24 SIT did not meet the criteria shown in OECD draft test guideline for skin irritation testing, because the prediction results for the chemical 1-bromohexane, which is an *in vivo* Category 2 substance, were shown as negative in the original LabCyte EPI-MODEL24 SIT. Various modifications of the original LabCyte EPI-MODEL24 SIT have been examined by the kit supplier in order to solve the 1-bromohexane problem, with the result that they were able to solve the problem by modifying the washing protocol for the testing chemicals (the modified LabCyte EPI-MODEL24 SIT). When the predictive potency of the modified LabCyte EPI-MODEL24 SIT was evaluated with the 20 test chemicals which were listed in the new OECD TG 439 for *in vitro* skin irritation testing, adopted in July, 2010, the sensitivity, the specificity and the overall accuracy were 90 %, 70 %, and 80%, respectively.. It was thought that these results met the acceptance criteria described in OECD TG 439. In order to confirm the performance of the modified LabCyte EPI-MODEL24 SIT according to OECD TG 439, a final validation study was executed from August to November, 2010. Results demonstrated high reliability and acceptable accuracy in the MTT assay, for use as a stand-alone assay to distinguish between skin irritants and non-irritants. Details of the results of the validation study will be described in a Japanese Center for the Validation of Alternative Methods (JaCVAM) final report.

2. Purpose of this document

The principle of *in vitro* skin irritation testing using a reconstructed human epidermis (RhE) model as described in the new OECD test guideline 439 (OECD TG 439; Attachment 1) for *in vitro* skin irritation testing, is to detect the initiating events in the following cascade of skin irritation. Chemical-induced skin irritation, manifested by erythema and oedema, is the result of a cascade of events beginning with penetration of the stratum corneum and damage to the underlying layers of keratinocytes. The dying keratinocytes release mediators that begin the inflammatory cascade which acts on the cells in the dermis, particularly the stromal and endothelial cells. It is the dilation and increased permeability of the endothelial cells that produce the observed erythema and oedema. The RhE-based test methods measure the initiating events in the cascade.

The main purpose of this document is to propose a new *in vitro* skin irritation test method using LabCyte EPI-MODEL24 (Japan Tissue Engineering Co., Ltd, Japan; the LabCyte EPI-MODEL24 SIT) as a "me-too" test based on the validation test method (VRM) for EpiSkin™ (SkinEthic, France) according to OECD TG 439.

This document is composed of the following categories:

1. Description of the LabCyte EPI-MODEL24: It is confirmed that quality control for the LabCyte EPI-MODEL24 quality standards and/or the characteristics thereof meet with the criteria described in OECD TG 439.
2. Optimization of the LabCyte EPI-MODEL24 SIT and assessment of the original SIT (OPTIMIZATION STAGE): The optimal exposure period for the LabCyte EPI-MODEL24 SIT was investigated as a method based on VRM, according to the ECVAM performance standard (1). The reliability performance of the original (optimized) SIT was evaluated based on the OECD draft test guideline for skin irritation testing (2).
3. Modification of the original LabCyte EPI-MODEL24 SIT and assessment of the modified SIT (MODIFICATION STAGE): The original LabCyte EPI-MODEL24 SIT was modified because the original LabCyte EPI-MODEL24 SIT did not meet the criteria shown in the OECD draft test guideline (2). The performance of the modified SIT was evaluated with respect to reliability and accuracy, according to OECD TG 439.

In the end, through the above research stages it was confirmed that the performance of the LabCyte EPI-MODEL24 SIT meets the acceptance criteria shown in OECD TG 439 as a "me-too" test method similar to VRM.

3. Introduction

Trials to replace the Draize skin irritation test on rabbits *in vivo*, carried out according to the OECD TG 404 test guidelines (3), have been underway for many years worldwide, especially in the European Union (EU). These efforts have been accelerated by the enforcement of the 7th Amendment to the Cosmetics Directive and of EU regulations for the registration, evaluation, and authorization of chemicals (REACH). Investigation of various *in vitro*/ex vivo test systems showed that the best results were achieved by a system using a three-dimensional reconstructed human epidermal model (4, 5). The European Centre for the Validation of Alternative Methods (ECVAM) therefore evaluated two reconstructed human epidermal models, EpiSkin™ (SkinEthics, Nice, France) and EpiDerm™ (MatTek, MA, USA), during a prevalidation study of *in vitro* skin irritation tests during 2000–2001 (5, 6). However, since the predictive performance of the two models in the ECVAM prevalidation study did not meet the acceptance criteria set by the Management Team, further investigations were required to improve the test protocols (6). Subsequently, during 2003–2004 a common protocol for the *in vitro* evaluation of skin irritation potential was developed, optimized, and allowed to proceed to a formal ECVAM validation study (7, 8). As a result of the study, an *in vitro* skin irritation test (SIT) using the EpiSkin™ reconstructed human epidermal model (EpiSkin™ test method) has been scientifically validated as a stand-alone method of distinguishing skin irritants from non-irritants according to the EU classification (9). Furthermore, the ECVAM performance standards for applying a new human epidermis model to *in vitro* skin irritation have been documented based on the VRM (1). The ECVAM performance standards can then be used to evaluate the accuracy and reliability of other analogous test methods, also known as "me-too" tests, either based on similar scientific principles and measures or to predict the same biological or toxic effect.

Various other available *in vitro* three-dimensional epidermis equivalents have been developed, such as the LabCyte EPI-MODEL24 (Japan Tissue Engineering Co. Ltd., Japan). The LabCyte EPI-MODEL24 is a new reconstructed human epidermis model, which is grown for 13 days in a chemically defined medium using normal human keratinocytes (10). The tissue model consists of a fully differentiated epidermis, including a basal cell layer, stratum spinosum, stratum granulosum and stratum corneum (10, 11). Since the launch of commercially released kits in 2005, the LabCyte EPI-MODEL24 has mainly been used for skin irritation studies (11-15) or for skin corrosion (16), but it is also commonly used for UV-related experiments (17-19), for DNA microarray (20,21) and for various experiments about skin function (22-26).

In order to develop a test method according to the VRM (EpiSkin™ test methods), which is able to discriminate between non-irritant versus irritant test substances using the LabCyte EPI-MODEL24, the optimal exposure period for LabCyte EPI-MODEL24 was determined before the assessment of its performance. Then, the performance of the original LabCyte EPI-MODEL24 SIT (15 minute exposure) after the optimization study was evaluated for its relevance and its ability to meet ECVAM minimum performance standards criteria.

The validation study of the original LabCyte EPI-MODEL24 SIT was executed from 2008 to 2009. The results for validation studies of the original LabCyte EPI-MODEL24 SIT were summarized in JSAAE REPORTS (Attachment 2, 3).

During the validation study of the original LabCyte EPI-MODEL24 SIT, two important decisions were presented by ECVAM. One of them changed the test chemical exposure period for the modified EpiDerm™ SIT, from 15 minutes to 60 minutes, while the other stated that the SIT using SkinEthic RHE (SkinEthics, Nice, France), which was a new reconstructed human epidermal model, was regarded by ECVAM as sufficiently similar in comparison with the VRM and was admitted as a validation study on the basis of the ECVAM performance standards (27). Furthermore, the ECVAM performance standards

were revised (the ECVAM performance standards (updated)); 28,29) in 2009. It was stated that the *in vivo* classification of test chemicals would be changed from referring to the EU DSD (European Classification System based on the Dangerous Substance Directive) to the UN GHS (United Nations Globally Harmonized System of Classification and Labelling of Chemicals). This change was reflected in the OECD draft test guideline for skin irritation testing (2).

It should be noted that, the JSAAE REPORTS (Attachment 2, 3) about the validation study for the original LabCyte EPI-MODEL24 SIT were assessed by a peer review panel in OECD according to the acceptance criteria as shown in the OECD draft test guideline. Unfortunately, the OECD peer review panel pointed out that the performance of the original LabCyte EPI-MODEL24 SIT did not meet the criteria shown in the OECD draft test guideline for skin irritation testing (2), because the prediction results of the chemical 1-bromohexane, which is an *in vivo* Category 2 substance, was shown as negative in the original LabCyte EPI-MODEL24 SIT by five out of six laboratories in the validation exercise. In order to solve the problem where 1-bromohexane showed a false-negative, various modification trials for LabCyte EPI-MODEL24 SIT were examined by the kit supplier. From the results of their examination, it was found that the problem could be solved by the improvement of the washing protocol (the modified LabCyte EPI-MODEL24 SIT). Using the 20 test chemicals listed in the OECD TG 439 (Attachment 1), which was adopted as the new guideline for the *in vitro* skin irritation testing by OECD in July, 2010, the predictive potency of the modified LabCyte EPI-MODEL24 SIT was evaluated, with the result that the sensitivity, specificity and overall accuracy were 90%, 70% and 80%, respectively. It was thought that these results met the acceptance criteria described in the OECD TG 439. In order to confirm the performance of the modified LabCyte EPI-MODEL24 SIT according to OECD TG 439, the final and formal validation study of the modified LabCyte EPI-MODEL24 SIT was performed from August to November, 2010.

4. Description of LabCyte EPI-MODEL24

4-1. Condition of the functional reconstructed human epidermis, LabCyte EPI-MODEL24

LabCyte EPI-MODEL24 is a new, commercially available reconstructed human cultured epidermal model produced by Japan Tissue Engineering Co. Ltd. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand the human keratinocytes while maintaining their phenotype, they are cultured with 3T3-J2 cells as a feeder layer (30,31). Reconstruction of human cultured epidermis is achieved by cultivating proliferating keratinocytes on an inert filter substrate (surface 0.3 cm^2) at the air-liquid interface for 13 days, with an optimized medium containing 5% fetal bovine serum. The result is a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum (Fig.4-1). The LabCyte EPI-MODEL24 is embedded in an agarose gel containing a nutrient solution and shipped in 24-well plates at around 18°C.

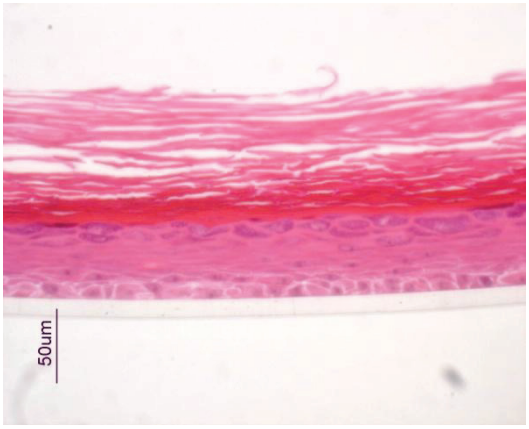


Fig.4-1. Histological cross-sectional views of the LabCyte EPI-MODEL24 with H&E staining (original magnification: ×40).

Histological examination shows a completely stratified epidermis containing all major epidermal layers, including a stratum basal (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC) (Fig.4-1). In addition, specific epidermal differentiation markers and basement membrane constituents are expressed in the appropriate regions, as seen in human skin (Fig.4-2).

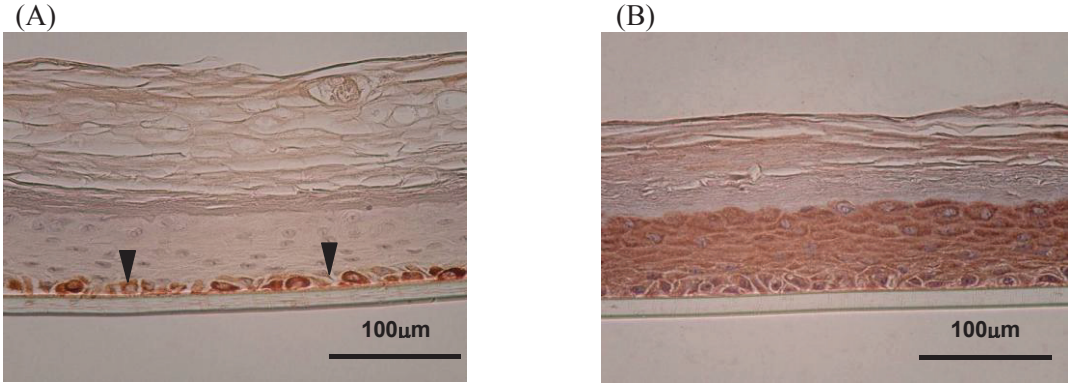


Fig.4-2. Histological cross-sectional views of LabCyte EPI-MODEL24 with

immunohistochemical staining using Laminin (a) and Transglutaminase (b).

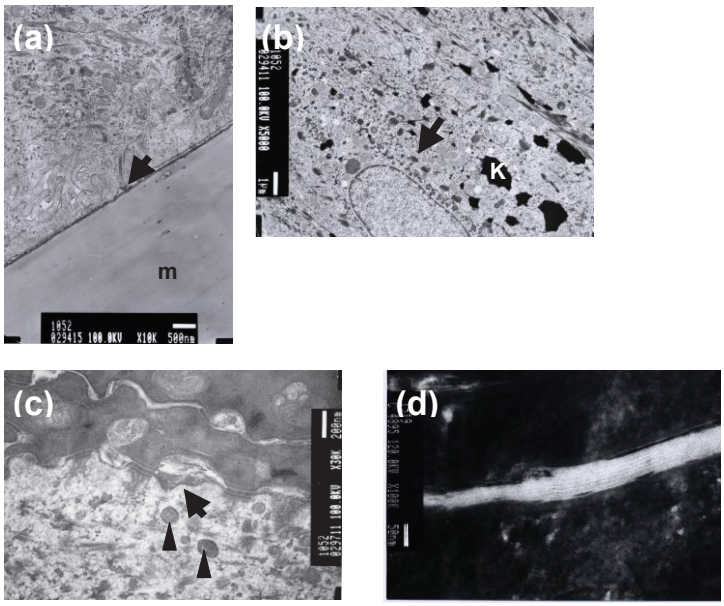


Fig.4-3. Transmission electron micrographs of the LabCyte EPI-MODEL24:

(a) Basement membrane (original magnification; ×10,000). Note anchoring filament (arrows).

(b) Keratohyalin granules in SG cells which are connected by desmosome (original magnification; ×5000). Note keratohyalin granules (K), desmosome (arrows).

(c) Lipid lamella bodies in the interface between SG and SC (original magnification; ×30,000).
Note lipid lamellar bodies (arrow head) and their extrusion (arrows).

(d) Lipid bilayer at SC (original magnification; ×100,000). Additional ruthenium tetroxide fixation was used.

Ultra-structurally, it was possible to observe a fully developed basement membrane zone, consisting of a highly developed lamina densa, lamina lucida, and anchoring filaments. Extrusion of lamellar bodies was observed at the interface between the SG and SC (Fig.4-3). Lipid lamellae, showing a characteristic electron dense and electron lucent pattern, were present. Keratohyalin granules were ubiquitously present in the granular cells at the SG (Fig.4-3).

Their histological evaluation was summarized in a scientific report (10).

The synthesis of specific lipids, including ceramides, which are known to be responsible for the water barrier of the stratum corneum, was detected in the LabCyte EPI-MODEL (22).

4-2. Quality control for LabCyte EPI-MODEL24

4-2a. Quality control procedures for LabCyte EPI-MODEL24

LabCyte EPI-MODEL24 is manufactured according to a defined standard operation procedure (SOP). All batches of the LabCyte EPI-MODEL24 production are checked for their viability, barrier function and morphology.

The product is released following stringent quality control procedures.

The quality of the final product is assessed by the following protocol and decision criteria;

1. Tissue viability: MTT assay
   Three LabCyte EPI-MODEL24 tissues were subjected to an MTT assay as follows. Tissues were put in the wells of 24-well plates containing 0.5 ml of MTT medium (0.5 mg/ml; Dojindo Co., Kumamoto, Japan) and were incubated for three hours (37°C, 5% CO_2, humidified atmosphere). Formazan produced in the tissues was extracted with isopropanol (300 µl) and the extract (200 µl) was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank.
   The mean of the OD values indicates tissue viability for each LabCyte EPI-MODEL24 batch. QC acceptance criteria: OD ≥ 0.8

2. Barrier function: 50% inhibitory concentration (IC_{50}) assay.
   To evaluate whether the stratum corneum in the LabCyte EPI-MODEL24 tissue resists the rapid penetration of the cytotoxic marker chemical sodium lauryl sulphate (SLS), the viability of the epidermis tissue was estimated in terms of the half maximal inhibitory concentration (IC_{50}). Various concentrations (0.1, 0.2, 0.3, and 0.4%(w/v)) of SLS (25 µl) were applied to the LabCyte EPI-MODEL24, and cell viability was measured after 18 hours using an MTT assay. All experiments were performed in triplicate. The acceptable range for the LabCyte EPI-MODEL24 is shown in Table 4-1.

   Table 4-1: QC acceptable range of barrier function

	Lower limit	Mean	Upper limit
LabCyte EPI-MODEL24 IL_{50}(18 hours SLS)	0.14 (w/v)% (1.4mg/ml)	0.26 (w/v)% (2.6mg/ml)	0.40 (w/v)% (4.0mg/ml)

   The test protocol for the barrier function of the LabCyte EPI-MODEL24 was established in May, 2008. In order to set an acceptable QC range, data for a barrier function database was collected from June to December, 2008. From June 2008 to October 2010, there was no batch with results outside the QC acceptance criteria.

3. Morphology.
   A piece of LabCyte EPI-MODEL24 was fixed with 4% parafolmaldehyde and 2% sucrose in 0.1M phosphate buffered saline (PBS) (pH 7.4) for more than three hours and processed for embedding in paraffin. Five-micrometer vertical sections were cut and stained with hematoxylin and eosin for light-microscopic examination.

   QC acceptance criteria: Confirmation of the formation of multilayered epidermis-like tissue containing a stratum corneum.

   Since the start of the commercial release of the product until October 2010, no batch has been outside the QC acceptance criteria..

4-2b. Batch control information for LabCyte EPI-MODEL24

Quality control data for the tissue viability and barrier function of each LabCyte EPI-MODEL24 batch is shown in Table 4-2.

Table 4-2: Batch information for LabCyte EPI-MODEL24
Tissue viability: October, 2005 – October, 2010
Barrier function: June, 2008 – October, 2010

Year	Month	Tissue viability		Barrier function	
		Mean±SD (OD)	CV[1)] (%)	Mean±SD (%)	CV[1)] (%)
2005	October	1.03±0.18	17.6		
	November	1.21±0.15	12.7		
	December	1.23±0.12	10.0		

Table 4-2: Continued

Year	Month	Tissue viability		Barrier function	
		Mean±SD (OD)	CV[1)] (%)	Mean±SD (%)	CV[1)] (%)
2006	January	1.34±0.06	4.3		
	February	1.14±0.16	13.7		
	March	1.24±0.26	21.3		
	April	1.48±0.13	8.7		
	May	1.49±0.18	11.9		
	June	1.37±0.17	12.4		
	July	1.41±0.15	10.6		
	August	1.43±0.07	5.2		
	September	1.52±0.12	8.1		
	October	1.93±0.09	4.6		
	November	1.98±0.19	9.7		
	December	1.68±0.13	7.6		
2007	January	1.70±0.08	4.7		
	February	1.62±0.09	5.3		
	March	1.67±0.11	6.9		
	April	1.51±0.09	6.1		
	May	1.52±0.09	6.1		
	June	1.66±0.10	6.3		
	July	1.50±0.22	15.0		
	August	2.07±0.40	19.3		
	September	1.48±0.18	12.4		
	October	1.70±0.15	8.6		
	November	1.92±0.19	9.7		
	December	1.70±0.05	3.2		
2008	January	1.74±0.20	11.4		
	February	1.37±0.23	17.0		
	March	1.35±0.18	13.7		
	April	1.42±0.15	10.8		
	May	1.33±0.14	10.7		
	June	1.28±0.03	2.0	0.23±0.02	10.1
	July	1.32±0.03	2.3	0.24±0.04	17.9
	August	1.26±0.04	3.5	0.27±0.01	5.2
	September	1.31±0.06	4.6	0.28±0.03	9.1
	October	1.55±0.06	3.7	0.26±0.01	4.7
	November	1.54±0.07	4.2	0.26±0.00	1.1
	December	1.51±0.09	6.1	0.26±0.02	6.8
2009	January	1.23±0.29	23.8	0.25±0.01	4.1
	February	1.41±0.12	8.7	0.25±0.01	5.0
	March	1.45±0.09	6.1	0.28±0.01	4.7
	April	1.44±0.23	15.7	0.23±0.02	6.6
	May	1.60±0.17	10.8	0.24±0.01	4.2
	June	1.34±0.10	7.4	0.26±0.04	15.9
	July	1.36±0.07	4.9	0.25±0.03	12.7
	August	1.47±0.08	5.4	0.24±0.02	9.0
	September	1.40±0.11	8.0	0.24±0.01	4.7

October	1.54±0.11	6.9	0.29±0.04	13.6
November	1.58±0.05	3.4	0.26±0.02	6.1
December	1.52±0.12	8.0	0.24±0.02	6.8

Table 4-2. Continued

Year	Month	Tissue viability		Barrier function	
		Mean±SD (OD)	CV[1] (%)	Mean±SD (%)	CV[1] (%)
2010	January	1.51±0.15	9.9	0.26±0.00	1.6
	February	1.60±0.12	7.5	0.25±0.01	3.4
	March	1.52±0.08	5.0	0.28±0.03	9.4
	April	1.44±0.13	8.9	0.25±0.01	4.6
	May	1.63±0.03	1.9	0.25±0.01	5.1
	June	1.58±0.12	7.6	0.24±0.02	8.9
	July	1.58±0.08	4.8	0.25±0.01	4.0
	August	1.49±0.10	6.8	0.24±0.01	5.2
	September	1.47±0.12	8.1	0.25±0.01	4.1
	October	1.42±0.08	5.5	0.25±0.02	8.8
Tissue viability Barrier function	October, 2005 to October, 2010 June, 2008 to October, 2010	1.49±0.23	15.5	0.25±0.02	9.2

1) Coefficient Variation.

As shown in Table 4-2, the mean SD of tissue viability and barrier function (IC_{50}) from the evaluation of continuous batches of LabCyte EPI-MODEL24 (with tissue viability data taken from October, 2005 to October, 2010 and barrier function data taken from June, 2008 to October, 2010) were 1.49 ± 0.23% and 0.25 ± 0.02%, respectively. The tissue viability and barrier function of LabCyte EPI-MODEL24 tissue have remained constant, indicating reproducibility (low coefficient variation (CV): 15.5% and 9.2%, respectively) and monthly consistency is high.

4-3. Shipment of LabCyte EPI-MODEL24 out of Japan

4-3a. Purpose

To examine whether LabCyte EPI-MODEL24 is exportable, it was sent to China and viability and barrier function tests were performed.

4-3b. Quality check of the exported LabCyte EPI-MODEL24

1. Viability

   Three LabCyte EPI-MODEL24 tissues were subjected to an MTT assay as follows. Tissues were put in the wells of 24-well plates containing 0.5 ml of MTT medium (*0.5 mg/ml*) and were incubated for three hours (37°C, 5% CO_2, humidified atmosphere). Formazan produced in the tissues was extracted with isopropanol (300 μl) and the extract (200 μl) was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank. The mean OD value indicates the tissue viablility for each LabCyte EPI-MODEL24 batch.

   Acceptance criteria:
   The mean OD ≥ 0.8

2. Barrier function

   The following test is performed using 15 tissue models.
   To evaluate whether the stratum corneum in the LabCyte EPI-MODEL24 tissue resists the rapid penetration of the cytotoxic marker chemical sodium lauryl sulphate (SLS), the viability of the epidermis tissue was estimated in terms of the half maximal inhibitory concentration (IC_{50}).

Various concentrations (0.1, 0.2, 0.3, and 0.4%(w/v)) of SLS (25 μl) are applied to the LabCyte EPI-MODEL24, and cell viability is measured after 18 hours by an MTT assay. All experiments are performed in triplicate. The MTT assay is performed using three tissue models and their viabilities are determined.

Acceptance criteria:
$0.14\% \leq IC50 \leq 0.4\%$

LabCyte EPI-MODEL24 was exported three times and it was confirmed that results were within the acceptance criteria in all batches.

4-3c. Shipment schedule

1st shipment: August 24, 2009.
2nd shipment: August 31, 2009.
3rd shipment: September 14, 2009

4-3d. Test facility

Shanghai Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China
Technical Center For Animal, Plant And Food Inspection and Quarantine Head of Animal and Toxicology Lab. (Shanghai, China)

4-3e. Results and discussion

Shipment results are shown in Table 4-3.

Table 4-3: Results of Shipments to China

Lot No.	Shipping date	Delivery date	Transportation period
LCE24-090824-A	Aug. 24, 2009	Aug. 26, 2009	2 days
LCE24-090831-A	Aug. 31, 2009	Sep. 2, 2009	2 days
LCE24-090914-A	Sep. 14, 2009	Sep. 16, 2009	2 days

The QC results for the exported LabCyte EPI-MODEL24 from the Shanghai Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China are shown in Table 4-4.

Table 4-4: QC results for LabCyte EPI-MODEL24 exported to China

Lot No.	Starting date for QC testing	Viability			Barrier function (IC50(%))	QC result
		Tissue (570nm/650nm)	Blank (570nm)	Fold		
LCE24-090824-A	Aug. 26, 2009	0.871±0.035	0.040±0.000	21.8	0.32	Pass
LCE24-090831-A	Sep. 2, 2009	1.011±0.033	0.037±0.000	27.1	0.27	Pass
LCE24-090914-A	Sep. 16, 2009	1.054±0.034	0.038±0.000	28.0	0.25	Pass

All three batches of the LabCyte EPI-MODEL24 shipped to China were within the QC acceptance criteria as described in the Test Protocol, suggesting that the quality of the LabCyte EPI-MODEL24 was maintained during the overseas shipment.

5. Optimization of the LabCyte EPI-MODEL24 SIT and assessment of the original SIT (OPTIMIZATION STAGE)

5-1. Summary of the OPTIMIZATION STAGE

The aim of the optimization study was to develop a test method able to discriminate non-irritant versus irritant test chemicals using the LabCyte EPI-MODEL24 as a "me-too" test based on the VRM (EpiSkin^{TM} test method) according to the ECVAM performance standard (1). The VRM consists of a 15 minute exposure period of test chemicals, followed by a rinsing step and a 42 hour post-incubation period before quantitative measurement of cell viability using an MTT reaction.

First of all, we examined a suitable exposure period for the LabCyte EPI-MODEL24 SIT and the optimized exposure period was set at 15 minutes. The performance of the original LabCyte EPI-MODEL24 SIT, which was reflected in the results of the optimization study, has been confirmed by intra-laboratory study.

After that, the original LabCyte EPI-MODEL24 SIT was evaluated for relevance and the ability to meet minimum criteria described in the ECVAM performance standards in the phase 1, 2 and 3 formal validation studies. The results of validation studies for the original LabCyte EPI-MODEL24 SIT were summarized in JSAAE REPORTS (Attachments 2,3) and then their reports were assessed by a peer review panel in OECD according to the OECD draft test guideline for skin irritation testing which was presented in 2009 (2).

5-2. Materials and methods

5-2a. Test chemicals

Nineteen test chemicals shown in Table 5-1 were selected among the twenty reference chemicals of the ECVAM performance standard (1). By definition, reference chemicals are used to determine if the performance of a new *in vitro* human skin model system for skin irritation testing is comparable to that of the VRM in the ECVAM performance standard.

Unfortunately, tri-isobuthyl phosphate (No.13) was not used in the examination because it was unavailable in Japan.

Table 5-1: Selected test chemicals for the optimization of a suitable exposure period.

No.	Test chemicals				
	Name	CAS number[1)]	*In vivo* class[2)]	PII[3)]	Supplier
1	1-bromo-4-chlorobutane	6940-78-9	NI	0	Wako chemical
2	diethyl phthalate	84-66-2	NI	0	Wako chemical
3	di-propylene glycol	25265-71-8	NI	0	Wako chemical
4	naphthalen acetic acid	86-87-3	NI	0	Wako chemical
5	allyl phenoxyacetate	7493-74-5	NI	0.3	Wako chemical
6	isopropanol	67-63-0	NI	0.3	Wako chemical
7	4-methyl-thio-benzaldehyde	3446-89-7	NI	1.0	Wako chemical
8	methyl stearate	112-61-8	NI	1.0	Kanto chemical
9	allyl heptanoate	142-19-8	NI	1.7	Wako chemical
10	heptyl butyrate	5870-93-9	NI	1.7	Aldrich
11	hexyl salicylate	6259-76-3	I	2.0	Sigma Fluka
12	terpinyl acetate	80-26-2	I	2.0	Alfa Aesar
(13)	(tri-isobutyl phosphate)	126-71-6	I	2.0	Unavailable in Japan

14	cyclamen aldehyde	103-95-7	I	2.3	Wako chemical
15	1-decanol	112-30-1	I	2.3	Wako chemical
16	1-bromohexane	111-25-1	I	2.7	Wako chemical
17	α-terpineol	98-55-5	I	2.7	Kanto chemical
18	di-n-propyl disulphide	629-19-6	I	3.0	Wako chemical
19	butyl methacrylate	97-88-1	I	3.0	Wako chemical
20	heptanal	111-71-7	I	3.3	Kanto chemical

1) CAS No.: Chemical abstracts service registry number.
2) I: irritant, NI: non irritant
3) PII: primary irritation index.

5-2b. Experimental protocol for a suitable exposure period for the LabCyte EPI-MODEL24 SIT

LabCyte EPI-MODEL24 tissues were shipped from the supplier on Mondays and delivered to recipients on Tuesdays. Upon receipt, the tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 ml) and incubated overnight (37°C, 5%, CO_2, humidified atmosphere). On the next day, the tissues were topically exposed to the test chemicals. Liquids (25 µl) were applied with a micropipette, and solids (25 mg) were applied from microtubes and moistened with 25 µl sterile water. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied by using a cell-saver-type tip with a micropipette. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were treated with 25 µl distilled water, and three tissues serving as positive controls were exposed to 5% SLS. After 10, 15, 20 or 30 minutes of exposure, each tissue was carefully rinsed with PBS (Invitrogen, CA, USA) ten times using a washing bottle to remove any remaining test chemical from the surface. The blotted tissues were then transferred to new wells on 24-well plates containing 1 ml of fresh assay medium.

The treated and control tissues were post-incubated for 42 hours (37°C, 5%, CO_2, humidified atmosphere). When the 42-hour post-incubation period was is completed, blotted tissues were transferred to new wells on 24-well plates containing 0.5 ml of freshly prepared MTT medium (0.5 mg/ml) for the MTT assay. Tissues were incubated for three hours (37°C, 5% CO_2, humidified atmosphere) and were then transferred to microtubes containing 300 µl isopropanol, completely immersing the tissue. Formazan extraction was performed at room temperature and the tissues were allowed to stand overnight. Subsequently, 200 µl extracts were transferred to a 96-well plate. The optical density was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank.

The tissue viability was calculated as a percentage relative to the viability of negative controls. The mean of the three values from identically treated tissues was used to classify a chemical according to the prediction model.

In this study, the prediction model for skin irritation potential with LabCyte EPI-MODEL24 was set with reference to the conditions for the VRM (EpiSkin™ test method) described in the ECVAM performance standards. This prediction model is described in Table 5-2. In the event that the three independent results within an individual batch were not in agreement, the result that occurred twice was used.

Table 5-2: Prediction models for the LabCyte EPI-MODEL24 SIT

Cell viability	Judgment
mean ≤ 50%	Irritant
mean > 50%	Non irritant

5-2c. Protocol for the original LabCyte EPI-MODEL24 SIT

The test protocol for the LabCyte EPI-MODEL24 SIT is described in section 5-2b based on the

EpiSkin™ VRM. From the result of the investigation of a suitable exposure period (see section 5-2b and 5-3b), the exposure period in the original LabCyte EPI-MODEL24 SIT was set at 15 min. The overall test protocol is described in the original SOP for the LabCyte EPI-MODEL24 SIT (ver. 4.0).

Three independent tests were performed on different batches of the LabCyte EPI-MODEL24, with three tissues per test chemical. After the 42-hour post-incubation period was completed, conditioned medium was collected for analysis of interleukin-1 alpha (IL-1α) release according to the VRM.

The tissue viability was calculated as a percentage relative to the viability of negative controls. The mean of the three values from identically treated tissues was used to classify a chemical according to the prediction model.

The amount of IL-1α release in the conditioned medium collected after 42 hours was determined using the IL-1α ELISA kit (Invitrogen, CA, USA), according to the detailed instructions of the manufacturer.

In this study, the prediction model for skin irritation potential with the LabCyte EPI-MODEL24 was set in reference to the conditions for the VRM (EpiSkin™ test method) described in the ECVAM performance standards. This prediction model is described in Table 5-4. In the event that the three independent results within an individual batch were not in agreement, the result that occurred twice was used.

Table 5-4: Prediction models for the original LabCyte EPI-MODEL24 SIT

Cell viability (1st)	IL-1α ELISA (2nd)	Judgment
mean ≤ 50%		Irritant
mean > 50%	IL-1α content (mean) ≥ 120 pg/tissue	
mean > 50%	IL-1α content (mean) < 120 pg/tissue	Non irritant

5-3. Results

5-3a. Investigation of a suitable exposure period for the LabCyte EPI-MODEL24 SIT

The results of the investigation of a suitable exposure period for the LabCyte EPI-MODEL24 SIT were evaluated by cell viability, as shown in Table 5-5.

Table 5-5: Results obtained from the experiment with various exposure periods

Test chemical		Exposure period (minutes)							
		10		15		20		30	
Name	*In vivo* class[1)]	viability (%)	*In vitro* class[1)]	viability (%)	*In vitro* class[1)]	viability (%)	*In vitro* class[1)]	viability (%)	*In vitro* class[1)]
1-bromo-4-chlorobutane	NI	44.0±12.8	I	49.6±5.0	I	25.3±7.5	I	14.5±6.5	I
diethyl phthalate	NI	93.3±8.4	NI	108.0±3.9	NI	118.5±1.6	NI	92.4±12.6	NI
di-propylene glycol	NI	90.3±2.8	NI	108.1±6.4	NI	95.3±5.7	NI	90.5±3.3	NI
naphthalen acetic acid	NI	92.4±7.7	NI	103.3±1.8	NI	100.8±7.0	NI	84.9±4.2	NI
allyl phenoxy-acetate	NI	87.5±11.3	NI	100.6±5.4	NI	90.7±6.4	NI	91.6±2.8	NI
isopropanol	NI	83.5±6.3	NI	102.1±1.6	NI	68.7±11.9	NI	9.6±1.1	I
4-methyl-thio-benzaldehyde	NI	15.0±2.7	I	17.2±1.6	I	10.9±2.6	I	15.5±2.6	I
methyl stearate	NI	94.4±2.0	NI	113.0±2.6	NI	89.6±7.0	NI	117.2±4.0	NI
allyl heptanoate	NI	92.9±3.1	NI	115.9±8.6	NI	83.3±3.6	NI	117.2±5.4	NI
heptyl butyrate	NI	95.2±9.5	NI	126.3±1.2	NI	90.9±4.6	NI	121.2±5.3	NI
hexyl salicylate	I	97.5±5.6	NI	107.4±4.8	NI	87.6±2.4	NI	113.4±3.5	NI
terpinyl acetate	I	37.7±11.8	I	52.3±16.3	NI	22.4±3.3	I	22.3±3.7	I
cyclamen aldehyde	I	16.9±0.3	I	16.0±3.2	I	13.6±2.3	I	14.6±1.9	I
1-decanol	I	16.1±1.0	I	19.2±1.3	I	14.2±0.9	I	15.7±2.4	I

1-bromohexane	I	92.2±4.0	NI	102.0±5.9	NI	103.5±16.3	NI	70.8±15.8	NI
α-terpineol	I	7.9±1.5	I	10.7±0.2	I	10.0±0.7	I	8.5±1.4	I
2-chloromethyl-3,5-dimethyl-4-methoxypyridine HC	I	5.8±0.2	I	5.6±0.3	I	4.3±0.7	I	5.1±0.7	I
di-n-propyl disulfide	I	98.0±3.5	NI	96.7±6.0	NI	93.4±8.0	NI	90.7±4.0	NI
butyl methacrylate	I	73.2±7.5	NI	19.3±3.2	I	14.6±0.6	I	15.3±1.5	I
Heptanal	I	22.2±5.5	I	12.6±4.5	I	11.3±1.8	I	12.1±0.7	I

In the case of a 10 minute exposure period, butyl methacrylate was evaluated as a non-irritant, though it had been classified as an irritant *in vivo* (shown in red). Because the sensitivity of the prediction with a 10 minute exposure period was lower than that for the group with an exposure period of 15 minutes or longer, it was thought that a 10 minute exposure period was not long enough.

With the 30 minute exposure period, on the other hand, isopropanol was indicated as an irritant, though it had been classified as non-irritant *in vivo* (shown in blue). It was decided that the 30 minute exposure period was too long and unsuitable, because the specificity of prediction was lower than that for groups with exposure periods of 20 minutes or less.

In the case of a 15 minute exposure period, 1-bromo-4-chlorobutane (non-irritant *in vivo* class) and terpinyl acetate (irritant *in vivo* class) resulted in cell viabilities of around 50%, while both of them were apparently indicated as an irritant in the 20 minute exposure. *In vivo* skin irritancy of 1-bromo-4-chlorobutane is very low, as its primary irritation index is 0 and it was thought that the 15 minute exposure period was better than the 20 minute exposure period because the cell viability for the 15 minute exposure period was higher. Also, *in vivo* skin irritancy of terpinyl acetate is at the borderline between non-irritant and irritant, and it was thought that the 15 minute exposure period was better than the 20 minute exposure because cell viability was around 50%.

From the above considerations, it was concluded that the suitable exposure period was 15 minutes. The original LabCyte EPI-MODEL24 SIT, which reflected the optimization study for a suitable exposure period, was referred to in the SOP for the ver.4.0 of the LabCyte EPI-MODEL24 SIT.

5-3b. Assessment of the original LabCyte EPI-MODEL24 SIT by intra-laboratory study

Nineteen test chemicals were tested using the LabCyte EPI-MODEL24 and the common protocol described in the ECVAM performance standard. Three independent runs were performed on different batches of LabCyte EPI-MODEL24, with three tissues per test chemical (Table 5-1). When only cell viability is used as an indicator, negative predictions for three chemicals, hexyl salicylate (No. 11), 1-bromohexane (no. 16), and di-n-propyl disulphide (no. 18), were not concordant with the results of the *in vivo* classification in the group of nine irritants and also two chemicals, 1-bromo-4-chlorobutane (no. 1) and 4-methyl-thio-benzaldehyde (no. 7), were classified as positive in the group of ten non-irritants, (Table 5-6).

Table 5-6. Results of skin irritation tests by cell viability and IL-1α release evaluation.

Test chemical			The original SIT using LabCyte EPI-MODEL24										
			Cell viability (%)				*In vitro* prediction by cell viability only[1)]	IL-1α(pg/tissue)				*In vitro* prediction by cell viability and IL-1a release[1)]	
No.	Name	Run	Mean	±	SD	Judgment[1)]		Mean	±	SD	Judgment[1)]		
1	1-bromo-4-chlorobutane	1	44.8	±	6.7	I	I	90.5	±	4.7	NI	I	I
		2	34.6	±	11.8	I		129.5	±	13.7	I	I	
		3	25.1	±	7.6	I		178.6	±	17.4	I	I	
2	diethyl phthalate	1	95.4	±	3.2	NI	NI	29.2	±	33.0	NI	NI	NI
		2	100.7	±	7.7	NI		15.5	±	5.0	NI	NI	
		3	102.5	±	2.5	NI		51.7	±	10.4	NI	NI	

Table 5-6. Continued

Test chemical			The original SIT using LabCyte EPI-MODEL24										
			Cell viability (%)				*In vitro* prediction by only cell viability[1)]	IL-1α(pg/tissue)				*In vitro* prediction by cell viability and IL-1a release[1)]	
No.	Name	Run	Mean	±	SD	Judgement[1)]		Mean	±	SD	Judgement[1)]		
3	di-propylene glycol	1	95.4	±	2.8	NI	NI	6.5	±	3.6	NI	NI	NI
		2	103.9	±	8.4	NI		16.7	±	6.3	NI	NI	
		3	99.0	±	5.1	NI		16.0	±	12.1	NI	NI	
4	naphthalen acetic acid	1	96.8	±	5.2	NI	NI	2.9	±	1.5	NI	NI	NI
		2	97.0	±	2.0	NI		5.7	±	2.9	NI	NI	
		3	101.8	±	2.5	NI		12.0	±	8.2	NI	NI	
5	allyl phenoxy-acetate	1	87.7	±	5.3	NI	NI	36.3	±	22.8	NI	NI	NI
		2	75.1	±	4.8	NI		115.0	±	20.3	NI	NI	
		3	99.2	±	2.3	NI		27.8	±	6.8	NI	NI	
6	isopropanol	1	86.2	±	2.1	NI	NI	57.7	±	9.9	NI	NI	NI
		2	86.1	±	1.4	NI		94.7	±	8.8	NI	NI	
		3	94.5	±	2.6	NI		99.8	±	19.8	NI	NI	
7	4-methyl-thio -benzaldehyde	1	25.7	±	2.4	I	I	93.9	±	10.5	NI	I	I
		2	22.7	±	5.4	I		119.3	±	29.1	NI	I	
		3	20.9	±	1.2	I		102.9	±	2.9	NI	I	
8	methyl stearate	1	107.2	±	3.2	NI	NI	6.7	±	0.8	NI	NI	NI
		2	110.4	±	1.8	NI		7.9	±	1.7	NI	NI	
		3	107.1	±	3.0	NI		5.6	±	1.2	NI	NI	
9	allyl heptanoate	1	45.9	±	42.4	I	NI	495.8	±	257.3	I	I	NI
		2	93.7	±	14.4	NI		86.3	±	99.4	NI	NI	
		3	118.9	±	5.8	NI		11.4	±	3.3	NI	NI	
10	heptyl butyrate	1	97.2	±	4.1	NI	NI	8.8	±	3.4	NI	NI	NI
		2	109.8	±	2.7	NI		7.6	±	1.8	NI	NI	
		3	99.3	±	3.7	NI		13.2	±	3.1	NI	NI	
11	hexyl salicylate	1	103.1	±	4.4	NI	NI	10.6	±	4.7	NI	NI	NI
		2	110.8	±	3.2	NI		4.9	±	2.2	NI	NI	
		3	101.1	±	4.1	NI		13.5	±	9.6	NI	NI	
12	terpinyl acetate	1	18.0	±	10.0	I	I	293.4	±	30.3	I	I	I
		2	36.3	±	6.5	I		233.6	±	83.5	I	I	
		3	39.1	±	8.1	I		233.6	±	83.5	I	I	
14	1-decanol	1	15.5	±	0.8	I	I	175.9	±	13.8	I	I	I
		2	21.6	±	1.5	I		166.5	±	27.2	I	I	
		3	17.2	±	1.8	I		164.4	±	21.9	I	I	
15	cyclamen aldehyde	1	19.7	±	1.8	I	I	144.2	±	5.8	I	I	I
		2	21.8	±	1.4	I		110.4	±	13.3	NI	I	
		3	17.0	±	3.6	I		173.2	±	38.1	I	I	
16	1-bromohexane	1	16.9	±	0.9	I	NI	309.3	±	96.5	I	I	I
		2	59.0	±	10.0	NI		164.8	±	33.3	I	I	
		3	84.1	±	7.9	NI		39.0	±	36.7	NI	NI	
17	α-terpineol	1	12.2	±	1.3	I	I	216.8	±	20.3	I	I	I
		2	14.5	±	0.3	I		167.7	±	4.8	I	I	
		3	7.3	±	0.7	I		239.0	±	53.4	I	I	
18	di-n-propyl disulphide	1	93.9	±	10.7	NI	NI	19.1	±	16.6	NI	NI	NI
		2	103.3	±	4.3	NI		6.5	±	2.6	NI	NI	
		3	104.2	±	9.0	NI		21.4	±	15.0	NI	NI	
19	butyl methacrylate	1	18.1	±	11.3	I	I	130.5	±	39.6	I	I	I
		2	44.9	±	2.1	I		112.3	±	11.5	NI	I	
		3	32.7	±	2.2	I		148.9	±	10.0	I	I	
20	heptanal	1	11.1	±	2.1	I	I	154.6	±	10.3	I	I	I
		2	14.3	±	1.6	I		136.6	±	3.5	I	I	
		3	9.8	±	0.8	I		189.5	±	24.8	I	I	

1) I: irritant, NI: non irritant

Dermal irritation is the production of reversible damage with inflammation. IL-1α is a key cytokine released in the inflammation process of skin irritation. In order to evaluate whether the IL-1α release could be used as an assay to predict the irritation potential of the test chemicals, we measured the IL-1α content in the conditioned medium from tissues onto which the chemicals were applied. The results of the IL-1α assay were used as a second endpoint to support the classification based on the MTT assay results. In this assay, 1-bromohexane (no. 16) was classified as an irritant based on the complementary evaluation of IL-1α release, although it was classified as a non-irritant in the MTT assay (Table 5-6).

Summarizing the above data, the sensitivity and specificity of predictions improved to 77.8% and 80% (Table 5-7). The overall accuracy was 78.9% (Table 5-7).

Table 5-7: Contingency table for skin irritation tests by cell viability and IL-1α release evaluation

		In vivo classification		
		Irritant	Non-irritant	Total
In vitro prediction	Irritant	7	2	9
	Non-irritant	2	8	10
	Total	9	10	19

Sensitivity (%)	77.8
Specificity (%)	80.0
Accuracy (%)	78.9

5-4. Discussion

The first purpose of the optimization stage is to set a suitable exposure period for the LabCyte EPI-MODEL24 SIT similar to VRM, as a "me-too" test. From the results of examining various exposure periods ranging from 10 to 30 min, it was judged that the exposure period of 15 minutes was the optimal period for the LabCyte EPI-MODEL24 SIT. The original LabCyte EPI-MODEL24 SIT, which reflected an optimization study of a suitable exposure period, was referred to in the SOP for ver.4.0 of the LabCyte EPI-MODEL24 SIT.

The second aim of this study was to evaluate whether the commonly used irritation protocols described in the ECVAM performance standards (1) were applicable to the original LabCyte EPI-MODEL24 SIT. To compare the performances of the original LabCyte EPI-MODEL24 SIT and VRM, we tested with the 19 test chemicals described in the ECVAM performance standards. Only the prediction for 1-bromohexane differed between the original LabCyte EPI-MODEL24 SIT and VRM, when viability was the endpoint used as an indicator for prediction. This chemical was erroneously predicted as a non-irritant by the LabCyte original EPI-MODEL24 SIT, whereas VRM classified it in concordance with the *in vivo* class. As demonstrated in a previous study (32), the IL-1α assay can improve prediction and support the viability assay. In this study of the original LabCyte EPI-MODEL24 SIT, the prediction for 1-bromohexane was improved, so that it was truly shown as an irritant through the combination of the MTT and IL-1α assays. As a result of this improvement, the sensitivity and the specificity of this prediction model using the LabCyte EPI-MODEL24 fulfilled both conditions described in the ECVAM performance standards: the sensitivity of a "me-too" test must be equal to or higher than 70% and the specificity equal to or higher than 80%. The ECVAM performance standard must demonstrate similarity and/or equivalence to the original LabCyte EPI-MODEL24 SIT in a formal inter-laboratory study, on the basis of the VRM. From these considerations, the original LabCyte EPI-MODEL24 SIT was evaluated in a validation study under blind conditions and supported by the Japanese Society for Alternatives to Animal Experiments (JSAAE).

The phase 1 (confirmation of technical transfer) and phase 2 (multisite) validation studies for the original SIT using the LabCyte EPI-MODEL24 were performed according to the ECVAM performance

standards. During the phase 1 and phase 2 validation studies, assay acceptance criteria regarding negative and positive control for the original LabCyte EPI-MODEL24 SIT were set based on the results of the phase 1 validation study. The acceptance criteria are referred to in the SOP for ver.5.0 of the LabCyte EPI-MODEL24 SIT. Details of the results of the validation study are described in the JSAAE report "VALIDATION STUDY OF *IN VITRO* SKIN IRRITATION TEST USING LABCYTE EPI-MODEL24" (Attachment 2). The conclusion described in the validation report is as follows:

Based on the EU classification, 9 irritants (one skin irritant could not be purchased in Japan) and 10 non-irritants in the ECVAM Performance Standards were tested by the same 7 labs. The assay demonstrated acceptable reliability of the positive control (100%) and accuracy (71% overall accuracy, 64% overall sensitivity, 79% overall specificity) in the MTT assay for use as a stand-alone assay to distinguish between skin irritants and non-irritants. In addition, the IL-1α endpoint was determined to be unnecessary.

Because it was decided that the IL-1α endpoint was not necessary after the phase 2 validation study, this conclusion was reflected in the SOP for ver.6.01 of the LabCyte EPI-MODEL24 SIT (Attachment 4).

In the ECVAM performance standards (updated) (28) that were revised in 2009, the *in vivo* classification of test chemicals was changed from referring to the EU DSD (European Classification System based on the Dangerous Substance Directive) to referring to the UN GHS (United Nations Globally Harmonised System of Classification and Labelling of Chemicals). This change was reflected in the OECD draft test guideline for skin irritation (2). With the changes for *in vivo* criteria, the set of reference chemicals described in the performance standard (updated) or the OECD draft test guideline was updated, and 6 chemicals were replaced. In line with these changes, the phase 3 validation study of the original SIT using LabCyte EPI-MODEL24 was performed with newly replaced 6 reference chemicals. Details of the results for the phase 3 validation study are described in the JSAAE report "VALIDATION STUDY OF *IN VITRO* SKIN IRRITATION TEST USING LABCYTE EPI-MODEL24 (2ND REPORT)" (Attachment 3). The content of the conclusion described in the validation report is as follows:

Based on the GHS-EU classification, 5 irritants and 1 non-irritant in the new ECVAM Performance Standards were tested by 6 labs using LabCyte EPI-MODEL24. The assay demonstrated high reliability with and without laboratories in the MTT assay, for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

The validation reports (Attachment 2,3) were assessed by a peer review panel in OECD. Unfortunately, the OECD peer review panel pointed out that the prediction results of the chemical 1-bromohexane, which is an *in vivo* Category 2 substance, in the original LabCyte EPI-MODEL24 SIT, did not meet the specific requirement of paragraph 12, Annex 2 of the OECD draft test guideline (2). Miss-classification of 1-bromohexane was not allowed in the OECD draft test guideline. However, the chemical 1-bromohexane was classified as a false-negative by five out of six laboratories in the validation exercise for the original LabCyte EPI-MODEL24 SIT. Therefore, a trial for the improvement of the original LabCyte EPI-MODEL24 SIT to solve this problem became indispensable.

6. Modification of the original LabCyte EPI-MODEL24 SIT and assessment of the modified SIT (MODIFICATION STAGE)

6-1. Summary of the MODIFICATION STAGE

As recommended by the OECD peer review panel, improvement of the original LabCyte EPI-MODEL24 SIT to solve the problem that 1-bromohexane was shown as a false-negative is indispensable in enabling its use as a "me-too" test similar to VRM (see Section 5). It is surmised that one reason 1-bromohexane showed a false negative in the original LabCyte EPI-MODEL24 SIT (SOP ver.6.01) is because 1-bromohexane did not remain in the reconstructed human epidermis tissues at the post-incubation for 42 hours. In order to solve the 1-bromohexane problem, we tried to find a condition where the 1-bromohexane would be retained in the stratum corneum of the LabCyte EPI-MODEL24 tissues at the post incubation. More concretely, the following item were examined.

a. Applied amount of test chemical.
b. Incubation temperature at exposure to the test chemicals.
c. Washing protocol for test chemicals on the tissue surface.

The 1-bromohexane problem was not solved by modifying chemical application, such as changing the amount of test chemicals applied or changing the temperature of incubation at exposure to the test chemicals. Only by modifying the washing protocol, were we able to solve the 1-bromohexane problem. The modified washing protocol that solved the 1-bromohexane problem was reflected in the SOP for ver.8.2 of the LabCyte EPI-MODEL24 SIT.

When the predictive potency of the modified LabCyte EPI-MODEL24 SIT was evaluated with the 20 test chemicals which were listed in the new OECD TG 439 for *in vitro* skin irritation testing, adopted in July, 2010, the sensitivity, the specificity and the overall accuracy were 90 %, 70 %, and 80%, respectively. It was thought that these results met the acceptance criteria described in the OECD TG 439. Furthermore, a catch-up validation study was performed with the modified LabCyte EPI-MODEL24 SIT. Results demonstrated high reliability and acceptable accuracy in the MTT assay, for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

6-2. Materials and methods

6-2a. Reference chemicals described in OECD TG 439

Twenty reference test chemicals shown in Table 6-1 were selected from the list in OECD TG 439 (Attachment 1). Reference chemicals are used to determine the reliability and accuracy of a proposed similar or modified test method, proven to be sufficiently similar to the VRM, both structurally and functionally.

Table 6-1: Reference chemicals list in the OECD TG 439

No.	Test chemical				
	Name	CAS number[1)]	*In vivo* class[2)]	PII[3)]	Supplier
1	1-bromo-4-chlorobutane	6940-78-9	NI	0	Wako chemical
2	Diethyl phthalate	84-66-2	NI	0	Wako chemical
3	naphthalen acetic acid	86-87-3	NI	0	Wako chemical
4	allyl phenoxy-acetate	7493-74-5	NI	0.3	Wako chemical
5	isopropanol	67-63-0	NI	0.3	Wako chemical
6	4-methyl-thio-benzaldehyde	3446-89-7	NI	1.0	Wako chemical
7	methyl stearate	112-61-8	NI	1.0	Kanto chemical
8	heptyl butyrate	5870-93-9	NI	1.7	Aldrich
9	hexyl salicylate	6259-76-3	NI	2.0	Sigma Fluka
10	Cinnamaldehyde	104-55-2	NI	2.0	Aldrich
11	1-decanol	112-30-1	I	2.3	Wako chemical
12	Cyclamen aldehyde	103-95-7	I	2.3	Wako chemical
13	1-bromohexane	111-25-1	I	2.7	Wako chemical
14	2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	86604-75-3	I	2.7	Wako chemical
15	di-n-propyl disulphide	629-19-6	I	3.0	Wako chemical
16	Potassium Hydroxide 5%	1310-58-3	I	3.0	Wako chemical
17	Benzene thiol 5-(1,1-dimethylethyl)-2-methyl	7340-90-1	I	3.3	Tokyo Chemical
18	1-methyl-3-phenyl-1-piperazine	5271-27-2	I	3.3	Tokyo Chemical
19	heptanal	111-71-7	I	3.3	Kanto chemical
20	1,1,1 Trichloroethane	71-55-6	I	4.0	Wako chemical

1) CAS No.: Chemical abstracts service registry number.
2) I: irritant, NI: non irritant
3) PII: primary irritation index.

6-2b. Wide-range of test chemicals

Fifty-four test chemicals over a wide range of classes were used for evaluation of the Modified LabCyte EPI-MODEL SIT, as shown in Table 6-2. These chemicals were referred to in the report of Kandárová et. Al (33). This selected test set consists of chemicals tested in EpiDerm and EPISKIN optimization studies preceding the ECVAM skin irritation validation study and also includes chemicals from the ECVAM validation study (32). Available information on irritation potential and classification according to the UN GHS systems are provided in Table 6-2.

Table 6-2: Fifty-four test chemicals over a MTT of classes

No.	Test chemicals				
	Name	CAS number[1)]	*In vivo* class[2)]	PII[3)]	Supplier
1	1-bromo-4-chlorobutane	6940-78-9	No Cat.	0	Wako chemical
2	diethyl phthalate	84-66-2	No Cat.	0	Wako chemical
3	di-propylene glycol	25265-71-8	No Cat.	0	Wako chemical
4	naphthalen acetic acid	86-87-3	No Cat.	0	Wako chemical
5	3-chloronitrobenzene	121-73-3	No Cat.	0	LGC STANDARDS
6	3,3-dithiodipropionic acid	1119-62-6	No Cat.	0	Wako chemical
7	4,4-methylenebis(2,6-di-tert-buthylphenol)	118-82-1	No Cat.	0	Tokyo Chemical
8	4-amino-1,2,4-triazole	584-13-4	No Cat.	0	Wako chemical
9	benzyl benzoate	120-51-4	No Cat.	0	Wako chemical
10	sodium bicarbonate	144-55-8	No Cat.	0	Kanto chemical
11	erucamide	112-84-5	No Cat.	0	Wako chemical
12	1,5-hexadiene	592-42-7	No Cat.	0	Aldrich
13	Polyethylene glycol 400	25322-68-3	No Cat.	0	Wako chemical
14	glycerol	56-81-5	No Cat.	0	Wako chemical
15	3,3-dimethylpentane	562-49-2	No Cat.	0	Aldrich
16	allyl phenoxy-acetate	7493-74-5	No Cat.	0.3	Wako chemical
17	isopropanol	67-63-0	No Cat.	0.3	Wako chemical
18	benzyl salicylate	118-58-1	No Cat.	0.3	Wako chemical
19	lauric acid	143-07-7	No Cat.	0.3	Kanto chemical
20	4-methyl-thio-benzaldehyde	3446-89-7	No Cat.	1.0	Wako chemical
21	methyl stearate	112-61-8	No Cat.	1.0	Kanto chemical
22	benzyl acetate	140-11-4	No Cat.	1.0	Wako chemical
23	hydroxycitronellal	107-75-5	No Cat.	1.0	Wako chemical
24	isopropyl myristate	110-27-0	No Cat.	1.0	Aldrich
25	isopropyl palmitate	142-91-6	No Cat.	1.0	Wako chemical
26	n-buthyl propionate	590-01-2	No Cat.	1.0	Wako chemical
27	sodium bisulphite	7631-90-5	No Cat.	1.0	Kanto chemical
28	benzyl alcohol	100-51-6	No Cat.	1.3	Aldrich
29	allyl heptanoate	142-19-8	No Cat.	1.7	Wako chemical
30	heptyl butyrate	5870-93-9	No Cat.	1.7	Aldrich

Table 6-2. Continued

No.	Test chemicals				
	Name	CAS number[1)]	*In vivo* class[2)]	PII[3)]	Supplier
31	2-ethoxy ethyl methacrylate	2370-63-0	No Cat.	1.7	Wako chemical
32	hexyl salicylate	6259-76-3	No Cat. (Cat. 3)	2.0	Sigma Fluka
33	linalyl acetate	111-95-7	No Cat. (Cat. 3)	2.0	Wako chemical
34	terpinyl acetate	80-26-2	No Cat. (Cat. 3)	2.0	Alfa Aesar
35	Linalool	78-70-6	No Cat. (Cat. 3)	2.0	Kanto chemical
36	cinnamaldehyde	104-55-2	No Cat. (Cat. 3)	2.0	Aldrich
37	eugenol	97-53-0	No Cat. (Cat. 3)	2.0	Wako chemical
38	cyclamen aldehyde	103-95-7	Cat. 2	2.3	Wako chemical
39	1-decanol	112-30-1	Cat. 2	2.3	Wako chemical
40	1-bromohexane	111-25-1	Cat. 2	2.7	Wako chemical
41	α-terpineol	98-55-5	Cat. 2	2.7	Kanto chemical
42	1-bromopentane	110-53-2	Cat. 2	2.7	Kanto chemical
43	2-chloromethyl-3,5-dimethyl-4-methoxypyridine HC	86604-75-3	Cat. 2	2.7	Wako chemical
44	butyl methacrylate	97-88-1	Cat. 2	3.0	Wako chemical
45	di-n-propyl disulphide	629-19-6	Cat. 2	3.0	Wako chemical
46	potassium hydroxide 5%	1310-58-3	Cat. 2	3.0	Wako chemical
47	heptanal	111-71-7	Cat. 2	3.3	Kanto chemical
48	benzene thiol, 5-(1,1-dimethylethyl)-2-methyl	7340-90-1	Cat. 2	3.3	Tokyo Chemical
49	1-methyl-3-phenyl-1-piperazine	5271-27-2	Cat. 2	3.3	Tokyo Chemical
50	SLS (20% aq)	151-21-3	Cat. 2	4.0	Wako chemical
51	1,1,1 trichloroethane	71-55-6	Cat. 2	4.0	Wako chemical
52	tetrachlotroethylene	127-18-4	Cat. 2	4.0	Wako chemical
53	capric acid (decanoic acid)	334-48-5	Cat. 2	4.0	Wako chemical
54	SLS (5% aq)	127-18-4	Cat. 2		Wako chemical

1) CAS No.: Chemical abstracts service registry number.
2) No Cat.: No Category, Cat. 2: Category 2, Cat. 3: Category 3
3) PII: primary irritation index.

6-2c. Test Protocol for modification of the original LabCyte EPI-MODEL24 SIT

The test protocol for modification of the original LabCyte EPI-MODEL24 SIT is described in section 5-2b. From the conclusion that an IL-1α endpoint was determined to be unnecessary in the validation studies, the content of IL-1α was not analyzed in this study. Three independent tests were performed on different batches of LabCyte EPI-MODEL24, with three tissues per test chemical. The overall test protocol is described in the original SOP for the LabCyte EPI-MODEL24 SIT (ver. 6.01).

6-2d. Protocol of the modified LabCyte EPI-MODEL24 SIT (SOP ver.8.2)

Prediction of the skin irritation potential of test chemicals by the modified LabCyte EPI-MODEL24 SIT was performed according to the modified protocol described in the SOP for ver.8.2 and described as

follows. LabCyte EPI-MODEL24 tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 ml) and incubated overnight (37°C, 5%, CO_2, humidified atmosphere). On the next day, the tissues were topically exposed to the test chemicals. Liquids (25 µl) were applied with a micropipette, and solids (25 mg) were applied from microtubes and moistened with 25 µl sterile water. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied using a cell-saver-type tip with a micropipette. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were treated with 25 µl distilled water, and three tissues serving as positive controls were exposed to 5% SLS. After 15 minutes, of exposure, each tissue was carefully rinsed with PBS at least ten times or more with a washing bottle to completely remove remaining test chemical from the surface. Furthermore, the leftover PBS outside the culture insert was gently removed with a sterile cotton bud, but the residue of PBS inside the insert was not touched. The blotted tissues were then transferred to new wells on 24-well plates containing 1 ml of fresh assay medium.

The treated and control tissues were post-incubated for 42 hours (37°C, 5% CO_2, humidified atmosphere). When the 42-hour post-incubation period was completed, blotted tissues were transferred to new wells on 24-well plates containing 0.5 ml of freshly prepared MTT medium *(0.5 mg/ml)* for the MTT assay. Tissues were incubated for three hours (37°C, 5% CO_2, humidified atmosphere) and were then transferred to microtubes containing 300 µl isopropanol, completely immersing the tissue. Formazan extraction was performed at room temperature and the tissues were allowed to stand overnight. Subsequently, 200 µl extracts were transferred to a 96-well plate. The optical density was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank.

Three independent tests were performed on different batches of LabCyte EPI-MODEL24, with three tissues per a test chemical. The prediction model is shown in Table 6-3. In the event that the three independent results within an individual batch were not in agreement, the result that occurred twice was used.

Table 6-3: The prediction models for the LabCyte EPI-MODEL24 SIT

Cell viability	Judgment
mean ≤ 50%	Irritant
mean > 50%	Non irritant

6-2e. Detecting chemical interference with MTT endpoints and correction procedures

A possible limitation of this skin irritation protocol described in OECD TG 439 might be due to the effect of a small amount of test chemicals on the MTT endpoints directly. The following two types of test chemicals can interfere with the MTT assay.

A. Chemicals that stain epidermis tissue.
B. Chemicals that can directly reduce MTT.

Test chemicals that stain the epidermis tissue could transfer from the epidermis tissue to the extraction solution and affect the optical density (OD) measurements. Test chemicals that can directly reduce MTT can also affect the optical density (OD) measurements, if the test chemical is present in the epidermis tissues when the MTT viability test is performed. The overall test protocol is described in the original SOP for the LabCyte EPI-MODEL24 SIT (ver. 8.2).

A. Detection of chemicals that stain the tissue
   Step 1 (preliminary test)
   Twenty-five µl (Liquid) or 25mg (Solid) of the test chemical was added to wells on a 24-well assay plate preliminarily filled with 0.5mL of distilled water. Untreated distilled water is used as a control. The mixture was incubated in a CO_2 incubator for 15 minutes. After incubation, the

mixture was evaluated for staining of the distilled water macroscopically. If the colour of the solution changes significantly, the test chemical is presumed to have the potential to stain the tissue and a functional check on viable tissues (Step2) should be performed. If the colour of the solution does not change significantly, it is determined that the test chemical has no potential to stain the tissue.

Step 2 (Functional check on viable tissue)
Twenty-five µl (Liquid) or 25mg (Solid) of the test chemical, which clearly changed the color of the distilled water (Step 1), was added onto the surface of the epidermis tissues. Distilled water is used as a negative control. All procedures of the original SIT using LabCyte EPI-MODEL24 described in section 6-2d were performed. However, the tissue was incubated for 3 hours in a culture medium without MTT instead of incubating in a medium containing MTT, in order to evaluate the staining of the epidermis tissues. The ratio of staining by a test chemical compared with the negative control was calculated.
If the ratio of staining by a test chemical is <5%, correction of the results is not necessary. If the ratio is between 5% and 30%, the corrected MTT OD is calculated.
If the ratio of staining by a test chemical is >30%, the test chemical must be considered incompatible with the test. However, when the cell viability (%) is <50%, the test chemical is determined as an irritant. Therefore, correction of the results or determination of incompatibility of the test chemical is not necessary in this case.

B. Detection of chemicals that directly reduce MTT
Step 3 (preliminary test)
Twenty-five µl (Liquid) or 25mg (Solid) of the test chemical was added to wells on a 24-well assay plate preliminarily filled with 0.5mL of MTT medium. Untreated MTT medium is used as a control. The mixture was incubated in a CO_2 incubator for 1 hour. After incubation, the mixture was evaluated for the staining of the MTT medium macroscopically. If the MTT medium turns significantly blue/purple, the test chemical can reduce MTT and an additional functional check (Step 4) must be performed. When the color of the solution does not change significantly, the test chemical is determined not to have the potential to reduce MTT.

Step 4 (Functional check on viable tissue)
Twenty-five µl (Liquid) or 25mg (Solid) of a test chemical, which clearly changed the color of the MTT medium to blue/purple (Step3), was added onto the surface of the epidermis tissues. Distilled water is used as a negative control. All procedures of the original SIT using LabCyte EPI-MODEL24 described in section 6-2d were performed. However, epidermis tissues that have been freeze-killed at -20 °C or lower for more than 24 hours were used instead of viable epidermis tissues. The ratio of staining by a test chemical compared with the negative control was calculated.
When the ratio of staining by a test chemical is <30%, the corrected MTT OD is calculated. When the ratio of staining by a test chemical is >30%, the test chemical must be considered incompatible with the test. However, when the cell viability (%) is <50%, the test chemical is determined as an irritant. Therefore, correction of the results or determination of incompatibility of the test chemical is not necessary in this case.

6-3. Results

6-3a. Modification of the original LabCyte EPI-MODEL24 SIT

First of all, in order to promote the penetration of 1-bromohexane to the stratum corneum, an increase in the applied amount of test chemical was examined. The applied amount of test chemical was 25μL in the original LabCyte EPI-MODEL24 SIT (SOP ver.6.01). Unfortunately, when the applied amount of 1-bromhexane was increased from 25μL to 50μL or 100μL, the cell viability of each tissue did not change at all (Fig.6-1). From these results, it was concluded that the 1-bromohexane problem could not be solved by changing the applied amount of test chemical.

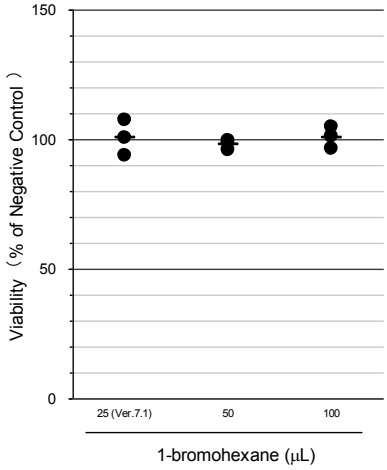


Fig.6-1 Influence of applied amount of chemical on cell viability of LabCyte EPI-MODEL24

For a purpose similar to the experiment with the applied amount, changing the temperature of incubation at exposure of the test chemical was examined. The incubation temperature at chemical exposure was room temperature in the original LabCyte EPI-MODEL24 SIT (SOP ver.6.01). 1-boromohexane was judged as a skin irritant with incubation at 37℃ for chemical exposure (Fig.6-2). However, isopropanol, which is a non irritant chemical in UN-GHS, showed a false-positive under the same condition (Fig.6-2). In the trial with the change in incubation temperature to 37℃ at exposure to the test chemicals, the 1-bromohexane problem was solved, but test chemicals that showed a false-positive increased, and the specificity of the LabCyte EPI-MODEL24 SIT decreased.

Therefore, it was thought that the 1-bromohexane problem could not be solved by changing the incubation temperature at exposure to the test chemicals.

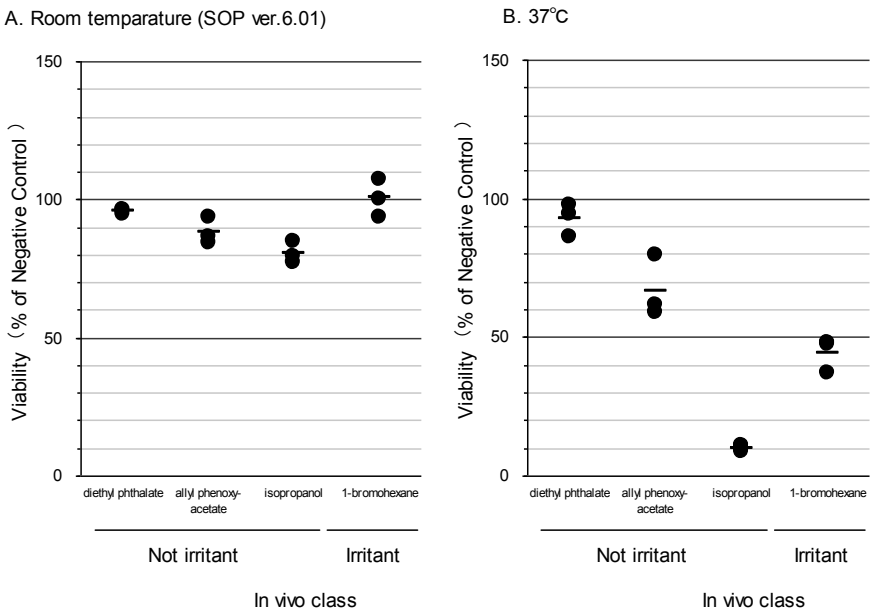


Fig.6-2 Influence of change in incubation temperature on the cell viability of LabCyte EPI-MODEL24

On the other hand, in order to retain chemical penetration into the stratum corneum, we modified the washing protocol as follows:

1. The PBS stream from the washing bottle doesn't hit directly on the tissue surface, but it hits the wall of the cell culture insert and the tissue surface is washed by the PBS current.
2. PBS inside the cell culture insert may be removed by gently tapping only once at the top of the beaker after each rinsing if necessary.
3. When removing the leftover PBS from the culture insert with a cotton bud, the inner side of the cell culture insert is not used, in order to prevent test chemicals that have penetrated the stratum corneum from being extracted by the cotton pad.

With the modification of the washing protocol, 1-bromohexane was then judged as a skin irritant (Fig.6-3). On the other hand, diethyl phthalate, allyl phenoxy-acetate, and isopropanol, which are all non irritant chemicals in the UN-GHS classification, were judged as non irritants under the same conditions (Fig.6-3).

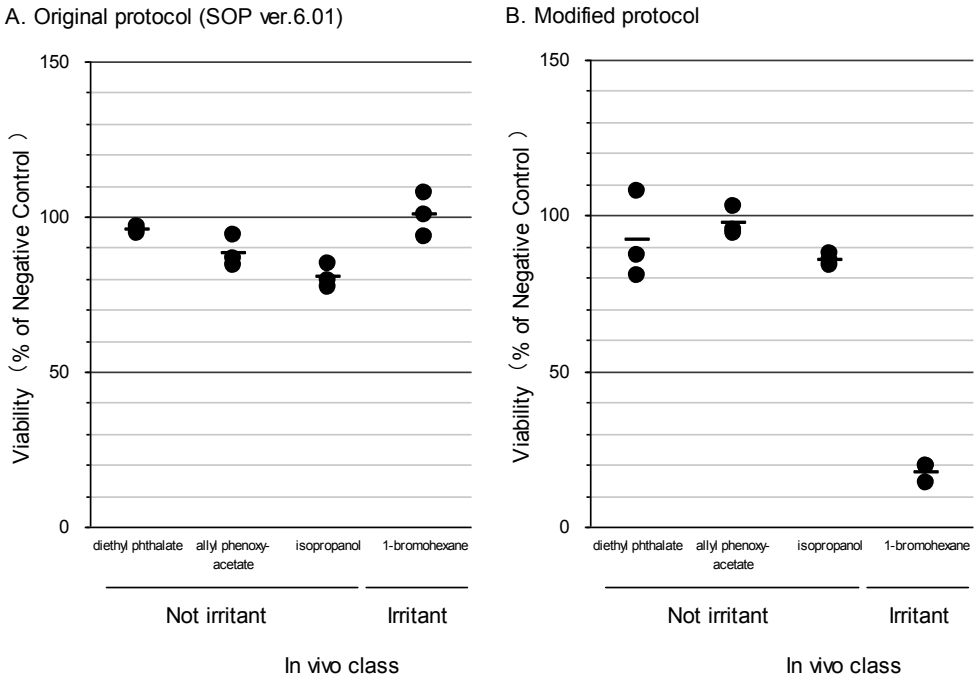


Fig.6-3 Influence of change to washing protocol on cell viability of LabCyte EPI-MODEL24

From these results, it was suggested that the 1-bromohexane problem could be solved by modifying the washing protocol.

Therefore, the modified washing protocol that where 1-bromohexane could be judged as a skin irritant was reflected in the SOP for ver.8.2 of the LabCyte EPI-MODEL24 SIT (Attachment 7).

Points in the washing protocol from the SOP for ver.6.01 which were modified in the SOP for ver.8.2 are shown in Table 6-3.

Table 6-4: Washing protocol modifications from the ver. 6.01 SOP to the ver.8.2 SOP

Modifications	SOP ver.6.01 (Original SIT)	SOP ver.8.2 (Modified SIT)
1. The PBS stream from the washing bottle	There were no instructions about the PBS stream.	It was instructed that the PBS stream must avoid hitting directly on the tissue surface.
2. Removal of PBS by tapping	Instructions stated that one must tap as much as possible after each rinsing.	It was instructed to tap only once if necessary
3. How to use the cotton pad	Instructions stated that the cotton pad should be used on the inner and outer sides of the cell culture insert.	It was instructed to use the cotton pad only on the outer side of the cell culture insert.

6-3b. Assessment of the modified LabCyte EPI-MODEL24 SIT based on the OECD TG 439 for intra-laboratory study

For 10 irritant chemicals (no.11 to no.20) *in vivo*, the results for di-n-propyl disulphide (no. 15) were not concordant with those of the *in vivo* classification (Table 6-4). 1-bromohexane (no.13) was judged as an irritant as shown by the modified LabCyte EPI-MODEL24 SIT (Table 6-4). On the other hand, for

10 non-irritants (from no.1 to no.10), 3 chemicals, 1-bromo-4-chlorobutane (no. 1), 4-methyl-thio-benzaldehyde (no. 6) and cinnamaldehyde (no. 10) were classified as positive by the modified LabCyte EPI-MODEL24 SIT (Table 6-4).

Table 6-5: Result of the modified LabCyte EPI-MODEL24 SIT with 20 test chemicals listed in *OECD TG 439*.

Test chemical			The modified LabCyte EPI-MODEL24 SIT			
			Cell viability (%)			*In vitro*
no.	Name	Run	Mean ± SD	Decision		class
1	1-bromo-4-chlorobutane	1	17.3 ± 4.4	I		I
		2	23.7 ± 1.5	I		
		3	18.6 ± 2.1	I		
2	diethyl phthalate	1	104.3 ± 4.4	NI		NI
		2	103.1 ± 1.5	NI		
		3	55.4 ± 13.8	NI		
3	naphthalen acetic acid	1	92.7 ± 1.2	NI		NI
		2	107.8 ± 2.0	NI		
		3	98.5 ± 3.8	NI		
4	allyl phenoxy-acetate	1	94.7 ± 1.8	NI		NI
		2	96.3 ± 3.6	NI		
		3	56.0 ± 3.8	NI		
5	isopropanol	1	83.5 ± 5.6	NI		NI
		2	87.7 ± 2.8	NI		
		3	82.7 ± 8.5	NI		
6	4-methyl-thio-benzaldehyde	1	24.6 ± 5.8	I		I
		2	21.4 ± 1.1	I		
		3	21.8 ± 0.8	I		
7	methyl stearate	1	98.6 ± 4.8	NI		NI
		2	113.1 ± 4.5	NI		
		3	101.5 ± 4.9	NI		
8	heptyl butyrate	1	105.9 ± 2.0	NI		NI
		2	108.8 ± 10.4	NI		
		3	109.7 ± 1.3	NI		
9	hexyl salicylate	1	88.4 ± 8.2	NI		NI
		2	119.5 ± 2.0	NI		
		3	112.2 ± 2.2	NI		
10	cinnamaldehyde	1	21.4 ± 4.1	I		I
		2	30.1 ± 5.9	I		
		3	35.6 ± 4.6	I		
11	1-decanol	1	24.9 ± 3.8	I		I
		2	29.8 ± 6.5	I		
		3	42.1 ± 2.9	I		
12	cyclamen aldehyde	1	30.1 ± 11.2	I		I
		2	24.2 ± 7.7	I		
		3	36.0 ± 4.5	I		
13	1-bromohexane	1	36.3 ± 8.2	I		I
		2	40.3 ± 10.8	I		
		3	43.0 ± 3.8	I		

Table 6-5. Continued

Test chemical			The modified LabCyte EPI-MODEL24 SIT			
			Cell viability (%)			*In vitro*
no.	Name	Run	Mean ± SD		Decision	class
14	2-chloromethyl-3,5-dimethyl-4-methoxypyridine HC	1	10.7 ± 1.5		I	I
		2	11.0 ± 1.7		I	
		3	16.4 ± 2.5		I	
15	di-n-propyl disulphide	1	74.9 ± 8.6		NI	NI
		2	77.1 ± 8.9		NI	
		3	63.2 ± 7.6		NI	
16	Potassium hydroxide (5% aq.)	1	3.3 ± 0.4		I	I
		2	2.5 ± 0.9		I	
		3	3.4 ± 1.4		I	
17	benzene thiol, 5-(1,1-dimethylethyl)-2-methyl	1	22.2 ± 8.3		I	I
		2	23.3 ± 2.4		I	
		3	37.2 ± 16.8		I	
18	1-methyl-3-phenyl-1-piperazine	1	12.1 ± 4.5		I	I
		2	20.5 ± 5.7		I	
		3	13.0 ± 4.4		I	
19	Heptanal	1	13.7 ± 9.9		I	i
		2	18.2 ± 1.8		I	
		3	19.9 ± 2.5		I	
20	1,1,1-trichloroethane	1	13.4 ± 0.6		I	I
		2	12.0 ± 1.1		I	
		3	10.1 ± 4.3		I	

Summarizing the data, the sensitivity and specificity of predictions by the modified LabCyte EPI-MODEL24 SIT were 90.0 % and 70.0 %, respectively (Table 6-5). The overall accuracy was 80.0% (Table 6-5). We believe that these results meet the acceptance criteria described in *OECD TG 439* (Attachment 5).

Table 6-6: Prediction model for the modified LabCyte EPI-MODEL24 SIT according to the SOP for ver.8.2.

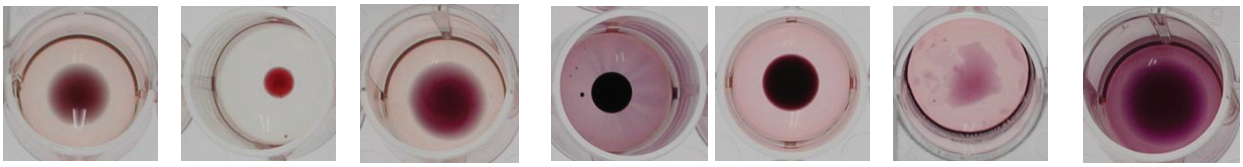
		In vivo classification		
		Irritant	Non-Irritant	Total
In vitro prediction	Irritant	9	3	12
	Non-irritant	1	7	8
	Total	10	10	20

Sensitivity (%)	90.0
Specificity (%)	70.0
Accuracy (%)	80.0

6-3c. Assessment of the modified LabCyte EPI-MODEL24 SIT using 54 test chemicals over a wide range of classification

First, 54 chemicals were examined to detect interference with MTT endpoints. Of the 54 tested

chemicals, there were no chemicals which directly stained epidermis tissue (data not shown). On the other hand, in the experiment for direct MTT reduction (6-2e, STEP 3), 4-methyl-thio-benzaldehyde (no. 20), benzyl alcohol (no. 28), cinnamaldehyde (no. 37), eugenol (no. 38), cyclamen aldehyde (no. 39), heptanal (no. 48) and benzene thiol 5-(1,1-dimethylethyl)-2-methyl (no. 49) were detected as potential MTT reducers (Fig. 6-4).



4-methyl-thio-benzaldehyde | benzyl alcohol | cinnamaldehyde | eugenol | cyclamen aldehyde | heptanal | 5-(1,1-dimethylethyl)-2-methyl

Fig.6-4 Direct MTT reduction ability (STEP 3).

Table 6-7: Detection of MTT reducer in 54 test chemicals.

Positive chemicals in step 3	Cell viability (%: not corrected)	Staining Ratio (% /NC[1)])	Corrected viability (%)
4-methyl-thio-benzaldehyd	21	13	20
benzyl alcohol	13	14	12
cinnamaldehyde	23	80	incompatible (16)
eugenol	34	145	incompatible (21)
cyclamen aldehyde	26	90	incompatible (19)
heptanal	26	16	25
benzene thiol 5-(1,1-dimethylethyl)-2-methyl	33	56	incompatible (28)

Therefore, we examined whether these substances reduced MTT using freeze-killed tissue instead of living tissue. As a result, the staining ratio (compared with a negative control) for 4-methyl-thio-benzaldehyde, benzyl alcohol, and heptanal were between 5% and 30 % (Table 6-7). Therefore corrected cell viabilities for these test chemicals had to be calculated (Table 6-7). However, the staining ratios for cinnamaldehyde, eugenol, cyclamen aldehyde, and benzene thiol 5-(1,1-dimethylethyl)-2-methyl were higher than 30 % and the cell viabilities of all potential MTT inducers were lower than 50%, so these chemicals were finally determined as irritants.

Next, the performance of the modified LabCyte EPI-MODEL24 SIT was evaluated with the 54 test chemicals. Results obtained with each chemical are given in 6-8. Sodium bisulphite (no. 27) and 2-ethoxy ethyl methacrylate (no. 31) showed different predictions in three independent test runs. Concordance with classifications obtained within-laboratory had a sufficient level of reproducibility for the modified LabCyte EPI-MODEL24 SIT at 96% (52/54).

As for 17 irritants (no.39 to no.54), the results for di-n-propyl disulphide (no. 46) were not concordant with those of the *in vivo* classification (Table 6-8). With regard to 37, non-irritants (from no.1 to no.37), 11 chemicals, 1-bromo-4-chlorobutane (no. 1), 4-methyl-thio-benzaldehyde (no. 20), benzyl acetate (no.

22), hydroxycitronellal (no. 23), n-buthyl propionate (no. 26),, benzyl alcohol (no. 28), 2-ethoxy ethyl methacrylate (no. 31), terpinyl acetate (no. 34), linalol (no.35), cinnamaldehyde (no. 36) and eugenol (no. 37) were classified as positive by the modified LabCyte EPI-MODEL24 SIT (Table 6-8).

In summary, based on the mean of the three independent runs (Table 6-8), 26 out of 37 non-irritants and 16 out of 17 irritants in the GHS classification were classified correctly by the modified LabCyte EPI-MODEL24 SIT. The statistical parameters describing assay performance are displayed in Table 6-9. Sensitivity and specificity of predictions by the modified LabCyte EPI-MODEL24 SIT were 94.1% and 70.3%, respectively. Overall accuracy was 77.8% (Table 6-9).

Table 6-8: Results for the modified LabCyte EPI-MODEL24 SIT tested with 54 chemicals.

Test chemical		The modified LabCyte EPI-MODEL24 SIT (SOP ver.8.2)						VRM	EpiDerm™ SIT
			Cell viability (%)				*In vitro* class		
No.	Name	Run	Mean	±	SD	Judgment			
1	1-bromo-4-chlorobutane	1	17.3	±	4.4	I	I	I	I
		2	23.7	±	1.5	I			
		3	18.6	±	2.1	I			
2	diethyl phthalate	1	104.3	±	4.4	NI	NI	NI	NI
		2	103.1	±	1.5	NI			
		3	55.4	±	13.8	NI			
3	di-propylene glycol	1	109.9	±	2.0	NI	NI	NI	NI
		2	100.2	±	10.2	NI			
		3	92.9	±	12.2	NI			
4	naphthalen acetic acid	1	92.7	±	1.2	NI	NI	NI	NI
		2	107.8	±	2.0	NI			
		3	98.5	±	3.8	NI			
5	3-chloronitrobenzene	1	104.3	±	4.4	NI	NI	NI	NI
		2	98.0	±	2.2	NI			
		3	95.2	±	6.9	NI			
6	3,3-dithiodipropionic acid	1	100.0	±	5.6	NI	NI	NI	NI
		2	98.9	±	2.5	NI			
		3	89.9	±	10.1	NI			
7	4,4-methylenebis (2,6-di-tert-buthylphenol)	1	103.9	±	10.6	NI	NI	NI	NI
		2	100.0	±	3.5	NI			
		3	100.0	±	9.9	NI			
8	4-amino-1,2,4-triazole	1	101.0	±	6.9	NI	NI	NI	NI
		2	97.4	±	6.7	NI			
		3	98.8	±	8.4	NI			
9	benzyl benzoate	1	105.7	±	2.0	NI	NI	NI	NI
		2	99.6	±	3.2	NI			
		3	100.3	±	10.1	NI			
10	sodium bicarbonate	1	99.6	±	3.7	NI	NI	NI	NI
		2	100.0	±	5.5	NI			
		3	100.3	±	12.4	NI			
11	Erucamide	1	102.5	±	2.2	NI	NI	NI	NI
		2	92.7	±	2.8	NI			
		3	90.0	±	11.0	NI			
12	1,5-hexadiene	1	85.6	±	12.2	NI	NI		NI
		2	92.5	±	7.2	NI			
		3	95.6	±	3.8	NI			
13	polyethlene glycol 400	1	106.6	±	8.7	NI	NI		NI
		2	98.2	±	5.4	NI			
		3	103.2	±	4.3	NI			
14	Glycerol	1	125.7	±	9.0	NI	NI		NI
		2	98.1	±	6.5	NI			
		3	103.3	±	6.5	NI			
15	3,3-dimethylpentane	1	72.3	±	3.2	NI	NI		NI
		2	76.3	±	9.7	NI			
		3	90.8	±	1.9	NI			
16	allyl phenoxy-acetate	1	94.7	±	1.8	NI	NI	NI	NI
		2	96.3	±	3.6	NI			
		3	56.0	±	3.8	NI			
17	isopropanol	1	83.5	±	5.6	NI	NI	NI	NI
		2	87.7	±	2.8	NI			
		3	82.7	±	8.5	NI			
18	benzyl salicylate	1	93.6	±	16.4	NI	NI	NI	NI
		2	98.2	±	3.6	NI			
		3	99.9	±	9.7	NI			
19	lauric acid	1	94.0	±	4.0	NI	NI	NI	I
		2	109.2	±	7.4	NI			
		3	110.0	±	6.3	NI			
20	4-methyl-thio-benzaldehyde	1	24.6	±	5.8	I	I	NI/I	I
		2	21.4	±	1.1	I			
		3	21.8	±	0.8	I			
21	methyl stearate	1	98.6	±	4.8	NI	NI	NI	NI
		2	113.1	±	4.5	NI			
		3	101.5	±	4.9	NI			
22	benzyl acetate	1	11.3	±	2.7	I	I	I	NI
		2	29.8	±	4.7	I			
		3	37.4	±	8.0	I			
23	hydroxycitronellal	1	19.8	±	11.2	I	I	NI	I
		2	20.4	±	1.8	I			
		3	32.3	±	6.0	I			
24	isopropyl myristate	1	107.9	±	3.7	NI	NI	NI	NI
		2	97.3	±	5.2	NI			
		3	97.2	±	12.4	NI			
25	isopropyl palmitate	1	104.1	±	10.6	NI	NI	NI	NI
		2	102.5	±	1.3	NI			
		3	115.9	±	2.2	NI			

Table 6-8. Continued

Test chemical		The modified LabCyte EPI-MODEL24 SIT (SOP for ver.8.2)						VRM	EpiDerm™ SIT
			Cell viability (%)				*In vitro* class		
no.	Name	Run	Mean	±	SD	Judgment			
26	n-buthyl propionate	1	23.1	±	4.7	I	I	I	I
		2	41.2	±	6.4	I			
		3	45.9	±	6.4	I			
27	sodium bisulphite	1	11.1	±	5.5	I	NI	NI	NI
		2	61.2	±	6.8	NI			
		3	74.7	±	9.7	NI			
28	benzyl alcohol	1	5.6	±	1.3	I	I	NI	I
		2	17.5	±	4.9	I			
		3	17.8	±	2.0	I			
29	allyl heptanoate	1	95.6	±	5.7	NI	NI	NI	NI
		2	108.9	±	10.7	NI			
		3	104.6	±	3.3	NI			
30	heptyl butyrate	1	105.9	±	2.0	NI	NI	NI	NI
		2	108.8	±	10.4	NI			
		3	109.7	±	1.3	NI			
31	2-ethoxy ethyl methacrylate	1	29.3	±	5.2	I	I	NI/I	I
		2	43.1	±	5.9	I			
		3	74.4	±	3.6	NI			
32	hexyl salicylate	1	88.4	±	8.2	NI	NI	NI	NI
		2	119.5	±	2.0	NI			
		3	112.2	±	2.2	NI			
33	linalyl acetate	1	101.2	±	1.1	NI	NI	NI/I	NI
		2	86.8	±	6.7	NI			
		3	92.6	±	12.6	NI			
34	terpinyl acetate	1	26.2	±	4.1	I	I	NI/I	NI
		2	33.6	±	3.3	I			
		3	36.3	±	10.9	I			
35	Linalool	1	7.9	±	0.7	I	I	I	I
		2	16.6	±	1.9	I			
		3	25.4	±	4.0	I			
36	cinnamaldehyde	1	21.4	±	4.1	I	I	I	I
		2	30.1	±	5.9	I			
		3	35.6	±	4.6	I			
37	Eugenol	1	18.5	±	2.2	I	I	I	I
		2	30.4	±	4.4	I			
		3	32.2	±	7.1	I			
38	cyclamen aldehyde	1	30.1	±	11.2	I	I	I	I
		2	24.2	±	7.7	I			
		3	36.0	±	4.5	I			
39	1-decanol	1	24.9	±	3.8	I	I	I	I
		2	29.8	±	6.5	I			
		3	42.1	±	2.9	I			
40	1-bromohexane	1	36.3	±	8.2	I	I	I	I
		2	40.3	±	10.8	I			
		3	43.0	±	3.8	I			
41	α-terpineol	1	13.6	±	1.3	I	I	I	I
		2	17.4	±	1.6	I			
		3	33.0	±	5.8	I			
42	1-bromopentane	1	24.3	±	15.1	I	I	I	I
		2	17.7	±	3.9	I			
		3	23.7	±	5.7	I			
43	2-chloromethyl-3,5-dimethyl -4-methoxypyridine HC	1	10.7	±	1.5	I	I	I	I
		2	11.0	±	1.7	I			
		3	16.4	±	2.5	I			
44	butyl methacrylate	1	28.6	±	6.1	I	I	I	I
		2	24.5	±	4.1	I			
		3	33.6	±	11.8	I			
45	di-n-propyl disulphide	1	74.9	±	8.6	NI	NI	I/NI	NI
		2	77.1	±	8.9	NI			
		3	63.2	±	7.6	NI			
46	potassium hydroxide 5%	1	3.3	±	0.4	I	I	I/NI	I
		2	2.5	±	0.9	I			
		3	3.4	±	1.4	I			
47	heptanal	1	13.7	±	9.9	I	I	I/NI	I
		2	18.2	±	1.8	I			
		3	19.9	±	2.5	I			
48	benzene thiol, 5-(1,1-dimethylethyl)-2-methyl	1	22.2	±	8.3	I	I	I	I
		2	23.3	±	2.4	I			
		3	37.2	±	16.8	I			
49	1-methyl-3-phenyl -1-piperazine	1	12.1	±	4.5	I	I	I	I
		2	20.5	±	5.7	I			
		3	13.0	±	4.4	I			

Table 6-8. Continued

Test chemical			The modified LabCyte EPI-MODEL24 SIT (SOP ver.8.2)					VRM	EpiDerm™ SIT
			Cell viability (%)				*In vitro* class		
no.	Name	Run	Mean	±	SD	Judgment			
50	SLS (20% aq)	1	9.4	±	0.9	I	I	NI	I
		2	13.2	±	1.3	I			
		3	13.4	±	4.2	I			
51	1,1,1 trichloroethane	1	13.4	±	0.6	I	I	I	I
		2	12.0	±	1.1	I			
		3	10.1	±	4.3	I			
52	tetrachlotroethylene	1	11.1	±	1.3	I	I	I	I
		2	17.1	±	0.9	I			
		3	22.6	±	2.1	I			
53	capric acid (decanoic acid)	1	6.1	±	2.8	I	I		I
		2	9.8	±	0.4	I			
		3	17.6	±	3.4	I			
54	SLS (5% aq)	1	14.5	±	1.9	I	I	I	I
		2	11.4	±	1.0	I			
		3	14.4	±	0.9	I			

Source of VRM and EpiDermTM SIT data (33)

Table 6-9. Prediction model for skin irritation test by cell viability evaluation with 54 test chemicals and based on the SOP for ver.8.2.

		In vivo classification		
		Irritant	Non-Irritant	Total
In vitro prediction	Irritant	16	11	27
	Non-irritant	1	26	27
	Total	17	37	54

Sensitivity (%)	94.1
Specificity (%)	70.3
Accuracy (%)	77.8

6-4. Discussion

As recommended by the OECD peer review panel, the original LabCyte EPI-MODEL24 SIT had to be improved in order to solve the problem where 1-bromohexane was being shown as a false-negative. We hypothesized that it was important to retain 1-bromohexane in the LabCyte EPI-MODEL24 tissue at the post incubation. Therefore, we modified the conditions for the application of test chemicals, such as the applied amount of test chemical and the temperature at exposure to test chemicals. However, unfortunately, the 1-bromohexane problem was not solved by changing conditions under which test chemicals were applied. In the end, it was found that the problem concerning 1-bromohexane could be solved by changing the washing protocol. By changing the washing protocol, 1-bromohexane penetration into the stratum corneum could be sufficiently retained. The judgments for the other 19 reference chemicals listed in OECD TG 439 excluding 1-bromohexane did not change between the original LabCyte EPI-MODEL24 SIT and the modified LabCyte EPI-MODEL24 SIT. With regard to the predictive potency of the modified LabCyte EPI-MODEL24 SIT with the 20 test chemicals listed in the OECD TG 439, the sensitivity, specificity and overall accuracy were 90 %, 70 %, and 80%, respectively. It was thought that these results met the acceptance criteria described in the OECD TG 439.

In the evaluation of the performance of the modified LabCyte EPI-MODEL24 SIT with the wide-range of 54 test chemicals, sensitivity, specificity and overall accuracy were 94.1 %, 70.3 %, and 77.8%,

respectively. Prediction potency was thought to be almost equal to the VRM (EpiSkin™ test method) or Epiderm™ SIT (33).

OECD TG 439 requires demonstration of similarity and/or equivalence to the SIT using the new reconstructed human epidermal model in a formal inter-laboratory study, based on the VRM. It was considered that the performance of the modified LabCyte EPI-MODEL24 SIT needed to be evaluated by a formal validation study under blind conditions, as stipulated by OECD TG 439. Therefore, a catch-up validation study of the modified LabCyte EPI-MODEL24 SIT was performed from September to November, 2010, supported by the Japanese Center for the Validation of Alternative Methods (JaCVAM). Results demonstrated high reliability and acceptable accuracy on the MTT assay for use as a stand-alone assay to distinguish between skin irritants and non-irritants. Details of the results of the validation study will be described in a JaCVAM final report.

7. Conclusion

Through a final, formal validation study based on OECD TG 439, this document demonstrates that the modified LabCyte EPI-MODEL24 SIT is a useful *in vitro* methodology for classification of skin irritation potential.

8. Acknowledgement

We would like to thank Ms. Lu Qiu at the Shanghai Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China for her technical support with the QC experiments for LabCyte EPI-MODEL24 after the export of shipments of LabCyte EPI-MODEL24 out of Japan. We would also like to thank Mr. Yoshihiro Yamaguchi and Ms. Maki Nakamura in Kobayashi Pharmaceuticals Co., Ltd. for their gracious support of this work.

9. Abbreviations

ECVAM	European Centre for the Validation of Alternative Methods
EU DSD	European Classification System based on the Dangerous Substance Directive (DSD)
GHS	Globally harmonised system on the classification and labelling of chemicals
IL-1α	Interleukin-1 alpha
JaCVAM	Japanese Center for the Validation of Alternative Methods
JSAAE	Japanese Society for Alternatives to Animal Experiments
J-TEC	Japan Tissue Engineering Co. Ltd.
MTT	3-(4,5-Dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide. A vital dye used to assess cell viability via a colorimetric assay. Cell viability is used as a proxy for predicting the skin irritancy potential of xenobiotic substances in human reconstructed epidermis models.
OECD	Organisation for Economic Cooperation and Development
QC	Quality control
PBS	Phosphate buffer saline
RhE	Reconstructed human epidermis
SD	Standard deviation
SIT	Skin irritation test(ing)
SLS	Sodium lauryl sulphate
TG	Test guideline
UN GHS	The GHS system for skin irritation as applicable to all authorities, i.e. using one irritant category.
VRM	Validated reference method

10. Attachments (not in this OECD Report)

Attachment 1. OECD TG 439: OECD guideline for the testing of chemicals. *In vitro* Skin Irritation: Reconstructed Human Epidermis Test Method. (2010) Website: http://browse.oecdbookshop.org/oecd/pdfs/browseit/9743901E.PDF

Attachment 2. LABCYTE VALIDATION MANAGEMENT TEAM: JSAAE DRAFT REPORT: VALIDATION STUDY OF *IN VITRO* SKIN IRRITATION TEST USING LABCYTE EPI-MODEL24. APRIL 15, 2009.

Attachment 3. LABCYTE VALIDATION MANAGEMENT TEAM: JSAAE REPORT: VALIDATION STUDY OF *IN VITRO* SKIN IRRITATION TEST USING LABCYTE EPI-MODEL24 (2ND REPORT). JULY 22, 2009

Attachment 4. Skin irritation test protocol using the reconstructed human model "LabCyte EPI-MODEL24" Ver.6.01

Attachment 5. Skin irritation test protocol using the reconstructed human model "LabCyte EPI-MODEL24" Ver.8.2

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Appendix 3

-JSAAE Skin Irritation Test Validation Study-

SKIN IRRITATION TEST
USING THE RECONSTRUCTED HUMAN MODEL "LABCYTE EPI-MODEL 24"
Ver. 8.2

LabCyte EPI-MODEL24 SKIN IRRITATION TEST$^{-42\ HOURS}$

S.O.P.

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MDS 2: PRE-INCUBATION OF LABCYTE EPI-MODEL 24 (Section 3.3.1)

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MDS 4: MTT ASSAY (Section 3.3.4)

MDS 5: FORMAZAN EXTRACTION AND MEASUREMENT (Section 3.3.5)

1. RATIONAL AND BACKGROUND

1.1 SKIN IRRITATION TEST using LabCyte EPI-MODEL 24 (SIT using LabCyte EPI-MODEL 24)

The SIT using LabCyte EPI-MODEL24 is designed for the prediction of acute skin irritation of chemicals by measurement of its cytotoxic effect, as reflected in the MTT assay, on the Reconstructed Human Epidermis (RHE) model. The SIT using LabCyte EPI-MODEL24 is not a kit; LabCyte EPI-MODEL24 tissues are commercially available per tissues item (with a minimum of 24 LabCyte EPI-MODEL24 tissues per order).

1.2 BACKGROUND OF SIT using LabCyte EPI-MODEL24

Performance standards for applying human skin models to in vitro skin irritation testing were also defined based on the validated test EpiSkin^{TM} test method (ECVAM SIVS, 2007). These performance standards can be then used to evaluate the accuracy and reliability of other analogous test methods (also referred to as "me-too" tests) that are based on similar scientific principles and measure or predict the same biological or toxic effect.

Based on the GHS-EU classification, 12 irritants and 13 non-irritants in the draft performance standards (ECVAM 2007) and the statement by ESAC (ESAC2009) were performed the validation study through the 7 labs SIT using LabCyte EPI-MODEL24. Results were summarized at JSAAE 1st report and 2nd report on this validation study.

1.3 BASIS OF THE METHOD

Chemical-induced skin irritation, manifested by erythema and oedema, is the results of a cascade of events beginning with penetration of the stratum corneum and damage to the underlying layers of keratinocytes. The dying keratinocytes release mediators that begin the inflammatory cascade which acts on the cells in the dermis, particularly the stromal and endothelial cells. It is the dilation and increased permeability of the endothelial cells that produce the observed erythema and oedema. The RhE-based test methods measure the initiating events in the cascade.

The relative viability of the treated tissues was measured at the end of the treatment exposure (15 minutes) followed by a post-exposure period (42 hours) using MTT [(3-4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazoliumbromide] assay. A cutoff value of 50% viability of the negative control value was considered and used to classify test substances as irritant (I) or non irritant (NI). The culture environment might allow the detection of very small quantities of cytokines secreted by the epidermis in response to topical application of test substances.

1.3.1 TEST SYSTEM DESCRIPTION

LabCyte EPI-MODEL24 is a new, commercially available RhE model produced by Japan Tissue Engineering Co. Ltd. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand human keratinocytes while maintaining their phenotype, they were cultured with 3T3-J2 cells as a feeder layer (Rheinwald and Green, 1975; Green, 1978). Reconstruction of human cultured epidermis is achieved by cultivating and proliferating keratinocytes on an inert filter substrate (surface 0.3 cm_2) at the air-liquid interface for 13 days with an optimized medium containing 5% fetal bovine serum. It constructs a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum. LabCyte EPI-MODEL24 is embedded in an agarose gel containing nutrient solution and shipped in 24-well plates at around 18°C.

1.3.1.1 Quality control of the test system

The LabCyte EPI-MODEL24 is manufactured according to defined quality assurance procedures. Each batch production was provided with quality controls such as storage conditions, RHE instructions for use, lot number and origin, histology (demonstration of human epidermis-like structure with multilayered stratum corneum), cell viability, barrier function integrity ($0.14 \leq IC50 \leq 0.4$).

1.3.1.2 Precautions

The epidermal cells are taken from healthy donor negative to HIV, and Hepatitis. Nevertheless, handling procedures for biological materials should be followed:

a) It is recommended to wear gloves during handling with the skin and kit components.
b) After use, the epidermis, the material and all media in contact with it should be decontaminated prior to disposal (e.g. using special containers or autoclaving).

1.3.2 ASSAY QUALITY CONTROL

1.3.2.1 Assay Acceptance Criterion 1: Negative Control

The absolute OD of the negative control (NC) tissues (treated with sterile DPBS) in the MTT assay is an indicator of tissue viability obtained in the testing laboratory after shipping and storing procedures and under specific conditions of use.

$0.7 \leq$ Mean OD (A570/650) measured value ≤ 2.5

1.3.2.2 Assay Acceptance Criterion 2: Positive Control

A 5% SDS (in H_2O) solution (see 7.6.3) is used as positive control (PC) and tested concurrently with the test chemicals. Concurrent means here the PC has to be tested in each assay, but not more than one PC is required per testing day. Viability of positive control should be within 95±1 % confidence interval of the historical data.

Mean tissue viability $\leq 40\%$

1.3.2.3 Assay Acceptance Criterion 3: Standard Deviation (SD)

Since in each test skin irritancy potential is predicted from the mean viability determined on 3 single tissues, the variability of tissue replicates should be acceptably low.

Standard Deviation (SD) of tissue viability of 3 identically treated replicates for negative control and positive control ≤ 18 %

1.4 LIMITATION OF THE METHOD

One limitation of this assay method is a possible interference of the test substance with the MTT endpoint. A colored test substance or one that directly reduces MTT (and thereby mimics dehydrogenase activity of the cellular mitochondria) may interfere with the MTT endpoint. However, these test substance are a problem only if at the time of the MTT test (i.e. 42 hours after test substance exposure) sufficient amounts of the test substance are still present on (or in) the tissues. In case of this unlikely event, the (true) metabolic MTT reduction and the contribution by a colored test material or (false) direct MTT reduction by the test material can be quantified by a procedure described in Section 3.2.

The method is not designed for testing of highly volatile test substances, gases and aerosols.

1.5 BRIEF BASIC PROCEDURE

On the day of receipt, LabCyte EPI-MODEL24 tissues are conditioned by incubation to release transportstress related compounds and debris overnight. After pre-incubation, tissues are topically exposed to the test chemicals for 15 minutes. Preferably, three tissues are used per test chemical (TC) and for the positive control (PC) and negative control (NC). Tissues are then thoroughly rinsed, blotted to remove the test substances, and transferred to fresh medium. After 42 hr incubation period, the MTT assay is performed by transferring the tissues to the well containing MTT medium (0.5 mg/ml). After 3 hr MTT incubation, the blue formazan salt formed by cellular mitochondria is extracted with 0.3 mL/tissue of isopropanol and the optical density of the extracted formazan is determined using a spectrophotometer at 570 nm and 650 nm as reference. Relative cell viability is calculated for each tissue as % of the mean of the negative control tissues. Skin irritation potential of the test material is predicted if the remaining relative cell viability is below 50%.

1.6 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)

According to the GHS classification (Category 2 or no label), an irritant is predicted if the mean relative tissue viability of three individual tissues exposed to the test substance is reduced below 50% of the mean viability of the negative controls.

In vitro results	In vivo prediction
Tissue viability is ≤ 50%	Irritant
Tissue viability is > 50%	Non Irritant

2. MATERIALS

2.1 LabCyte EPI-MODEL 24

2.1.1 LabCyte EPI-MODEL 24 KIT COMPONENTS

LabCyte EPI-MODEL 24 kit components are shown in Table 1.

Table 1 - LabCyte EPI-MODEL24 Kit Components

Component	Qty	Description
LabCyte EPI-MODEL 24 plate	1 plate	Contains 24 culture inserts with tissues fixed in nutritive agar medium for transport (usable area: 0.3cm^2).
Assay Medium	1 bottle	Basic medium for incubation (30mL). Store at refrigeration temperature.
24-well plate	1 plate	Blank plate for use in assay. Store at room-temperature.

2.1.2 SHIPMENT OF LabCyte EPI-MODEL24

LabCyte EPI-MODEL 24 is packed in a special container (Icompo/NIPPON EXPRESS CO., LTD) and delivered by NIPPON EXPRESS CO., LTD. After the Icompo is delivered, examine the contents and make sure that all kit components (LabCyte EPI-MODEL24 plate, assay medium, and 24-well assay plate) are included in the package. Confirm lot numbers and expiration dates also. Record details in the Methods Documentation Sheet (MDS) 1.

NIPPON EXPRESS will pick up the Icompo at a later date (generally, the day after the date of delivery), and we ask that you return it with a slip documenting receipt, as well as the insulating materials.

2.1.3 INSTRUCTIONS FOR USE OF LabCyte EPI-MODEL 24

Begin incubating all of the culture inserts after opening the package. Do not store the culture inserts again after opening.

The human epidermis cells used in LabCyte EPI-MODEL 24 originate from a normal donor and are HIV-, HBV-, HCV-, and HPV-negative. However, handle them with enough care and in accordance with the laboratory biosafety guidelines since they contain raw materials of human origin.

2.2 TEST CHEMICALS

Coded test chemicals are delivered to each laboratory.

2.3 CONSUMABLES

The following consumables are required.

* The described quantities are necessary so that 1 to 6 samples can be assayed once.

- Assay Medium, 100mL (J-TEC: 402250) 1 bottle
- MTT, 25mg (J-TEC: 403026) 1 bottle
- Wide orifice cell saver tips for micro-pipettes (sterile) 96 tips 1 box
- 24-well assay plate (Becton,Dickinson and Company: 353047) 7 plates
- 96-well plate (Becton,Dickinson and Company: 353072) 1 plates
- Phosphate buffered saline (PBS) 500mL (Invitrogen: 14190-144) 2 bottles
- Isopropanol 500mL (Wako Pure Chemical Industries: 164-08335) 1 bottle
- SLS 25g (SIGMA:L4390) 1 bottle
- Sterile distilled water 20mL (Otsuka Pharmaceutical: 36A1X00001) 1 bottles
- Sterile cotton buds (JAPAN COTTON BUDS: 10A754D) 1 box

2.4 OTHERS

2.4.1 EQUIPMENT / INSTRUMENTS

- Safety cabinet (or clean bench)
- Water bath (37 °C)
- CO_2 incubator (37 °C, 5%CO_2, capable of maintaining high humidity)
- Autoclave
- 96-well multi-plate reader (required filters: 450nm, 570nm, 650nm)
- Precision balance (0.1mg)
- Aspirator
- Stop-watches
- Adjustable micro-pipette (10-200μL, 200-1000μL)
- Sharp-edged forceps (sterile)
- Micro spatula (sterile)
- Beaker (1~2L: sterile)
- Sterilizable poly wash bottle (500~1000mL: sterile)

2.4.2 CONSUMMABLE ITEMS

- Micro-pipette tips (sterile: 10~200μL, 200~1000μL)
- Microtubes (1.5mL)
- Scalpel (KEISEI MEDICAL INDUSTRIAL: Keisei Scalpel 11A)

3. TEST METHOD

*Perform operations in Section 3.1.1~3.1.4 and Section 3.3.1~3.3.2 aseptically in a safety cabinet (or clean bench).

*Operations other than above do not need to be performed with an aseptic technique. For these operations, refer to **Section 2.1.3 INSTRUCTIONS FOR USE OF LabCyte EPI-MODEL 24**

3.1 PREPARATIONS

3.1.1 MTT SOLUTION

(1) Dissolve MTT in the assay medium to prepare the MTT medium (final concentration: 0.5mg/mL) Use ultrasonic cleaning equipment or a vortex mixer as necessary in order to completely dissolve the MTT.

*Store in a dark, cold place and use it within 24 hours.

(2) Record details of step (1) above in the MDS 4.

3.1.2 POSITIVE CONTROL SUBSTANCE

(1) Weigh 500mg of SLS precisely.

(2) To prepare a positive control solution, put the SLS into a graduated cylinder or measuring flask and dilute to 10mL with distilled water (final concentration: 5% w/v)]

* Store in a dark, cold place and use it within 24 hours.

(3) Record details of steps (1) and (2) above in the MDS 3.

3.1.3 NEGATIVE CONTROL SUBSTANCE

(1) Use distilled water.

3.1.4 POLY WASH BOTTLE FOR PBS

(1) Sterilize poly wash bottle using an autoclave.

(2) Fill the sterilized poly wash bottle aseptically with sterile PBS.

3.2 TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH MTT ENDPOINT

There are two kinds of test chemicals that interfere with the MTT assay as follows.

(a) Chemical that stains epidermis tissues.
(b) Chemical that is able to directly reduce MTT.

Test chemical that stains the epidermis tissues has a possibility to transfer from the epidermis tissues to the extraction solution and to affect the optical density (OD) measurements.

Test chemical that is able to directly reduce MTT can affect the optical density (OD) measurements, if the test chemical is present in the epidermis tissues when the MTT viability test is performed. Detection procedure of these test chemicals is described below.

3.2.1 DETECTION OF THE CHEMICALS THAT STAIN THE TISSUE

3.2.1.1 STEP1 (PRELIMINARY TEST)

(1) Add 25μL (Liquid) or 25mg (Solid) of the test chemical into wells of 24-well assay plate preliminarily filled with 0.5mL of distilled water. Untreated distilled water is used as control.
(2) Close the lid of 24-well assay plate and incubate the mixture in CO_2 incubator for 15 minutes.
(3) After incubation, shake the mixture gently and evaluate the staining of the distilled water macroscopically.
(4) When the color of the solution changes significantly, the test chemical is presumed to have the potential to stain the tissue and a functional check on viable tissues (Step2) should be performed. When the color of the solution does not change significantly, the test chemical is determined not to have a potential to stain the tissue.

3.2.1.2 STEP2 (FUNCTIONAL CHECK ON VIABLE TISSUE)

(1) Add 25μL (Liquid) or 25mg (Solid) of the test chemical, which clearly changed the color of the distilled water (Step1), onto the surface of the epidermis tissues. Distilled water is used as negative control.
(2) Follow all procedures described in this SOP Section 3.3 EXECUTION OF THE TEST. However, incubate the tissue for 3 hours in culture media without MTT instead of incubating in media containing MTT to evaluate the staining of the epidermis tissues.
(3) Calculate ratio of staining by test chemical from the following formula.

$$\text{Ratio of staining by test chemical (\%)} = \frac{\text{OD test chemical} - \text{OD negative control}}{\text{OD negative control}} \times 100$$

(4) When the ratio of staining by test chemical is <5%, correction of the results is not necessary. When the ratio is between 5% and 30%, the corrected MTT OD is calculated using the following formula.test chemical

$$\text{Corrected MTT OD} = \text{OD stained tissue (MTT assay)} - \text{OD stained tissue (no MTT assay)}$$

When the ratio of staining by test chemical is >30%, the test chemical must be considered incompatible with the test. However, when the Cell viability (%), which is calculated according to the procedures described in this SOP Section 3.3.5.2, is <50%, the test chemical is determined as irritant. Therefore correction of the results or determination of incompatibility of the test chemical is not necessary.

3.2.2 DETECTION OF CHEMICALS THAT DIRECTLY REDUCE MTT

3.2.2.1 STEP3 (PRELIMINAY TEST)

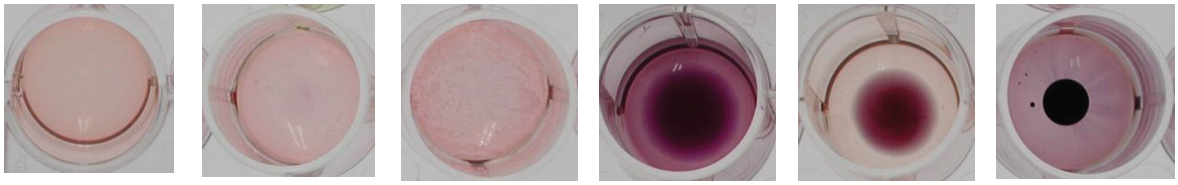
(1) Add 25μL (Liquid) or 25mg (Solid) of the test chemical into wells of 24-well assay plate

preliminarily filled with 0.5mL of MTT medium. Untreated MTT medium is used as control.

(2) Close the lid of 24-well assay plate and incubate the mixture in CO_2 incubator for 1 hour.

(3) After incubation, shake the mixture gently and evaluate the staining of the MTT medium macroscopically.

(4) When the MTT medium turns blue/purple significantly, the test chemical can reduce MTT and additional functional check (Step4) must be performed.



5% SLS	1-bromo hexane	1,1,1-tetrachloroethane	5-(1,1-dimethylethyl)-2-methyl	cinnamaldehyde	eugenol

Photo 1 – Example of test for direct MTT reduction ability (STEP 3). Test substances 5-(1,1-dimethylethyl)-2-methyl, cinnamaldehyde and eugenol and have directly reduced MTT. In these cases, Step 4 must be performed.

3.2.2.2 STEP4 (FUNCTIONAL CHECK ON VIABLE TISSUE)

(1) Add 25µL (Liquid) or 25mg (Solid) of the test chemical, which clearly changed the color of the MTT medium into blue/purple (Step3), onto the surface of the epidermis tissues. Distilled water is used as negative control.

(2) Follow all procedures described in this SOP Section 3.3 EXECUTION OF THE TEST. However, use the epidermis tissues that has been freeze-killed at -20 °C or lower for more than 24 hours instead of viable epidermis tissues.

(3) Calculate ratio of staining by test chemical from the following formula.

$$\text{Ratio of staining by test chemical (\%)} = \frac{\text{OD test chemical} - \text{OD negative control}}{\text{OD negative control}} \times 100$$

(4) When the ratio of staining by test chemical is <30%, correct OD data using the following formula.

$$\text{Corrected OD} = \text{OD (viable tissue) test chemical} - [\text{OD (freeze-killed tissue) test chemical} - \text{OD (freeze-killed tissue) negative control}]$$

When the ratio of staining by test chemical is >30%, the test chemical must be considered incompatible with the test. However, When the Cell viability (%), which is calculated according to the procedures described in this SOP Section 3.3.5.2, is <50%, the test chemical is determined as irritant. Therefore correction of the results or determination of incompatibility of the test chemical is not necessary.

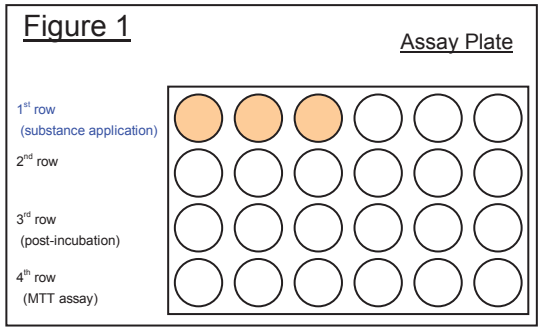
3.3 EXECUTION OF THE TEST

3.3.1 PREPARATION OF LabCyte EPI-MODEL 24 (DAY -1)

(1) Pre-warm the assay medium for 30 minutes to 37 °C using a water bath.

(2) Fill 3 wells of the 1st row of each 24-well assay plate with the pre-warmed assay medium (0.5mL/well).
→ Figure 1

Figure 1



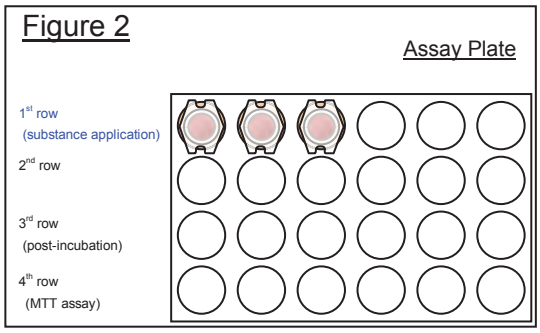
(3) Open the LabCyte EPI-MODEL 24 aluminum package.

(4) Open the LabCyte EPI-MODEL 24 plate lid and pick up the culture inserts using sterile forceps.
*Do not touch the epidermis surface of culture inserts.
*Use forceps to remove agar medium sticking to the outside of the culture inserts.

(5) Transfer the culture inserts into assay medium filled wells of the 1st row using sterile forceps.
→ Figure 2

*Avoid air bubble formation under the tissue inserts.

Figure 2



(6) Place the plate (lid on) in a CO_2 incubator.

(7) Incubate overnight (15~30 hours) until Section 3.2.2 "APPLICATION OF TEST CHEMICALS AND RINSING."

(8) Record details of steps (1) - (7) above in the MDS 2.

3.3.2 APPLICATION OF TEST CHEMICALS AND RINSING (DAY 0)

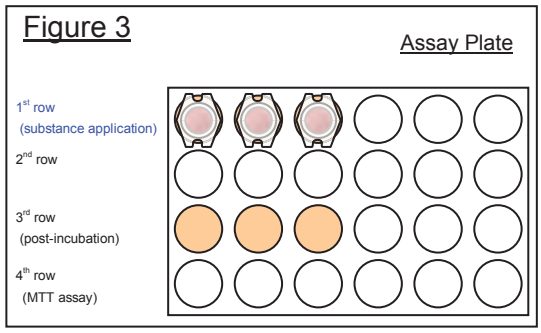
3.3.2.1 PREPARATION OF WELLS FOR POST-INCUBATION (3RD ROW)

(1) Pre-warm the assay medium for 30 minutes to 37 °C using a water bath.

(2) Remove the assay plate from the CO_2 incubator.

(3) Open the lid of the assay plate, and fill 3 wells of the 3rd row with the pre-warmed assay medium (1.0mL/well) using a micropipette.
→ Figure 3

Figure 3



(4) Place the plate (lid on) in a CO_2 incubator.

(5) Incubate until application of test chemicals (0~12 hours).

(6) Record details of steps (1) – (5) above in the MDS 3.

3.3.2.2 APPLICATION OF TEST CHEMICALS

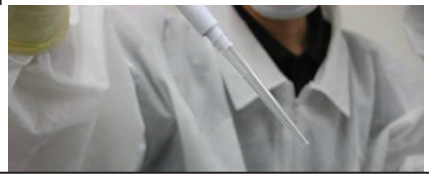
(1) Remove the assay plate from the CO_2 incubator.

(2) Apply test chemicals onto the surface of epidermis tissues in the 1st row of the assay plate. Use 3 wells per test chemical (N=3).

FOR LIQUIDs: Carefully apply 25µL of the test chemical onto the central part of each epidermis using a micropipette. After applied, close the lid of the assay plate and tap the side of the plate outside the safe cabinet (or clean bench) in order for the liquid to spread out over the entire epidermis surface. If necessary, use a micro spatula to coat the unapplied surface with liquids. Do not push the epidermis

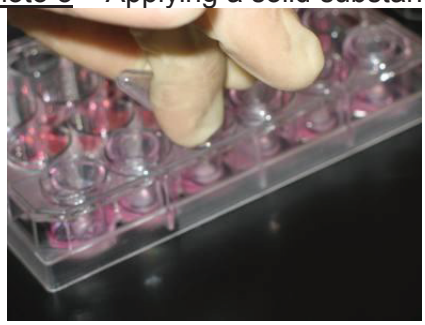
surface too hard with the spatula.
*Use wide orifice cell saver tips for viscous liquids.
→ Photo 2
Use a pipette, etc. to familiarize yourself with the nature of the test chemicals in advance.

Photo 2 - Pipette tips for viscous liquids



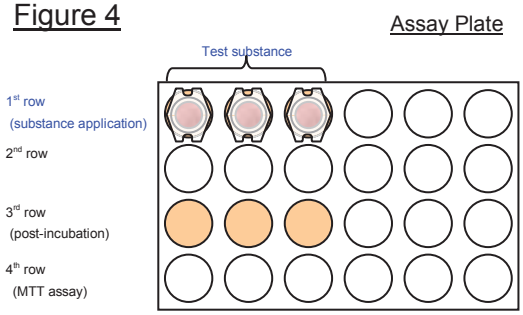
FOR SOLIDs: Weigh out 25mg (±1mg) of the solid chemical with a precision balance in advance. Apply first 25µL of distilled water and then the weighed test chemical onto the epidermis surface. Use a micro spatula if necessary to gently spread the test chemical.
→ Photo 3
*One 24-well assay plate should be used to assay only one test chemical.
→ Figure 4
(1 samples x 3(n) = 3 (culture inserts))

Photo 3 – Applying a solid substance



(3) Apply test chemical onto each well at 1~3-minute intervals.
(4) Incubate each well for 15 minutes in the cabinet (lid on between the intervals).
*Close the lid of the assay plate at all times except when applying samples. It might affect the amount of test sample if the lid is kept open, due to air circulation in the safe cabinet (or clean bench).
(5) Record details of steps (1) - (4) above in the MDS 3.

Figure 4



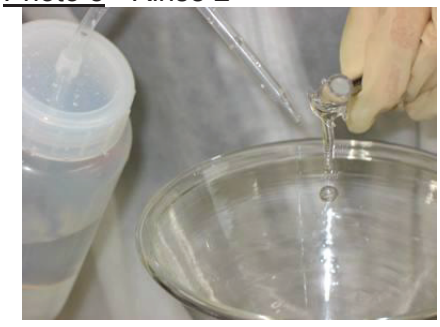
3.3.2.3 REMOVAL OF THE TEST CHEMICALS

(1) 15 minutes (±30 seconds) after applying a chemical, open the assay plate and pick up a culture insert with sterile forceps.
(2) Fill the culture insert with PBS using a PBS filled poly wash bottle. Hit the PBS stream from the washing bottle on the side-wall of the culture insert and wash on the tissue surface by the PBS current.
→ Photo 4
Attention: Must not to hit the PBS stream on the tissue surface directly. Be careful not to damage the tissue surface.

Photo 4 - Rinse 1

(3) Discard the PBS into a beaker by tilting the insert. If necessary, remove the PBS inside the culture insert by tapping it above the beaker only once.
→ Photo 5
(4) Repeat steps (2) and (3) at least 10 times or more as much as possible, and remove all residual test chemical on the tissue surface almost completely.
(5) Gently remove the leftover PBS outside the culture insert with a sterile cotton bud. But don't touch inside the culture insert by a cotton bud.

Photo 5 - Rinse 2



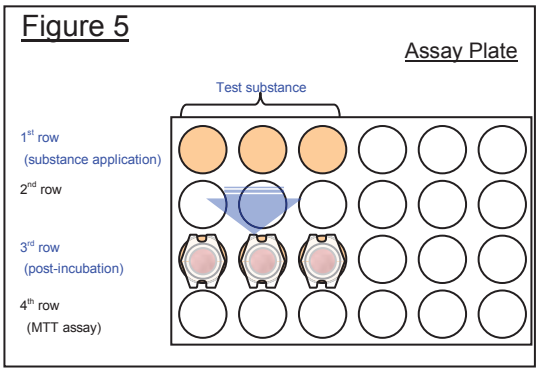
→ Photo 6

Attention: Even if residues of washing PBS remain on the tissue surface, don't do at all because it is not necessary to remove them.

Photo 6 - Rinse 3

(6) If test material remains on the epidermis surface, repeat steps (2) ~ (5) again.
(7) Transfer the blotted culture insert to a well in the 3rd row of the same column (for post-incubation).
→ Figure 5
*Avoid air bubble formation under the culture inserts.
(8) Repeat steps (1) ~ (7) for all the culture inserts at 1~3-minute intervals.
(9) Record details of steps (1) – (8) above in the MDS 3.

Figure 5



3.3.3 POST TREATMENT INCUBATION (DAY 0~2)

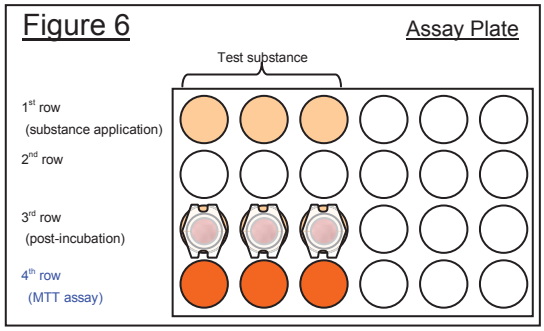
(1) Close the lid of the assay plate and place it in a CO_2 incubator.
(2) Incubate for 42 hours.

3.3.4 MTT ASSAY (DAY 2)

3.3.4.1 PREPARATION OF WELLS FOR MTT ASSAY

(1) Pre-warm MTT medium for 30 minutes to 37°C using a water bath.
(2) Remove the assay plate from the CO_2 incubator.
(3) Open the lid of the assay plate, and fill each well of the 4th row with the pre-warmed MTT medium (0.5mL/well) using a micropipette.
→ Figure 6
(4) Close the lid of the assay plate and place it in the CO_2 incubator.
(5) Incubate until starting MTT assay (about 0 ~ 12 hours).
(6) Record details of steps (1) – (5) above in the MDS 4.

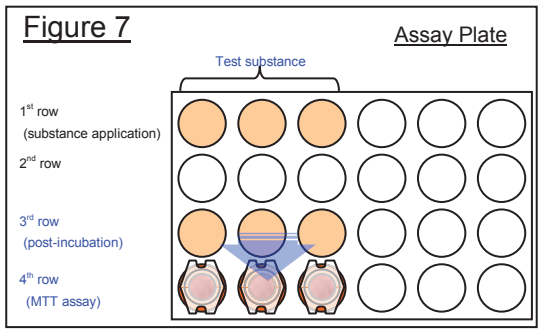
Figure 6



3.3.4.2 MTT ASSAY

(1) Remove the assay plate from the CO_2 incubator after 42 hours (±1 hour) of post-incubation.
(2) Transfer each culture insert from the 3rd row to the 4th row of the corresponding column.
→ Figure 7
*Avoid dripping from the base end surface of the culture insert into other wells.
*Avoid air bubble formation under the culture inserts.
(3) Close the lid of the assay plate and place it in the CO_2 incubator.
(4) Incubate for 3 hours.
(5) Record details of steps (1) – (4) above in the MDS 4.

Figure 7



3.3.5 FORMAZAN EXTRACTION AND MEASUREMENT (DAY 2~3)

3.3.5.1 FORMAZAN EXTRACTION

(1) Remove the assay plate(s) from the CO_2 incubator 3 hours (±5 minutes) after the MTT assay.

(2) Open the lid of the assay plate and pinch the cultured epidermis from each culture insert of the 4th row with forceps.
→ Photo 7
*Use a micro spatula to scratch up the epidermis or a scalpel to cut the membrane filter on the base of the culture insert if the cultured epidermis cannot be pinched due to damage from a test chemical.

Photo 7 - Detachment of epidermis

(3) Transfer the epidermis tissue into a 1.5mL micro tube.

(4) Add 300µL of isopropanol to the micro tubes and soak the entire epidermis tissue in the isopropanol.

(5) Incubate the micro tubes in a dark cold place (or refrigerator) overnight (more than 15 hours) in order to completely extract pigments.
*Tighten the micro tube seal.
*Periodically shaking the micro tubes will contribute to a more efficient extraction.

(6) Shake the micro tubes to mix the solution.
*If split epidermis tissues are suspended, wait until they sink or gently centrifuge them (if a centrifuge is available).

(7) Transfer 200µL of the solution in each micro tube into each well on a 96-well plate.
*One well of 200µL of isopropanol should be set as a blank.
*Figure 8 shows an example of allocation in a 96-well plate.

Figure 8 – Allocation for a 96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank											
B	DW-1	Sample 1-1	Sample 3-1	Sample 5-1	Sample 7-1	Sample 9-1	Sample 11-1	Sample 13-1	Sample 15-1	Sample 17-1	Sample 19-1	
C	DW-2	Sample 1-2	Sample 3-2	Sample 5-2	Sample 7-2	Sample 9-2	Sample 11-2	Sample 13-2	Sample 15-2	Sample 17-2	Sample 19-2	
D	DW-3	Sample 1-3	Sample 3-3	Sample 5-3	Sample 7-3	Sample 9-3	Sample 11-3	Sample 13-3	Sample 15-3	Sample 17-3	Sample 19-3	
E	5% SLS-1	Sample 2-1	Sample 4-1	Sample 6-1	Sample 8-1	Sample 10-1	Sample 12-1	Sample 14-1	Sample 16-1	Sample 18-1	Sample 20-1	
F	5% SLS-2	Sample 2-2	Sample 4-2	Sample 6-2	Sample 8-2	Sample 10-2	Sample 12-2	Sample 14-2	Sample 16-2	Sample 18-2	Sample 20-2	
G	5% SLS-3	Sample 2-3	Sample 4-3	Sample 6-3	Sample 8-3	Sample 10-3	Sample 12-3	Sample 14-3	Sample 16-3	Sample 18-3	Sample 20-3	
H												

(8) Record details of steps (1) – (7) above in the MDS 5.

3.3.5.2 OPTICAL DENSITY MEASUREMENTS OF THE EXTRACTS

(1) Using a 96-well plate reader, measure optical densities (OD) at 570nm and 650nm and determine the measured OD by subtracting the 570nm OD from the 650nm OD.
The equation is shown below:

$$\text{Measured OD} = [570\text{nm OD}_{\text{sample}} - 570\text{nm OD}_{\text{blank}}] - [650\text{nm OD}_{\text{sample}} - 650\text{nm OD}_{\text{blank}}]$$

*Set the plate reader-calculated value as the measured OD if the 96-well plate reader performs automatic calculations.

(2) Calculate the cell viability of a sample using the equation below. Furthermore, calculate the variability (SD) of tissue replicates.

(3) Record details of steps (1) and (2) above in the MDS 5.

$$\text{Cell Viability (\%)} = \frac{\text{Measured OD}_{\text{sample}}}{\text{Mean Measured OD}_{\text{NC}}} \times 100$$

4. ASSESSMENT

4.1 CONDITIONS FOR A SUCCESSFUL STUDY

The skin irritation test should be considered successful if both of the following criteria have been met.

- Tissue viability: 0.7 ≤ mean OD (A570/650) measured value for negative control ≤ 2.5.
- Positive control: mean tissue viability for 5% SLS (positive control) ≤ 40%.
- SD: SD (negative control and positive control) of tissue viability of 3 identically treated replicates ≤ 18 %

4.2 ASSAY CRITERIA

The criteria for in vitro interpretation are shown below.

The test must be performed 3 times per a sample in total. Sort the tissue viabilities obtained from the repeated tests in ascending order, and classify the irritancy based on the median of those tissue viabilities.

Tissue Viability (primary)	Classification
Tissue viability is ≤ 50%	Irritant
Tissue viability is > 50%	Non Irritant

[FLOWCHART] ASSESSMENT FLOWCHART

(1) Tissue viability in negative control → (either criterion is not met) → Assay Failure
0.7 ≤ Mean OD measured value ≤ 2.5
Positive control (5% SLS) result should be "irritant"
Mean tissue viability ≤ 40%
SD"
SD (negative control and positive control) of tissue viability of 3 identically treated replicates ≤ 18 %
↓
(All criteria are met)
↓
(2) Assessment of test samples (3-time repeated tests: all tests satisfy (1))
The median of the 3 tissue viabilities (%) ≤ 50% → (Yes) → Classified as irritant
↓
(No)
↓
Classified as non irritant

MDS 1:
RECEIPT OF LABCYTE EPI-MODEL 24

Laboratory name: _____ Test name: _____ Test No. : _____

1. LabCyte EPI-MODEL 24

Date received : _____

Lot No. : _____

Exouration date : _____
(MM/DD/YYYY)

Accessories : Assay medium, 30mL □ (Lot No. : __ Expiration date : _____)
(MM/DD/YYYY)
24 well assay plate □

Note

2. Assay medium

Date received : _____

Lot No. : _____

Expiration date : _____
(MM/DD/YYYY)

Note

Date:_____Operator:_____ Check date:_____ Study director:_____
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:_____ Name:_____
(MM/DD/YYYY)

MDS 2:
PRE-INCUBATION OF LABCYTE EPI-MODEL 24 (Section 3.3.1)

Laboratory name: _____ Test name: _____ Test No. : _____

1. Warm up the assay medium and add 0.5mL of the assay medium to the wells of the 1st row on the 24-well assay plate.

Assay medium： （Lot No.：_____ Expiration date：_____）
(MM/DD/YYYY)
Warm for 30 min. □
Add 0.5mL of assay medium to each well □
Number of plates：_____

2. Transfer culture inserts to wells in the 1st row on the 24-well assay plate.

LabCyte EPI-MODEL 24：（Lot No.：_____ Expiration date：_____）
(MM/DD/YYYY)
Time/date executed：_____
(MM/DD/YYYY HH:MM)

Confirm that there are no bubbles under the cell culture insert. □

3. LabCyte EPI-MODEL 24 is cultured in CO_2 incubator overnight.

.

Time/date of culture start：_____
(MM/DD/YYYY HH:MM)

Planned time/date of exposure to test chemical：_____
(MM/DD/YYYY HH:MM)

Note

Date:_____Operator:_____ Check date:_____ Study director:_____
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:_____ Name:_____
(MM/DD/YYYY)

MDS 3-1:
APPLICATION OF TEST CHEMICALS, RINSING AND POST-INCUBATION (Section 3.1.2, 3.3.2 ~ 3.3.3)

Laboratory name: _____ Test name: _____ Test No.: _____

1. Preparation of positive control.
   Weight of SLS ____mg__ Preparation vol. _____mL Operation date： _____
   (MM/DD/YYYY HH/MM)

2. Warm up the assay medium and add 1.0mL of the assay medium to the wells of the 3rd row on the 24-well assay plate.
   Assay medium： （Lot No.： _____ Expiration date： _____）
   (MM/DD/YYYY)
   Warm for 30 min. □ Add 1.0mL of assay medium. □
   Time/date executed： _____
   (MM/DD/YYYY HH/MM)

3. Apply test chemicals to the LabCyte EPI-MODEL 24.
   Time/date execution started： _____ Time/date completed： _____
   (MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

4. After exposure to test chemical for 15 min., wash out the LabCyte EPI-MODEL 24 and transfer the culture inserts to the 3rd row on the 24-well assay plate.
   PBS： （Lot No.： _____ Expiration date： _____）
   (MM/DD/YYYY)
   Time/date execution started： _____ Time/date completed： _____
   (MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

   Confirm that there are no bubbles under the cell culture insert. □

5. Test chemical information

Test chemical code No.	Lot No.	Physical state	Test chemical vol.(weight) (measured weight)	Time of application	Exposure time （15min.）
Distilled Water (Negative control)		Liquid	25μL	:	□
5%SLS (Positive control)		Liquid	25μL	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous,	25μL, (mg, mg, mg)	:	□

		solid			
		Liquid, viscous, solid	25µL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25µL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25µL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25µL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25µL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25µL, (mg, mg, mg)	:	□

Date:_____Operator:_____ Check date:_____ Study director:_____
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:_____ Name:_____
(MM/DD/YYYY)

MDS 3-2:
APPLICATION OF TEST CHEMICALS, RINSING AND POST-INCUBATION
(Section 3.3.2~3.3.3)

Laboratory name: ________________ Test name: ________________ Test No. : ____________

5. Test chemical information (continued)

Test chemical code No.	Lot No.	Physical state	Test chemical vol.(weight) (measured weight)	Time of application	Exposure time (15min.)
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□

6. Culture LabCyte EPI-MODEL 24 in CO_2 incubator for 42 hrs.
Time/date post-incubation started : ____
(MM/DD/YYYY HH:MM)
Time/date post-incubation completed : ____
(MM/DD/YYYY HH:MM)

Note

Date:_________Operator:___________________ Check date:________ Study director:__________
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:___________________ Name:____________________________________

(MM/DD/YYYY)

MDS 4:
MTT ASSAY (Section 3.3.4)

Laboratory name: _____ Test name: _____ Test No. : _____

1. Preparation of MTT medium
   Preparation vol. _____mL Lot No. _____ Time/date executed : _____
   (MM/DD/YYYY HH/MM)

2. Warm up the MTT medium and add 0.5mL of the MTT medium to the wells in the 4th row on the 24-well assay plate.

   MTT medium. : （Lot No. : _____ Expiration date : _____）
   (MM/DD/YYYY)
   Warm for 30 min. □ Add 0.5mL of the MTT medium. □ Time/date executed : _____
   (MM/DD/YYYY HH/MM)

3. After post-incubation, the LabCyte EPI-MODEL24 transfer to wells of 4th row of 24-well assay plate.

   Time/date started : _____ Time/date completed : _____
   (MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

   Confirm that there are no bubbles under the cell culture insert. □

4. Store LabCyte EPI-MODEL 24 culture overnight in CO_2 incubator for 42 hrs.

Information on MTT reaction time

Test chemical code No.	Lot No.	MTT reaction start time	Time when MTT reaction ends	Test chemical code No..	Lot No.	MTT reaction start time	Time when MTT reaction ends
Distilled Water (Negative control)		:	:			:	:
5%SLS (Positive control)		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:

Note

Date:_____Operator:_____ Check date:_____ Study director:_____
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:_____ Name:_____
(MM/DD/YYYY)

MDS 5:
FORMAZAN EXTRACTION AND MEASUREMENT (Section 3.3.5)

Laboratory name: _____ Test name: _____ Test No. : _____

1. After MTT reaction, use forceps to pick up the cultured epidermis from the cell culture insert and put it in a 1.5mL microtube.

   Did you use a scalpel to cut out the cultured epidermis? □
   Date of execution : _____
   (MM/DD/YYYY)

2. Add isopropanol (300μL) to microtube so that the cultured epidermis is completely immersed in isopropanol.

   Isopropanol Lot No.___ To add isopropanol (300μL) □
   Immersion of the cultured epidermis in isopropanol. □
   Date of execution : _____
   (MM/DD/YYYY)

3. For MTT formazan extraction, allow micro tube to stand in a cold and dark space.
   Place micro tube in a cold and dark space. □

4. Extract solution (200mL) is transferred to each well on the 96-well plate.

   Transfer to the 96-well plate. □
   Time/date executed : _____
   (MM/DD/YYYY HH/MM)

   Sample location on 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank											
B	Distilled Water-1											
C	Distilled Water-2											
D	Distilled Water-3											
E	5% SLS-1											
F	5% SLS-2											
G	5% SLS-3											
H												

5. Analyze extract OD at 570nm and 650nm, and calculate the OD(570nm-650nm).

Analyze OD at 570nm and 650nm.	□
Calculate the OD(570nm-650nm).	□
Calculate cell viability and SD.	□
Cell viability and SD are recorded on a separate data sheet.	□
The data sheet is attached to the back of this sheet.	□
Check for input errors.	□

Time/date executed : _____
(MM/DD/YYYY HH/MM)

Note

Date:_____Operator:_____ Check date:_____ Study director:_____
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:_____ Name:_____
(MM/DD/YYYY)

REVISION HISTORY

Rev.	Content	Date Revised
Ver.1	1) First version	27/02/2008
Ver.2	1) Revised clerical error.	28/02/2008
Ver.3	1) Revised the post-incubation time and assessment criteria in compliance with the EpiSkin method described in "Performance Standards for Applying Human Skin Models to in vitro Skin Irritation Testing" 2) Added photos and figures for instruction.	17/03/2008
Ver.4	1) Added MDS 1~6. 2) Added instruction and operational steps regarding the IL-1α ELISA kit. 3) Added subsections "Delivery of LabCyte EPI-MODEL24" and "Instruction For Use of LabCyte EPI-MODEL24" to Section 2. 4) Added the description regarding test chemicals to Section 2. 5) To Section 2, added the description of materials provided by J-TEC separately from other materials. 6) Stated the specific calculation procedures in Section 3.2.5.2 "OPTICAL DENSITY MEASUREMENTS OF EXTRACTS".	15/05/2008
Ver.4.1	1) Moved scalpel from Section 2.4 "MATERIALS PROVIDED BY J-TEC" to Section 2.5 "MATERIALS NOT PROVIDED WITH THE J-TEC KITS". 2) Removed the description regarding how to execute procedures alone. 3) Moved IL-1α ELISA reagents from Section 3.1 "PREPARATIONS" to Section 3.2 "TEST METHOD". 4) Added a flowchart for the IL-1α ELISA procedures. 5) Changed from "in a cold dark place" to "in a cold dark place (or refrigerator)" regarding formazan extraction. 6) Added the description ~~of~~ "ultrasonic cleaning equipment or vortex mixer" as an example of an MTT dissolution method. 7) Changed the exposure time column from entering actual time to checkboxes on the MDS 3.	21/05/2008
Ver.5.0	1) Corrected typing errors in the section number for IL-1α ELISA reagents. 2) Removed the space for SLS lot numbers on the MDS 3. 3) Removed the space for PBS lot numbers on the MDS 3. 4) Added the space for isopropanol lot numbers on the MDS 5. 5) Added a checkbox about using a scalpel when removing tissues in the MDS 5. 6) Added the space for IL-1α ELISA kit lot numbers on the MDS 6. 7) Changed the applicable parts of product codes and kit components in Section 2.2, with the change of IL-1α ELISA kit types to a 96 well test only. 8) Decreased the volume by half to 10mL and changed the storage condition from within 1 month to within 24 hours in Section 3.1.2 "POSITIVE CONTROL SUBSTANCE". 9) Added the manufacturers and product codes of the 24-well plate and 96-well plate in Section 2.4 "MATERIALS PROVIDED BY J-	27/08/2008

	TEC". 10) Added specific time frames for incubation or culturing. 11) Added the conditions for a successful study in Section 4 "ASSESSMENT" 12) Changed the specific method of applying liquids in Section 3.2.2.2 "APPLICATION OF TEST CHEMICALS". 13) Added descriptions in English on the MDS Sheets. 14) Changed the application time interval from 1 minute to 1~3minute(s). 15) Numbered figures and flowcharts. 16) Increased the size of spaces for lot numbers on the MDS Sheets. 17) Changed spaces for dates from MM/DD to MM/DD/YYYY. 18) Added director check date, study director, secretariat check date and name at the end of each MDS. 19) Changed the size of matrixes for sample allocation to a 96-well plate in the MDS 5 & 6. 20) Changed the test chemical name to test chemical code in the MDS 3 & 4. 21) Divided the MDS 3 into MDS 3-1 and 3-2, and added spaces for date, operator, check date, study director at the end of the MDS 3-1, and spaces for laboratory name, test name and test no. at the beginning of the MDS 3-2.	
Ver. 6.0	1) Removed the descriptions regarding the measurement of IL-1α production, since the validation committee decided to use cell viabilities only as an index for the skin irritancy test at the meeting in 2009. 2) Revised the expression "the materials provided by J-TEC" for the validation study to that for a standard skin irritancy test preparation. 3) Clearly stated the cell viability equation to use the mean of measured values. 4) Clearly stated to use the median of cell viabilities from the three-time repeated tests as assay criteria.	27/02/2009
Ver. 6.01	1) In order to avoid the possible influence of volatile test chemicals on the results of other test chemicals, the types of test chemicals per plate was changed from 2 chemicals to just 1 chemical.	23/03/2009
Ver. 7.01	1) Test for detecting chemicals that interfere with MTT endpoint was added to Section 3.2.	03/07/2009
Ver. 7.2	1) Revised clerical error.	30/09/2009
Ver. 8.1	1) Added the description about rational and background as following chapters in Section 1. LabCyte EPI-MODEL 24 SKIN IRRITATION TEST (SIT using LabCyte EPI-MODEL24) BACKGROUND OF SIT using LabCyte EPI-MODEL24 BASIS OF THE METOD LIMITATION OF THE METHOD BRIEF BASIC PROCEDURE DATA INTERPRETATION PROCEDURE (PREDICTION	30/06/2010

	MODEL) 2) Added photo about chemicals that directly reduce MTT in Section 3. 3) Added the washing protocol more detail in Section 3. 4) Added assessment about SD.	
Ver.8.2	1) Changed description about the washing protocol in Section 3. 2) Changed unit of consumable reagents and vessels from per a validation study to per a test.	17/08/2010

Appendix 4

JaCVAM Report:
Me-too Validation Study of *in vitro* Skin Irritation Test using LabCyte EPI-MODEL24
(LabCyte EPI-MODEL24 SIT)

February 28, 2011

LabCyte Validation Management Team

Members of LabCyte Validation Management Team

Mr. Hajime Kojima, JaCVAM: Japanese Centre for the Validation of Alternative Methods
Mr. Masakazu Katoh, Japan Tissue Engineering Co. Ltd.
Mr. Toshiro Yokouchi, Japan Tissue Engineering Co. Ltd.

Biostatistian group

Mr. Takashi Omori, Doshisya Univ.

Participant laboratories

KOBAYASHI Pharmaceutical Co., Ltd. (Mr. Yoshihiro Yamaguchi and Ms. Maki Nakamura)
Fancl Corp. (Ms. Tamie Suzuki and Ms. Runa Izumi)
Drug Safety Testing Center Co., Ltd. (Mr. Shinsuke Shinoda and Ms. Saori Hagiwara)

Abbreviations

CAS: Chemical Abstracts Service
ECVAM: European Centre for the Validation of Alternative Methods
ESAC: ECVAM Scientific Advisory Committee
GHS: Globally Harmonised System
GLP: Good Laboratory Practice
ISO: International Organization for Standardization
JaCVAM: Japanese Centre for the Validation of Alternative Methods
JSAAE: Japanese Society for Alternatives to Animal Experiments
J-TEC: Japan Tissue Engineering Co. Ltd.
NIHS: National Institute of Health Sciences
OECD: Organisation for Economic Co-operation and Development
QC: Quality control
PBS: Phosphate buffered saline
PS: Performance Standard
RhE: Reconstructed human epidermis
SD: Standard deviation
SLS: Sodium lauryl sulphate
SPSF: Standard Project Submission Form
TG: Test Guideline
UN: United Nations
VMT: Validation management team
VRM: Validated reference method
WNT: National Coordinators of the Test Guideline Project

Contents

Appendices:

1. Validation study of in vitro skin irritation test using LabCyte EPI-MODEL24 (FINAL report)
2. Summary report of the peer review panel on LabCyte EPI-MODEL24 in vitro test method for the assessment of skin irritation potential of chemicals
3. Additional validation of the RhE tests: LabCyte EPI-MODEL24 acute skin irritation prediction
4. Skin Irritation test protocol using the reconstructed human model "LabCyte EPI-MODEL24" (ver.8.2)
5. Original data in the catch-up validation study
6. Test records and data sheet in KOBAYASHI Pharmaceutical Co., Ltd. (example: in Japanese)
7. Result of QC for each lot of LabCyte EPI-MODL24
8. Detailed review documents on the "LabCyte EPI-MODEL24"
9. Masakazu Katoh, Fumiyasu Hamajima, Takahiro Ogasawara, and Ken-ichiro Hata (2009) Assessment of the Human Epidermal Model LabCyte EPI-MODEL for *In Vitro* Skin Irritation Testing According to the ECVAM-Validated Protocol , Journal of Toxicological Science, 34(3) 327-334.
10. OECD TG 439, *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method

1. Goal statement

- The ultimate goal of the test strategy is to replace the regulatory Draize skin irritation test to meet the OECD (Organisation for Economic Co-operation and Development) TG (Test Guideline) 404 (OECD, 2002).
- The primary goal of this catch-up validation study is to evaluate the ability of *in vitro* tests to reliably discriminate skin irritant (I) from non-irritant (NI) chemicals, as defined by the OECD and UN (United Nations) proposal for GHS (Globally Harmonised System) for the classification and labelling of skin irritation (category 1/category 2; no category; Anon., 2003).

2. Objective

The OECD Working group of the National Coordinators of the Test Guideline Project (WNT) accepted the TG No.439: *in vitro* skin irritation test guideline in March 2010. This TG addresses the human health endpoint of skin irritation. Three validated test methods currently adhere to this TG. Prevalidation, optimization and validation studies have been completed for an *in vitro* test method that uses a Reconstructed human epidermis (RhE) model. This method is commercially available as EpiSkin™ and has been designated as the Validated Reference Method (VRM). Two other commercially available *in vitro* skin irritation RhE test methods, namely the EpiDerm™ SIT (EPI-200) and SkinEthic™ RHE test methods, have shown similar results to the VRM according to Performance Standard (PS) - based validation.

On the other hand, another *in vitro* test system that employs a RhE model (LabCyte EPI-MODEL24) has progressed through protocol optimization as a skin irritation test. A multi-laboratory assessment of this system was performed according to several ECVAM (European Centre for the Validation of Alternative Methods) performance standards (ESAC: ECVAM Scientific Advisory Committee statement, 2007, 2008, 2009). The present objective added the Japanese RhE and other similar models to adhere to the OECD TG 439. A me-too validation study was conducted to assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of this test system. The study included a challenging set of 20 test chemicals that would meet the performance standard set forth in the TG No.439. The validation study was undertaken in accordance with the principles and criteria documented in the OECD *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* (No. 34, OECD, 2005) and according to the Modular Approach to validation (Hartung *et al.* 2004).

3 ·Background

Researchers in Japan aimed to include the Japanese RhE (LabCyte EPI-MODEL24: LabCyte EPI-MODEL24 SIT) *in vitro* skin irritation test in the TG as an addition to other similar models in the SPSF (Standard Project Submission Form) that were submitted to OECD WNT by EU delegate in April 2008. The validation study described herein was performed between April 2008 and January 2009 by the Validation Management Team (VMT), with financial support from the Japanese Society for Alternative to Animal Experiments (JSAAE).

The study conducted by VMT referenced the original ECVAM performance standard (ECVAM 2007), in which a range of appropriate models was described as one of the acceptance criteria. After completion of the first phase of the study in August 2008, the VMT discussed the criteria for the Labcyte EPI MODEL24 SIT. The VMT decided that the criteria were not set because there was not enough data to define this kind of range at that time. Furthermore, the pre-specification was considered to have too narrow a range and the draft OECD TG came under review at the time of the discussions. As a result, the reliability of the model was considered to be high. Therefore, the VMT decided that criteria for the range may not be needed for this model, while the check for variation should be done.

Based on validation results of the Labcyte EPI-MODEL24 SIT, a member of the Japanese WNT submitted an SPSF of it to OECD in January 2009 and the OECD WNT accepted this assay in its working

plan in May 2009. The VMT submitted the first validation report to the OECD secretary in July 2009. On the other hand, we confirmed an additional validation study in reference to the new ECVAM performance standards (ESAC statement, 2009) to be revised for the TECD TG between April and May of 2009 and submitted the second validation report and Background Review Documents to the OECD secretary in August 2009.

Using these documents, OECD performed a peer review and we received the peer review report from the OECD secretary in March 2010. The OECD peer review on the LabCyte EPI-MODEL24 SIT *in vitro* test method for the assessment of skin irritation potential of chemicals was performed with the validation report and background review documents. In the summary report, the peer review panel indicated that the issue of misclassifying 1-bromohexane should be resolved.

To resolve this issue, the protocol was revised by Japan Tissue Engineering (J-TEC). To confirm general versatility with the revised protocol, we planned an additional validation study according to the OECD performance standard.

4. Test methods

4-1. Reconstructed human cultured epidermal model

LabCyte EPI-MODEL24 is a new, commercially available RhE model produced by J-TEC. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand human keratinocytes while maintaining their phenotype, the cells are cultured with 3T3-J2 cells as a feeder layer (Rheinwald and Green, 1975; Green, 1978). Reconstruction of a human cultured epidermis is achieved by cultivating and proliferating keratinocytes on an inert filter substrate (surface area 0.3 cm^2) at the air-liquid interface for 13 days with an optimized medium containing 5% fetal bovine serum. The process generates a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum. LabCyte EPI-MODEL24 is embedded in an agarose gel containing nutrient solution and shipped in 24-well plates at around 18 °C (Kato, 2009: Appendix 4).

4-2. MODEL supplier

According to OECD Good Laboratory Practice (GLP) Consensus Document No.5 "*Compliance of Laboratory Suppliers with GLP Principles*", responsibility for the quality and fitness for use of equipment and materials rests entirely with the management of the test facility (OECD, 1999).

The acceptability of equipment and materials in laboratories complying with GLP must therefore be guaranteed to any regulatory authority to which studies are submitted. In some countries where GLP has been implemented, suppliers belong to national regulatory or voluntary accreditation schemes (for laboratory animals) that can provide users with additional documentation proving that they are using a test system of defined quality.

Audits performed during the study focused on procedures established to guarantee a defined quality of the tissue models.

5. Validation management structure

The management structure of the study is shown in Figure 1.

5-1. Validation management team

The VMT played a central role in overseeing the conduct of the validation study, including implementation of the following aspects of the study:

1) Goal statement

2) Project plan including objective
3) Study protocol / amendments
4) Outcome of QC (Quality Control) audits
5) Test chemicals
6) Data management procedures
7) Timeline / study progression
8) Data collection and analysis
9) Study interpretation and conclusions
10) Reports and publications
The VMT made the final decision on which laboratories would participate in the validation study.
Responsible VMT members:
Chair (Hajime Kojima, JaCVAM: Japanese Centre for the Validation of Alternative Methods)
The sponsor representative, LabCyte EPI-MODEL 24 suppliers and lead lab (Masakazu Katoh: J-TEC)

5-2. Chemical selection, acquisition, coding, and distribution

1) Definition of selection criteria
2) Chemical selection
3) Liaise with suppliers
4) Final check of chemicals provided
5) Acquisition
6) Coding
7) Distribution
Responsible VMT member: Hajime Kojima, JaCVAM

5-3 . Independent biostatisticians

1) Approve spreadsheets
Responsible VMT member: Takashi Omori: Doshisya Univ.

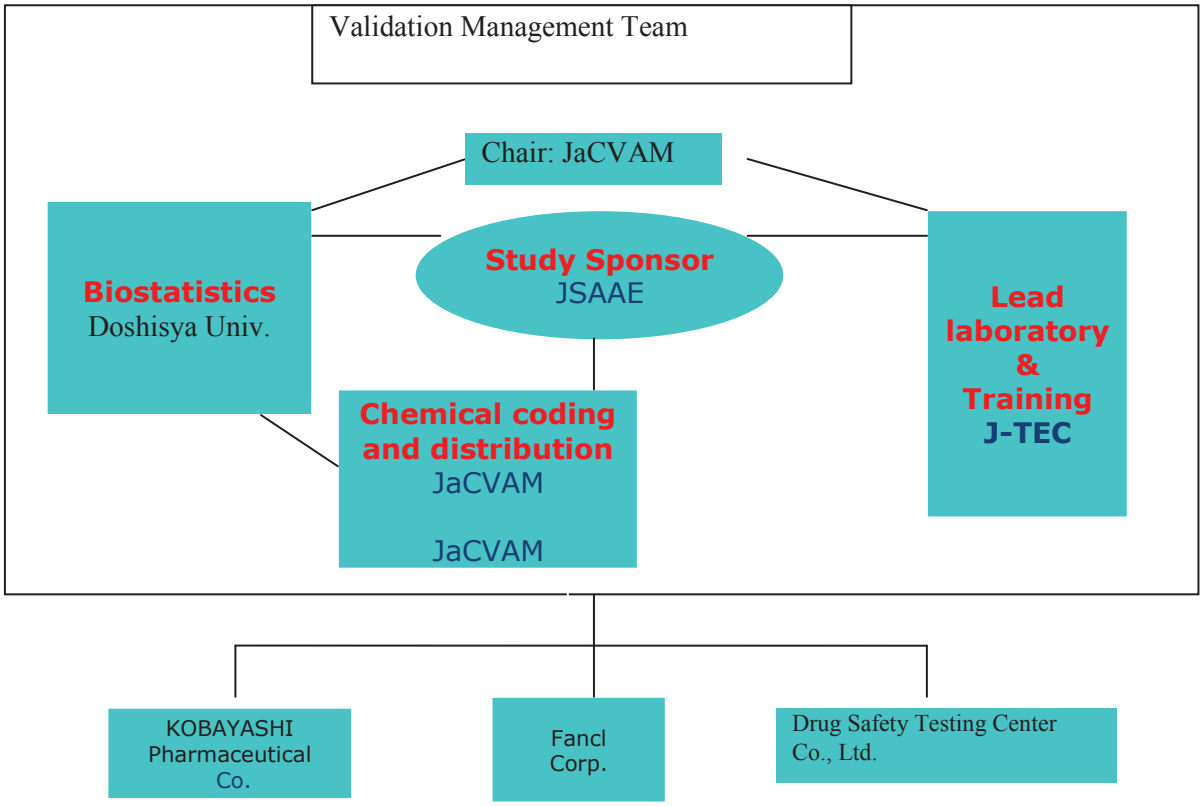


Fig. 1. Management structure of me-too validation study on the LabCyte EPI-MODEL24 SIT

5-4. Participating laboratories

The laboratories participating in the study are shown in **Fig. 1**.

The following three laboratories participated in the validation study for the evaluation of the LabCyte EPI-MODEL 24 assays:

- Laboratory a — KOBAYASHI Pharmaceutical Co., Ltd. (Yoshihiro Yamaguchi and Maki Nakamura)
- Laboratory b — Fancl Corp. (Tamie Suzuki and Runa Izumi)
- Laboratory c — Drug Safety Testing Center Co., Ltd. (Shinsuke Shinoda and Saori Hagiwara)

A lead laboratory was also identified as J-TEC (Mr. Masakazu Kato and Mr Toshihiro Yokouchi). This laboratory did not participate in the validation study.

Each laboratory was responsible for complying with GLP principles and specifying QC aspects of the study.

5-5. Sponsorship

The study was managed and financed by JaCAM and J-TEC.

1) JaCVAM financially supported the following activities:
- management of the study (VMT meetings)
- provision of independent statistical support (VMT meetings)
- purchase, coding, and distribution of chemicals to the laboratories

- independent QC audit of the data
- publication of the study results

2) J-TEC supported the following aspects of the study:
- the lead laboratories for the test method
- training of the participating laboratories
- independent QC audit of the LabCyte EPI-MODEL24 SIT
- financial assistance of the participated laboratories

6. Study design and test period

Before initiation of the validation study, J-TEC delegates conducted a training course on using LabCyte EPI-MODEL24 SIT with a revised protocol (ver.8.1) at the National Institute of Health Sciences (NIHS) on July 27, 2010. All technicians from each laboratory participated in this training course. Furthermore, all laboratories participated in preliminary testing. After this preliminary test, the protocol was revised to ver.8.2 to include detailed descriptions of the washing protocol. Three laboratories attended an additional validation study after one laboratory was not able to obtain a positive test result with 1-bromohexane.

The preliminary test was conducted by three laboratories between August and September of 2010. The duration of validation study was between September and November of 2010.

7. Test chemicals

7-1. Chemical selection

To meet the OECD performance standard, the VMT selected 20 chemicals for testing (Table 1). In the reference chemicals of OECD Test guideline No. 439, tetrachloroethylene was not selected. Instead of it, No.20, 1,1,1-trichloroethane was tested. Because tetrachloroethylene was changed from 1,1,1-trichloroethane at the final step before approving as the test guideline, the VMT escaped the attention of conclusive confirmation. The class of their chemicals is at the same level and the VMT judged not to become a serious problem in this validation study. The final approval of the chemicals proposed by JaCVAM was the responsibility of the VMT. To avoid any potential for bias in the final selection, laboratory representatives on the VMT did not participate in these discussions, nor were they made aware of the chemicals finally approved for testing in the validation study.

Table 1. Minimum List of Reference Chemicals for Determination of Accuracy and Reliability Values for Similar or Modified RhE Skin Irritation Test Methods and Codes

No.	Name	CAS number	UN GHS *in vivo* Cat.	Storage	Chemical code		
					Lab a	Lab b	Lab c

1	1-bromo-4-chlorobutane	6940-78-9	No Cat.	RT	B-261	D-281	G-301
2	diethyl phthalate	84-66-2	No Cat.	RT	B-262	D-282	G-302
3	naphthalene acetic acid	86-87-3	No Cat.	RT	B-263	D-283	G-303
4	allyl phenoxy-acetate	7493-74-5	No Cat.	RT	B-264	D-284	G-304
5	isopropanol	67-63-0	No Cat.	RT	B-265	D-285	G-305
6	4-methylthio-benzaldehyde	3446-89-7	No Cat.	RT	B-266	D-286	G-306
7	methyl stearate	112-61-8	No Cat.	RT	B-267	D-287	G-307
8	heptyl butyrate	5870-93-9	No Cat. (*Optional Cat. 3*)	RT	B-268	D-288	G-308
9	hexyl salicylate	6259-76-3	No Cat. (*Optional Cat. 3*)	RT	B-269	D-289	G-309
10	cinnamaldehyde	104-55-2	No Cat. (*Optional Cat. 3*)	2-8C	B-270	D-290	G-310
11	1-decanol	112-30-1	Cat.2	RT	B-271	D-291	G-311
12	cyclamen aldehyde	103-95-7	Cat.2	RT	B-272	D-292	G-312
13	1-bromohexane	11-25-1	Cat.2	RT	B-273	D-293	G-313
14	2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	86604-75-3	Cat.2	RT	B-274	D-294	G-314
15	di-n-propyl disulphide	629-19-6	Cat.2	RT	B-275	D-295	G-315
16	potassium hydroxide 5%	1310-58-3	Cat.2	RT	B-276	D-296	G-316
17	benzynethiol,5-(1,1-dimethylethyl)-2-methyl	7340-90-1	Cat.2	RT	B-277	D-297	G-317
18	1-methyl-3-phenyl-1-piperazine	5271-27-2	Cat.2	RT	B-278	D-298	G-318
19	heptanal	111-71-7	Cat.2	RT	B-279	D-299	G-319
20	1,1,1-trichloroethane	71-55-6	Cat.2	RT	B-280	D-300	G-320

1) CAS No.: Chemical abstracts service registry number.

7-2. Chemical coding and distribution

Independent coding and distribution of chemicals were contracted out by JaCVAM to an independent laboratory. The undeciphered information on this code was confirmed by the VMT after the validation study because this chemical code list was too easy and simple. The certification of chemicals was according to ISO (International Organization for Standardization) 9001 and GLP, and has proven experience of reliable services. The codes were provided by JaCVAM.

8. Protocol

8-1. Protocol of the skin irritation test with LabCyte EPI-MODEL24 SIT

According to the suggestion of the OECD peer review panel, J-TEC resolved the false-negative issues of 1-bromohexane. The SOP (Standard Operating Procedure: ver.8.1) included a modified washing protocol in a revision dated June 30, 2010. Modifications to the washing protocol are shown in Table 2 (Detailed process described in Appendix 7). Using the revised SOP, the validation study was performed to show clear data and addressed comments of the OECD peer review panel.

Table 2. Modification points of washing protocol between SOP ver.7.1 and SOP ver.8.2.

Modification points	SOP ver.7.1	SOP ver.8.2
1. Handling the PBS stream from washing bottle	It was not defined.	The revision specified to avoid hitting the tissue surface directly with the PBS tream.
2. Removal of PBS by tapping	It was not defined.	It was briefly defined.
3. Correct use of the cotton pad	It was not defined.	It was defined to avoid touching the tissue surface directly with the cotton pad.

LabCyte EPI-MODEL 24 tissues were shipped from the supplier on Mondays and delivered to recipients on Tuesdays. Upon receipt, the tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 mL), and incubated overnight (37°C, 5% CO_2 humidified atmosphere). On the following day, the tissues were topically exposed to the test chemicals. Liquids (25 µL) were applied with a micropipette, and solids (25 mg) were applied from microtubes and moistened with 25-µL sterile water. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied using a cell-saver-type tip with a micropipette. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were treated with 25-µL distilled water, and three tissues serving as positive controls were exposed to 5% SLS (sodium lauryl sulphate). After a 15-minute exposure, each tissue was carefully washed with PBS (phosphate buffered saline, Invitrogen, CA, USA) 10 times using a washing bottle to remove any remaining test chemical from the surface. The blotted tissues were then transferred to new 24-well plates containing 1 mL of fresh assay medium.

The treated and control tissues were incubated for 42 hours (37 °C, 5% CO_2 humidified atmosphere). When the 42-hour incubation period was complete, blotted tissues were transferred to new 24-well plates containing 0.5 mL of freshly prepared MTT medium (1 mg/mL; Dojindo Co., Kumamoto, Japan) for the MTT assay. Tissues were incubated for 3 hours (37 °C, 5% CO_2 humidified atmosphere) and then transferred to microtubes containing 0.3 mL isopropanol, which completely immersed the tissue. Formazan extraction was performed at room temperature, and the tissues were allowed to stand overnight.

Subsequently, 200-µL extracts were transferred to a 96-well plate. The optical density was measured at 570 nm and 650 nm as a reference absorbance, with isopropanol as a blank.
The tissue viability was calculated as a percentage relative to the viability of the negative controls. The median of three values from identically treated tissues was used to classify a chemical according to the prediction model.

8-2. Prediction model of skin irritation

In this study, the prediction model (acceptability criteria and positive criteria) of skin irritation potential with LabCyte EPI-MODEL24 SIT was set to refer to the conditions for the OECD TG 439 and its Performance Standards.

8-2-1) Acceptance criteria on the RhE test method components

According to paragraph 27; acceptability criteria in the OECD TG 439, tissues treated with the negative controls and positive controls, *i.e.* 5% aqueous SLS, should reflect their ability to respond to an irritant chemical under the conditions of the test method. Associated and appropriate measures of variability between tissue replicates should be defined.

1) OD_{NC} of the negative control is greater than 0.7.
2) The viability of the positive control (5% aqueous SLS) is less than 40%.
3) If standard deviations (SDs) are used they should be within the one-sided 95% tolerance interval calculated from historical data; for the VRM SD < 18%.

8-2-2) Positive criteria

The OD values obtained with each test sample can be used to calculate the percentage of viability normalized to the No Category, which is set to 100%. The cut-off value for percentage of cell viability distinguishing irritant from non-classified test chemicals and the statistical procedure(s) used to evaluate the results and identify irritant chemicals, should be clearly defined, documented, and proven to be appropriate. The cut-off values for the prediction of irritation are given below:

The test chemical is considered to be an irritant to skin in accordance with GHS category 2 if the tissue viability after exposure and post-treatment incubation is less than or equal (≤) to 50%.

Depending on the country and regional regulatory requirements, the test chemical may be considered as a No Category if the tissue viability after exposure and post-treatment incubation is more than (>) 50%.

8-2-3) Study acceptance criteria

It is possible that one or several tests with one or more test chemicals do not meet test acceptance criteria for the test and control chemicals or are not acceptable for other reasons. To complement missing data, a maximum number of two additional tests for each test chemical is admissible ("retesting"). Because retesting requires concurrent testing with a positive control and negative control, a maximum number of two additional runs may be conducted for each test chemical.

It is conceivable that even after retesting, the minimum number of three valid runs required for each tested chemical is not obtained for every Reference Chemical in every participating laboratory, leading to an incomplete data matrix. In such cases the following three criteria should all be met in order to consider the datasets acceptable:

1. All 20 Reference Chemicals should have at least one complete run sequence.
2. In each of at least three participating laboratories, a minimum of 85% of the run sequences need to be complete (for 20 chemicals, three invalid run sequences are allowed in a single laboratory).
3. A minimum of 90% of all possible run sequences from at least three laboratories need to be complete (for 20 chemicals tested in three laboratories, a total of six invalid run sequences are allowed).

8-2-4) Rules

The calculation of the reliability and accuracy values of the proposed test method should be done considering all four criteria below, ensuring that values for reliability and relevance are calculated in a predefined and consistent manner:

1. Only data of runs from complete run sequences qualify for calculation of within- and between-laboratory variability and predictive capacity (accuracy) of the test method.
2. The final classification for each Reference Chemical in each participating laboratory should be obtained by using the mean value of viability over the different runs of a complete run sequence.
3. Only data obtained for chemicals that have complete run sequences in all participating laboratories qualify for calculation of between-laboratory variability of the test method.
4. Calculation of the accuracy values should be done on the basis of individual laboratory predictions

obtained for the 20 Reference Chemicals by the different participating laboratories.
In this context, a **run sequence** consists of three independent runs from one laboratory for one test chemical. A **complete run sequence** is a run sequence from one laboratory for one test chemical where all three runs are valid. This means that any single invalid run invalidates an entire run sequence of three runs.

Within-laboratory reproducibility

An assessment of within-laboratory reproducibility should show that the concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of 20 Reference Chemicals within one single laboratory is equal to or higher than (≥) 90%.

Between-laboratory reproducibility

An assessment of between-laboratory reproducibility is not essential if the proposed test method is to be used in a single laboratory only. For methods to be transferred between laboratories, the concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of 20 Reference Chemicals between preferentially a minimum of 3 laboratories should be equal or higher than (≥) 80%.

Predictive capacity (accuracy)

The accuracy (sensitivity, specificity and overall accuracy) of the proposed similar or modified test method should be comparable or better to that of the VRM, taking into consideration information relating the species of interest (Table 3). The sensitivity should be equal to or higher than (≥) 80%. However, an additional restriction applies to the sensitivity of the proposed *in vitro* test method; only two *in vivo* Category 2 chemicals, *1-decanol* and *di-n-propyl disulphide,* may be misclassified as a No Category by more than one participating laboratory. The specificity should be equal to or higher than (≥) 70%. No restrictions with regard to specificity of the proposed *in vitro* test method were applied; any participating laboratory may misclassify any *in vivo* No Category chemical as long as the final specificity of the test method is within the acceptable range. The overall accuracy should be equal to or higher than (≥) 75%. Although the sensitivity of the VRM calculated for the 20 Reference Chemicals listed in Table 1 is equal to 90%, the defined minimum sensitivity value required for any similar or modified test method to be considered valid is set at 80% because both *1-decanol* (a borderline chemical) and *di-n-propyl disulphide* (a false negative of the VRM) are known to be non-irritant chemicals in humans, although they have been identified as irritants in the rabbit test. Since RhE models are based on cells of human origin, they may predict these chemicals as non-irritant (UN GHS No Category).

Table3: Required predictive values for sensitivity, specificity and overall accuracy for any similar or modified test method to be considered valid

Sensitivity	Specificity	Overall Accuracy
≥ 80%	≥ 70%	≥ 75%

8-3. Data collection, handling, and analysis

The independent biostatistician for the study collected and organized the data using specific data collection software (Datasheet5.0:20090430.xls). They worked in close collaboration with JaCVAM (Hajime Kojima). After decoding the data, JaCVAM performed statistical analyses. The data management procedures and statistical tools applied were approved by the VMT.

8-4. Quality assurance, GLP Laboratories

All participating laboratories conducted research following OECD GLP-like principles.

QC aspects

JaCVAM (Hajime Kojima) assured the quality of all the data and records. After the validation study, all study documents were submitted to the chairperson of VMT and only data sheets were forwarded by e-mail to the biostatistician. All data sheets from one participating laboratory, KOBAYASHI Pharmaceutical Co., Ltd. are provided as an example in Appendix 6. The chairperson reviewed the contents of the study documents and clarified illegible or unclear content by contacting each group by e-mail or telephone.

9. Results

9-1 Comments in the datasheets

A few comments from each laboratory are listed in Table 4. Application of potassium hydroxide (5%aq) (B276, B296, and B288) caused the model's layers to be desquamated. Upon application of B301, B304, B306, and B310, the cups were discoloured and crystallized. The VMT judged that these occurrences had no effect on the results of the study.

Table 4 Comments on the datasheets (Viability)

Lab	Exp.No.	Lot	Date	Comments
a	Main-1	LCE24-100906-A	9/9/10	The model's layers treated by B-276 were desquamated
b	Main-1	LCE24-100906-A	9/8/10	The model's layers treated by B-296 were desquamated
b	Main-2	LCE24-100913-A	9/15/10	The model's layers treated by B-288 & B-296 were desquamated
c	Main-1	LCE24-100830-A	9/6/10	Cups treated by B-301, 304, 306 and 310 were discoloured.
c	Main-2	LCE24-100913-A	9/20/10	Cups treated by B-301, 304, 306 and 310 were discoloured.
c	Main-3	LCE24-100920-A	9/27/10	Cups treated by B-301, 304, 306 and 310 were discoloured.

9-2 Negative control

Table 5 shows the absorbance values for the negative control. All data for the negative control met the acceptance criteria.

Table 5 Viability of negative control

Laboratory	Average OD/ triplicate tissues	Average, SD at all OD
Lab a	0.88	0.91±0.05
	0.87	
	0.92	
	0.98	
Lab b	1.03	1.03±0.06
	1.02	
	1.13	
	0.98	
	0.98	
Lab c	1.09	1.07±0.09
	0.98	
	1.01	
	1.06	
	1.20	

9-3 Positive control

Table 6 shows the absorbance values for the positive control. All data for the positive control met the acceptance criteria.

Table 6 Viability of the positive control

Laboratory	Average OD/ triplicate tissues	Average, SD at all OD
Lab a	2.29	2.65±0.68
	3.62	
	2.59	
	2.10	
Lab b	4.98	3.87±0.84
	3.22	
	4.50	
	3.02	
	3.62	
Lab c	2.87	2.69±0.49
	3.37	
	2.64	
	2.55	
	2.02	

9-4 Viability of chemicals

Table 7 shows the mean viability of testing chemicals at each tissue. Two data points at Lab a, eight data points at Lab b, and four data points at Lab c showed a SD > 18% and did not meet the acceptance criteria. Instead of generating insufficient data, each laboratory re-tested up to two additional runs. At Lab b, No. 15 resulted in a single invalid run, thereby invalidating an entire run sequence of three runs. In addition, the VMT did not accept all data from the fourth or fifth runs. The original data are shown in Appendix 5.

All study acceptance criteria were met as shown below.

1. All 20 Reference Chemicals had at least one complete run sequence at each laboratory.
2. In each of three participating laboratories, at least 95% of the run sequences were complete (One invalid run sequence was allowed in Lab b).
3. 99.4% of all possible run sequences from the three laboratories were complete (for 20 chemicals tested in three laboratories, a total of one invalid run sequence is allowed).

These experiments confirmed the feasibility of the LabCyte EPI-MODEL24 SIT test method.

Table 7. Mean viability of chemicals at each laboratory

		Exp.				
Chem.	Lab	1	2	3	4	5
01	a	12.4	11.3	19.0		
	b	16.5	10.7	10.6		
	c	9.0	9.8	9.8		
02	a	91.7	81.5	69.6	80.1	
	b	60.9	57.5	65.5	69.5	
	c	90.5	77.4	102.0	93.0	88.7
03	a	108.0	113.0	105.0		
	b	96.5	96.7	90.2		
	c	89.4	90.8	106.0	98.9	96.0
04	a	19.1	43.4	65.1	59.3	
	b	66.6	70.6	48.1	66.2	
	c	90.1	93.0	93.2		
05	a	89.6	77.0	67.6		
	b	75.9	57.5	74.8	77.1	
	c	68.5	86.6	66.4	67.2	74.4
06	a	16.2	15.9	17.0		
	b	17.3	13.5	11.4		
	c	15.5	16.1	12.0		
07	a	110.0	110.0	104.0		
	b	98.8	93.1	76.3		
	c	91.2	102.0	108.0		
08	a	109.0	122.0	111.0		
	b	93.1	106.0	86.6		
	c	95.5	106.0	119.0		
09	a	105.0	111.0	102.0		
	b	98.0	95.7	83.5		
	c	99.6	100.0	113.0		
10	a	15.7	20.3	16.0		
	b	11.5	15.9	11.4		
	c	17.3	14.1	14.9		
11	a	14.2	16.5	9.4		
	b	12.4	17.3	16.2		
	c	22.1	15.1	14.1		
12	a	8.9	15.9	10.0		
	b	11.0	7.8	9.0		
	c	6.0	7.4	5.7		
13	a	48.0	16.2	16.1	15.5	
	b	39.5	6.6	49.6	17.2	19.0
	c	17.5	17.0	16.2		
14	a	2.1	4.3	4.1		
	b	4.9	5.2	9.1		
	c	2.8	3.4	3.2		
15	a	19.9	95.9	83.5		
	b	39.1	28.0	52.7	17.5	18.5
	c	81.1	83.2	86.3		
16	a	0.9	1.7	1.6		
	b	4.6	2.0	3.3		
	c	0.9	3.1	11.6		
17	a	6.9	46.6	1.0		
	b	10.6	21.0	11.6		
	c	6.3	5.0	6.6		
18	a	6.7	4.5	3.6		
	b	9.8	10.9	11.0		
	c	1.3	1.8	2.2		
19	a	9.4	10.3	10.4		
	b	9.5	7.0	9.5		
	c	11.9	10.2	10.9		
20	a	8.7	12.0	7.8		
	b	9.1	7.9	37.6	17.4	
	c	7.6	7.0	6.8		

Red block
VRM SD > 18%, Not acccepted data

Light block
Not acceped for 4th dat

9-5. Classification of three independent viabilities at each laboratory

The classifications from individual viabilities and the mean of three independent viabilities are shown in Table 8. Lab a misclassified two data points (No. 4 and 15 at the first test), Lab b misclassified two data points (No. 15 at the first and second tests), and Lab c missed no classifications. As previously discussed, the third data point of the test with No. 15 at Lab b induced a single invalid run, thereby invalidating the entire run sequence of three runs. Therefore, the VMT judged "not detected" in the classification of No.15.

Table 8: Classification using three independent viabilities
P: Positive, N: Negative, F: Final detemination by median, ND: Not detected

No	UN GHS *in vivo* Cat.	Lab a				Lab b				Lab c			
		1	2	3	F	1	2	3	F	1	2	3	F
1	No	P	P	P	P	P	P	P	P	P	P	P	P
2	No	N	N	N	N	N	N	N	N	N	N	N	N
3	No	N	N	N	N	N	N	N	N	N	N	N	N
4	No	P	N	N	P	N	N	N	N	N	N	N	N
5	No	N	N	N	N	N	N	N	N	N	N	N	N
6	No	P	P	P	P	P	P	P	P	P	P	P	P
7	No	N	N	N	N	N	N	N	N	N	N	N	N
8	No	N	N	N	N	N	N	N	N	N	N	N	N
9	No	N	N	N	N	N	N	N	N	N	N	N	N
10	No	P	P	P	P	P	P	P	P	P	P	P	P
11	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
12	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
13	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
14	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
15	Cat.2	P	N	N	N	P	P	ND	ND	N	N	N	N
16	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
17	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
18	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
19	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
20	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P

10. Discussion

10-1. Reliability

Within-laboratory reproducibility

An assessment of within-laboratory reproducibility should show a concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of the 20 Reference Chemicals at each laboratory. As shown in Table 8 above, Lab a missed two classifications (No. 4 and 15) and the rate of within-laboratory reproducibility was 90.0% (18/20). Lab b missed one data point (No. 15) and the rate of reproducibility was 95.0% (19/20). Lab c missed no classifications and had a reproducibility rate of 100%. Therefore, results of all laboratories were sufficient, having a reproducibility rate equal to or higher than (≥) 90%.

Between-laboratory reproducibility

For methods to be transferred between laboratories, the concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of the 20 Reference Chemicals between three laboratories was evaluated. As shown in Table 8, all laboratories missed more than four classifications and the rate of between-laboratory reproducibility was 95.0% (19/20). Therefore, all laboratories had a sufficient between-laboratory reproducibility that was equal to or higher than (≥) 80%.

10-2. Predictivity

The accuracy (sensitivity, specificity, and overall accuracy) of the LabCyte EPI-MODEL24 SIT skin irritation test was evaluated by cell viabilities (MTT) as an indicator, and the UN-GHS classifications are shown in Table 9. The sensitivity, specificity, and accuracy of this prediction model at each laboratory were 90–100%, 60-70%, and 75–84.2%, respectively. Some deviations from the OECD Performance standard (sensitivity of 80%, specificity of 70%, and accuracy of 75%; shown in Table 3) were specific adaptations for the LabCyte EPI-MODEL excluding 60% of specificity at Lab a. Unfortunately, one of three laboratories is insufficient with acceptance criteria at the OECD Performance standard. Two of three laboratories, however, are sufficient with acceptance criteria at the OECD Performance standard and the VMT considered that this assay had acceptable reliability of accuracy.

Table 9. 2x2 tables

Lab a		*In vivo* classification		
		Irritant	Non-Irritant	Total
In vitro prediction	Irritant	9	4	13
	Non-irritant	1	6	7
	Total	10	10	20

Sensitivity (%)	90.0
Specificity (%)	60.0
Accuracy (%)	75.0

Lab b		*In vivo* classification		
		Irritant	Non-Irritant	Total
In vitro prediction	Irritant	9	3	12
	Non-irritant	0	7	7
	Total	9	10	19
Sensitivity (%)		100.0		
Specificity (%)		70.0		
Accuracy (%)		84.2		

Lab c		*In vivo* classification		
		Irritant	Non-Irritant	Total
In vitro prediction	Irritant	9	3	12
	Non-irritant	1	7	8
	Total	10	10	20
Sensitivity (%)		90.0		
Specificity (%)		70.0		
Accuracy (%)		80.0		

11. Conclusions

Based on the reference list in the OECD Performance Standards, a catch-up validation of the LabCyte EPI-MODEL24 SIT by three labs was performed. The assay demonstrated high reliability within and between laboratories, and acceptable reliability of accuracy (75–84.2% overall accuracy, 90–100% overall sensitivity, and 60-70% overall specificity) on the MTT assay excluding 60% of specificity at one laboratory. Two of three laboratories are sufficient with acceptance criteria at the OECD Performance standard and the VMT considered that this assay had acceptable reliability of accuracy for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

12. Acknowledgement

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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

In Vitro Skin Irritation: Reconstructed Human *Epidermis* Test Method

INTRODUCTION

1. Skin irritation refers to the production of reversible damage to the skin following the application of a test chemical for up to 4 hours [as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS)](1). This Test Guideline (TG) provides an *in vitro* procedure that may be used for the hazard identification of irritant chemicals (substances and mixtures) in accordance with UN GHS Category 2 (1) (2). In member countries or regions that do not adopt the optional UN GHS Category 3 (mild irritants), this Test Guideline can also be used to identify non-classified chemicals. Therefore, depending on the regulatory framework and the classification system in use, this Test Guideline may be used to determine the skin irritancy of chemicals either as a stand-alone replacement test for *in vivo* skin irritation testing or as a partial replacement test within a tiered testing strategy (4).

2. The assessment of skin irritation has typically involved the use of laboratory animals [OECD TG 404; adopted in 1981 and revised in 1992 and 2002] (4). In relation to animal welfare concerns, TG 404 in its supplement recommended a tiered testing strategy for the determination of skin corrosion/irritation, using validated *in vitro* and *ex vivo* test methods, thus avoiding pain and suffering of animals. Three validated *in vitro* test methods have been adopted as OECD TGs 430, 431 and 435 (5) (6) (7), to be used for the corrosivity part of the tiered testing strategy recommended in supplement to TG 404 (4).

3. This Test Guideline addresses the human health endpoint skin irritation. It is based on the *in vitro* test system of reconstructed human *epidermis* (RhE), which closely mimics the biochemical and physiological properties of the upper parts of the human skin, *i.e.* the *epidermis.* The RhE test system uses human derived non-transformed keratinocytes as cell source to reconstruct an epidermal model with representative histology and cytoarchitecture. Performance Standards (PS) developed by EC-ECVAM (8) (9) are available to facilitate the validation and assessment of similar and modified RhE-based test methods, in accordance with the principles of Guidance Document No. 34 (10) (See Annex 4).

4. Pre-validation, optimisation and validation studies have been completed for four commercially available *in vitro* test methods (11) (12) (13) (14) (15) (16) (17) (18) (19) (20) (21) (22) (23) (24) (35) (36) (37) (38) (39) based on the RhE test system. These four test methods are included in this TG and are listed in Annex 2, which also provides information on the type of validation study used to validate the respective test methods. As noted in Annex 2, three of these methods have been used to develop the present TG including the Performance Standards (Annex 4) and are, in Annex 2 and 4, referred to as Validated Reference Methods (VRM).

5. Mutual Acceptance of Data will only be guaranteed for test methods, validated according to the Performance Standards (Annex 4), if these test methods have been reviewed and adopted by OECD. The

test methods included in this TG can be used indiscriminately to address countries' requirements for test results from *in vitro* test method for skin irritation, while benefiting from the Mutual Acceptance of Data.

6. Definitions of terms used in this document are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

7. A limitation of the Test Guideline, as demonstrated by the full prospective validation study assessing and characterising RhE test methods (17), is that it does not allow the classification of chemicals to the optional UN GHS Category 3 (mild irritants) (1). Thus, the regulatory framework in member countries will decide how this Test Guideline will be used. When employed as a partial replacement test, follow-up *in vivo* testing may be required to fully characterize skin irritation potential (4). It is recognized that the use of human skin is subject to national and international ethical considerations and conditions.

8. This Test Guideline addresses the *in vitro* skin irritation component of the tiered testing strategy recommended in supplement to TG 404 on dermal corrosion/irritation (4). While this Test Guideline does not provide adequate information on skin corrosion, it should be noted that OECD TG 431 on skin corrosion is based on the same RhE test system, though using another protocol (6). This Test Guideline is based on RhE-models using human keratinocytes, which therefore represent *in vitro* the target organ of the species of interest. It moreover directly covers the initial step of the inflammatory cascade/mechanism of action (cell and tissue damage resulting in localised trauma) that occurs during irritation *in vivo*. A wide range of chemicals has been tested in the validation underlying this Test Guideline and the database of the validation study amounted to 58 chemicals in total (17) (19) (24). The Test Guideline is applicable to solids, liquids, semi-solids and waxes. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be ground to a fine powder before application; no other pre-treatment of the sample is required. Gases and aerosols have not been assessed yet in a validation study (25). While it is conceivable that these can be tested using RhE technology, the current Test Guideline does not allow testing of gases and aerosols. It should also be noted that highly coloured chemicals may interfere with the cell viability measurements and need the use of adapted controls for corrections (see paragraphs 24-26).

9. A single testing run composed of three replicate tissues should be sufficient for a test chemical when the classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements and/or mean percent viability equal to 50 ± 5%, a second run should be considered, as well as a third one in case of discordant results between the first two runs.

PRINCIPLE OF THE TEST

10. The test chemical is applied topically to a three-dimensional RhE model, comprised of non-transformed human-derived epidermal keratinocytes, which have been cultured to form a multilayered, highly differentiated model of the human *epidermis*. It consists of organized basal, spinous and granular layers, and a multilayered *stratum corneum* containing intercellular lamellar lipid layers representing main lipid classes analogous to those found *in vivo*.

11. Chemical-induced skin irritation, manifested mainly by erythema and oedema, is the result of a cascade of events beginning with penetration of the chemicals through the *stratum corneum* where they may damage the underlying layers of keratinocytes and other skin cells. The damaged cells may either release inflammatory mediators or induce an inflammatory cascade which also acts on the cells in the *dermis*, particularly the stromal and endothelial cells of the blood vessels. It is the dilation and increased permeability of the endothelial cells that produce the observed erythema and oedema (25). Notably, the

RhE-based test methods, in the absence of any vascularisation in the *in vitro* test system, measure the initiating events in the cascade, e.g. cell / tissue damage (17) (18), using cell viability as readout.

12. Cell viability in RhE models is measured by enzymatic conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1], into a blue formazan salt that is quantitatively measured after extraction from tissues (25). Irritant chemicals are identified by their ability to decrease cell viability below defined threshold levels (*i.e.* ≤ 50%, for UN GHS Category 2). Depending on the regulatory framework and applicability of the Test Guideline, chemicals that produce cell viabilities above the defined threshold level, may be considered non-irritants (*i.e.* > 50%, No Category).

DEMONSTRATION OF PROFICIENCY

13. Prior to routine use of any of the four validated test methods that adhere to this Test Guideline (Annex 2), laboratories should demonstrate technical proficiency, using the ten Proficiency Chemicals listed in Table 1.

14. As part of the proficiency testing, it is recommended that users verify the barrier properties of the tissues after receipt as specified by the RhE model producer. This is particularly important if tissues are shipped over long distance/time periods. Once a test method has been successfully established and proficiency in its use has been acquired and demonstrated, such verification will not be necessary on a routine basis. However, when using a test method routinely, it is recommended to continue to assess the barrier properties at regular intervals.

Table 1: Proficiency Chemicals[1]

Chemical	CAS NR	*In vivo* score[2]	Physical state	UN GHS Category
NON-CLASSIFIED CHEMIALS				
naphthalene acetic acid	86-87-3	0	Solid	No Cat.
isopropanol	67-63-0	0.3	Liquid	No Cat.
methyl stearate	112-61-8	1	Solid	No Cat.
heptyl butyrate	5870-93-9	1.7	Liquid	No Cat. (*Optional Cat. 3*)[3]
hexyl salicylate	6259-76-3	2	Liquid	No Cat. (*Optional Cat. 3*)[3]
CLASSIFIED CHEMICALS				
cyclamen aldehyde	103-95-7	2.3	Liquid	Cat. 2
1-bromohexane	111-25-1	2.7	Liquid	Cat. 2
potassium hydroxide (5% aq.)	1310-58-3	3	Liquid	Cat. 2
1-methyl-3-phenyl-1-piperazine	5271-27-2	3.3	Solid	Cat. 2
heptanal	111-71-7	3.4	Liquid	Cat. 2

[1] The Proficiency Chemicals are a subset of the chemicals used in the validation study.
[2] *In vivo* score in accordance with the OECD Test Guideline 404 (4).
[3] Under this Test Guideline, the UN GHS optional Category 3 (mild irritants) (1) is considered as No Category.

PROCEDURE

15. The following is a description of the components and procedures of a RhE test method for skin irritation assessment (See also Annex 3 for parameters related to each test method). Standard Operating Procedures (SOPs) for the four test methods complying with this TG are available (27) (28) (29) (40).

RHE TEST METHOD COMPONENTS

General conditions

16. Non-transformed human keratinocytes should be used to reconstruct the epithelium. Multiple layers of viable epithelial cells (basal layer, *stratum spinosum, stratum granulosum*) should be present under a functional *stratum corneum*. *Stratum corneum* should be multilayered containing the essential lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic benchmark chemicals, *e.g.* sodium dodecyl sulphate (SDS) or Triton X-100. The barrier function should be demonstrated and may be assessed either by determination of the concentration at which a benchmark chemical reduces the viability of the tissues by 50% (IC_{50}) after a fixed exposure time, or by determination of the exposure time required to reduce cell viability by 50% (ET_{50}) upon application of the benchmark chemical at a specified, fixed concentration. The containment properties of the RhE model should prevent the passage of material around the *stratum corneum* to the viable tissue, which would lead to poor modelling of skin exposure. The RhE model should be free of contamination by bacteria, viruses, mycoplasma, or fungi.

Functional conditions

Viability

17. The assay used for determining the magnitude of viability is the MTT-assay (26). The RhE model users should ensure that each batch of the RhE model used meets defined criteria for the negative control (NC). The optical density (OD) of the extraction solvent alone should be sufficiently small, *i.e.* OD< 0.1. An acceptability range (upper and lower limit) for the negative control OD values (in the Skin Irritation Test Method conditions) are established by the RhE model developer/supplier. Acceptability ranges for the 4 validated test methods are given in Table 2. It should be documented that the tissues treated with NC are stable in culture (provide similar viability measurements) for the duration of the test exposure period.

Table 2: Acceptability ranges for negative control OD values of the test methods included in this TG

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM)	≥ 0.6	≤ 1.5
EpiDerm™ SIT (EPI-200)	≥ 0.8	≤ 2.8
SkinEthic™ RHE	≥ 0.8	≤ 3.0
LabCyte EPI-MODEL24 SIT	≥ 0.7	≤ 2.5

Barrier function

18. The *stratum corneum* and its lipid composition should be sufficient to resist the rapid penetration of cytotoxic benchmark chemicals, *e.g.* SDS or Triton X-100, as estimated by IC_{50} or ET_{50} (Table 3).

Morphology

19. Histological examination of the RhE model should be provided demonstrating human *epidermis*-like structure (including multilayered *stratum corneum*).

Reproducibility

20. The results of the positive and negative controls of the test method should demonstrate reproducibility over time.

Quality control (QC)

21. The RhE model should only be used if the developer/supplier demonstrates that each batch of the RhE model used meets defined production release criteria, among which those for *viability* (paragraph 17), *barrier function* (paragraph 18) and *morphology* (paragraph 19) are the most relevant. These data should be provided to the test method users, so that they are able to include this information in the test report. An acceptability range (upper and lower limit) for the IC_{50} or the ET_{50} should be established by the RhE model developer/supplier. Only results produced with qualified tissues can be accepted for reliable prediction of irritation classification. The acceptability ranges for the four test methods included in this TG are given in Table 3.

Table 3: QC batch release criteria of the test methods included in this TG

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM) (18 hours treatment with SDS) (27)	IC_{50} = 1.0 mg/ml	IC_{50} = 3.0 mg/ml
EpiDerm™ SIT (EPI-200) (1% Triton X-100) (28)	ET_{50} = 4.0 hr	ET_{50} = 8.7 hr
SkinEthic™ RHE (1% Triton X-100) (29)	ET_{50} = 4.0 hr	ET_{50} = 10.0 hr
LabCyte EPI-MODEL24 SIT (18 hours treatment with SDS) (40)	IC_{50} = 1.4 mg/ml	IC_{50} = 4.0 mg/ml

Application of the Test and Control Chemicals

22. At least three replicates should be used for each test chemical and for the controls in each run. For liquid as well as solid chemicals, sufficient amount of test chemical should be applied to uniformly cover the *epidermis* surface while avoiding an infinite dose, *i.e.* ranging from 26 to 83 $\mu L/cm^2$ or mg/cm^2 (see Annex 3), should be used. For solid chemicals, the *epidermis* surface should be moistened with deionised or distilled water before application, to improve contact between the test chemical and the *epidermis* surface. Whenever possible, solids should be tested as a fine powder. A nylon mesh may be used as a spreading aid in some cases (see Annex 3). At the end of the exposure period, the test chemical should be carefully washed from the *epidermis* surface with aqueous buffer, or 0.9% NaCl. Depending on the RhE test methods used, the exposure period ranges between 15 and 60 minutes, and the incubation temperature between 20 and 37°C. These exposure periods and temperatures are optimized for each individual RhE test method and represent the different intrinsic properties of the test methods (*e.g.* barrier function) (see Annex 3).

23. Concurrent NC and positive controls (PC) should be used in each run to demonstrate that viability (with the NC), barrier function and resulting tissue sensitivity (with the PC) of the tissues are within a defined historical acceptance range. The suggested PC chemical is 5% aqueous SDS. The suggested NC chemicals are water or phosphate buffered saline (PBS).

Cell Viability Measurements

24. According to the test procedure, it is essential that the viability measurement is not performed immediately after exposure to the test chemical, but after a sufficiently long post-treatment incubation period of the rinsed tissue in fresh medium. This period allows both for recovery from weak cytotoxic effects and for appearance of clear cytotoxic effects. A 42 hours post-treatment incubation period was found optimal during test optimisation of two of the RhE-based test methods underlying this TG (12) (13) (14) (15) (16).

25. The MTT assay is a validated quantitative method which should be used to measure cell viability under this Test Guideline. It is compatible with use in a three-dimensional tissue construct. The tissue sample is placed in MTT solution of appropriate concentration (*e.g.* 0.3 - 1 mg/mL) for 3 hours. The MTT is converted into blue formazan by the viable cells. The precipitated blue formazan product is then extracted from the tissue using a solvent (*e.g.* isopropanol, acidic isopropanol), and the concentration of formazan is measured by determining the OD at 570 nm using a filter band pass of maximum ± 30 nm.

26. Optical properties of the test chemical or its chemical action on MTT (e.g. chemicals may prevent or reverse the colour generation as well as cause it) may interfere with the assay leading to a false estimate of viability. This may occur when a specific test chemical is not completely removed from the tissue by rinsing or when it penetrates the *epidermis*. If a test chemical acts directly on the MTT (*e.g.* MTT-reducer), is naturally coloured, or becomes coloured during tissue treatment, additional controls should be used to detect and correct for test chemical interference with the viability measurement technique. Detailed description of how to correct direct MTT reduction and interferences by colouring agents is available in the SOPs for the four validated test methods included in this Test Guideline (27) (28) (29) (40).

Acceptability Criteria

27. For each test method using valid RhE model batches (see paragraph 21), tissues treated with the NC should exhibit OD reflecting the quality of the tissues that followed shipment, receipt steps and all protocol processes. Control OD values should not be below historically established boundaries. Similarly, tissues treated with the PC, *i.e.* 5% aqueous SDS, should reflect their ability to respond to an irritant chemical under the conditions of the test method (see Annex 3 and for further information SOPs of the four test methods included in this TG (27) (28) (29) (40)). Associated and appropriate measures of variability between tissue replicates, i.e., standard deviations (SD) should fall within the acceptance limits established for the test method used (see Annex 3).

Interpretation of Results and Prediction Model

28. The OD values obtained with each test chemical can be used to calculate the percentage of viability normalised to NC, which is set to 100%. The cut-off value of percentage cell viability distinguishing irritant from non-classified test chemicals and the statistical procedure(s) used to evaluate the results and identify irritant chemicals should be clearly defined, documented, and proven to be appropriate (see SOPs of the test methods for information). The cut-off values for the prediction of irritation are given below:

- The test chemical is considered to be irritant to skin in accordance with UN GHS Category 2 if the tissue viability after exposure and post-treatment incubation is less than or equal (≤) to 50%.

- Depending on the regulatory framework in member countries, the test chemical may be considered as non-irritant to skin in accordance with UN GHS No Category if the tissue viability after exposure and post-treatment incubation is more than (>) 50%.

DATA AND REPORTING

Data

29. For each run, data from individual replicate tissues (*e.g.* OD values and calculated percentage cell viability data for each test chemical, including classification) should be reported in tabular form, including data from repeat experiments as appropriate. In addition means ± SD for each run should be reported. Observed interactions with MTT reagent and coloured test chemicals should be reported for each tested chemical.

Test Report

30. The test report should include the following information:

Test and Control Chemicals:
- Chemical name(s) such as CAS name and number, if known;
- Purity and composition of the chemical (in percentage(s) by weight);
- Physical-chemical properties relevant to the conduct of the study (*e.g.* physical state, stability, volatility, pH and water solubility if known);
- Treatment of the test/control chemicals prior to testing, if applicable (*e.g.* warming, grinding);
- Storage conditions;

Justification of the RhE model and protocol used

Test Conditions:
- Cell system used;
- Complete supporting information for the specific RhE model used including its performance. This should include, but is not limited to;
  i) viability
  ii) barrier function
  iii) morphology
  iv) reproducibility and predictivity
  v) Quality controls (QC) of the model
- Details of the test procedure used;
- Test doses used, duration of exposure and post treatment incubation period;
- Description of any modifications to the test procedure;
- Reference to historical data of the model. This should include, but is not limited to:
  i) acceptability of the QC data with reference to historical batch data
  ii) acceptability of the positive and negative control values with reference to positive and negative control means and ranges
- Description of evaluation criteria used including the justification for the selection of the cut-off point(s) for the prediction model;
- Indication of controls used for direct MTT-reducers and/or colouring test chemicals;

Results:

- Tabulation of data from individual test chemical for each run and each replicate measurement together with the mean, SD and overall classification;
- Results of controls used for direct MTT-reducers and/or colouring test chemicals;
- Description of other effects observed;

Discussion of the results

Conclusion

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ANNEX 1

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with "concordance" to mean the proportion of correct outcomes of a test method (10).

Cell viability: Parameter measuring total activity of a cell population *e.g.* as ability of cellular mitochondrial dehydrogenases to reduce the vital dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue), which depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

Chemical: means a substance or a mixture

Concordance: This is a measure of test method performance for test methods that give a categorical result, and is one aspect of relevance. The term is sometimes used interchangeably with accuracy, and is defined as the proportion of all chemicals tested that are correctly classified as positive or negative. Concordance is highly dependent on the prevalence of positives in the types of test chemical being examined (10).

ET_{50}: Can be estimated by determination of the exposure time required to reduce cell viability by 50% upon application of the marker chemical at a specified, fixed concentration, see also IC_{50}.

EU CLP (European Commission Regulation on the Classification, Labelling and Packaging of Substances and Mixtures): Implements in the European Union (EU) the UN GHS system for the classification of chemicals (substances and mixtures) (3).

GHS (Globally Harmonized System of Classification and Labelling of Chemicals by the United Nations (UN)): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

IC_{50}: Can be estimated by determination of the concentration at which a marker chemical reduces the viability of the tissues by 50% (IC_{50}) after a fixed exposure time, see also ET_{50}.

Infinite dose: Amount of test chemical applied to the *epidermis* exceeding the amount required to completely and uniformly cover the *epidermis* surface.

Me-too test: A colloquial expression for a test method that is structurally and functionally similar to a validated and accepted reference test method. Such a test method would be a candidate for catch-up validation. Interchangeably used with similar test method (10).

Mixture: means a mixture or a solution composed of two or more substances in which they do not react.

Performance standards (PS): Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are; (i) essential test method components; (ii) a minimum list of Reference Chemicals selected

from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals (10).

Reference chemicals: Chemicals selected for use in the validation process, for which responses in the *in vitro* or *in vivo* reference test system or the species of interest are already known. These chemicals should be representative of the classes of chemicals for which the test method is expected to be used, and should represent the full range of responses that may be expected from the chemicals for which it may be used, from strong, to weak, to negative. Different sets of reference chemicals may be required for the different stages of the validation process, and for different test methods and test uses (10).

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (10).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility (10).

Replacement test: A test which is designed to substitute for a test that is in routine use and accepted for hazard identification and/or risk assessment, and which has been determined to provide equivalent or improved protection of human or animal health or the environment, as applicable, compared to the accepted test, for all possible testing situations and chemicals (10).

Sensitivity: The proportion of all positive/active test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (10).

Skin irritation: The production of reversible damage to the skin following the application of a test chemical for up to 4 hours. Skin irritation is a locally arising reaction of the affected skin tissue and appears shortly after stimulation (30). It is caused by a local inflammatory reaction involving the innate (non-specific) immune system of the skin tissue. Its main characteristic is its reversible process involving inflammatory reactions and most of the clinical characteristic signs of irritation (erythema, oedema, itching and pain) related to an inflammatory process.

Specificity: The proportion of all negative/inactive test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (10).

Substance: means chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

Test chemical: means what is being tested

Tiered testing strategy: Testing which uses test methods in a sequential manner; the test methods selected in each succeeding level are determined by the results in the previous level of testing (10).

ANNEX 2

TEST METHODS INCLUDED IN THIS TG

Nr.	Test method name	Validation study type	References
1	**EpiSkin™**	Full prospective validation study (2003-2007). The test method components of this method were used to define the essential test method components of the original and updated ECVAM PS (8) (9) (22)*. Moreover, the method's data relating to identification of non-classified vs classified substances formed the main basis for defining the specificity and sensitivity values of the original PS*.	(2) (8) (9) (11) (12) (15) (16) (17) (18) (19) (20) (21) (22) (24) (27)
2	**EpiDerm™ SIT (EPI-200)**	EpiDerm™ (*original*): Initially the test method underwent full prospective validation together with Nr. 1. from 2003-2007. The test method components of this method were used to define the essential test methods components of the original and updated ECVAM PS (8) (9) (22)*. **EpiDerm™ SIT (EPI-200):** A modification of the original EpiDerm ™ was validated using the original ECVAM PS (22) in 2008*	(2) (8) (9) (11) (13) (14) (16) (17) (18) (19) (21) (22) (24) (28) (2) (22) (23) (24) (28)
3	**SkinEthic™ RHE**	Validation study based on the original ECVAM Performance Standards (22) in 2008*.	(2) (22) (23) (24) (29)
4	**LabCyte EPI-MODEL24 SIT**	Validation study (2011-2012) based on the Performance Standards (PS) of OECD TG 439 which are based on the updated ECVAM PS* (8) (9).	(8) (9) (35) (36) (37) (38) (39) (40) and PS of this TG*

*) The original ECVAM Performance Standards (PS) (22) were developed in 2007 upon completion of the prospective validation study (17) which had assessed the performance of test methods Nr 1 and 2 in reference to the classification system as described in the 28th amendment to the EU Dangerous Substances Directive (31). In 2008 the UN GHS was introduced (1) (3), effectively shifting the cut-off value for distinguishing non-classified from classified substances from an *in vivo* score of 2.0 to 2.3. To adapt to this changed regulatory requirement, the accuracy values and reference chemical list of the ECVAM PS were updated in 2009 (2) (8) (9). As the original PS, also the updated PS were largely based data from methods Nr. 1 and 2 (17), but additionally used data on reference chemicals from method Nr. 3. In 2010, the updated ECVAM PS were used for stipulating the PS as presented in this TG (Annex 4). As methods Nos. 1, 2 and 3 [i.e. EpiSkin™, EpiDerm™ SIT (EPI-200) and SkinEthic™ RHE] have served to define this TG including the PS, they are considered as Validated Reference Methods (VRM) (Annex 4). Detailed information on the validation studies, a compilation of the data generated as well as background to the

necessary adaptations of the PS as a consequence of the UN GHS implementation can be found in the ECVAM/BfR explanatory background document to this OECD TG (24).

SIT: Skin Irritation Test
RHE: Reconstructed Human Epidermis

ANNEX 3

PROTOCOL PARAMETERS SPECIFIC TO EACH OF THE TEST METHODS INCLUDED IN THIS TG

The RhE methods do show very similar protocols and notably all use a post-incubation period of 42 hours (27) (28) (29). Variations concern mainly three parameters relating to the different barrier functions of the test methods and listed here: A) pre-incubation time and volume, B) Application of test chemicals and C) Post-incubation volume.

	EpiSkin™ (SM)	**EpiDerm™ SIT (EPI-200)**	**SkinEthic RHE™**	**LabCyte EPI-MODEL24 SIT**
A) Pre-incubation				
Incubation time	18- 24 hours	18-24 hours	< 2 hours	15-30 hours
Medium volume	2mL	0.9mL	0.3mL	0.5mL
B) Chemical application				
For liquids	10µL (26µL/cm^2)	30µL (47µL/cm^2)	16µL (32µL/cm^2)	25µL (83µL/cm^2)
For solids	10mg (26mg/cm^2) + DW (5µL)	25mg (39mg/cm^2) + DPBS (25µL)	16mg (32mg/cm^2) + DW (10µL)	25mg (83mg/cm^2) + DW (25µL)
Use of nylon mesh	Not used	If necessary	Applied	Not used
Total application time	15 minutes	60 minutes	42 minutes	15 minutes
Application temperature	RT	a) at RT for 25 minutes b) at 37ºC for 35 minutes	RT	RT
C) Post-incubation volume				
Medium volume	2 mL	0.9mL x 2	2 mL	1 mL
D) Maximum acceptable variability				
Standard deviation between tissue replicates	SD≤18	SD≤18	SD≤18	SD≤18

RT: Room temperature
DW: distilled water
DPBS: Dulbecco's Phosphate Buffer Saline

ANNEX 4

PERFORMANCE STANDARDS FOR ASSESSMENT OF PROPOSED SIMILAR OR MODIFIED *IN VITRO* RECONSTRUCTED HUMAN *EPIDERMIS* (RhE) TEST METHODS FOR SKIN IRRITATION

(Intended for the developers of new or modified similar test methods)

1. Generally, the purpose of Performance Standards (PS) is to communicate the basis on which new test methods, both proprietary (*i.e.* copyrighted, trademarked, registered) and non-proprietary can be determined to have sufficient accuracy and reliability for specific testing purposes. The following PS were defined on the basis of three validated and accepted reference methods using RhE; the PS can be used to evaluate the reliability and accuracy of other analogous test methods (colloquially referred to as "me-too" tests) that are based on similar scientific principles and measure or predict the same biological or toxic effect (10).

2. Prior to adoption of modified test methods, *i.e.* proposed potential improvements to an approved test method, there should be an evaluation to determine the effect of the proposed changes on the test performance and the extent to which such changes affect the information available for the other components of the validation process. Depending on the number and nature of the proposed changes, the data generated and the supporting documentation for those changes, they should either be subjected to the same validation process as described for a new test, or, if appropriate, to a limited assessment of reliability and relevance using established PS (10).

3. Methods considered similar (me-too) to the Validated Reference Methods (VRM, see Annex 2) used to define the present Performance Standards or modifications of validated RhE methods should be evaluated prior to their inclusion in the Test Guideline to determine their reliability and accuracy using chemicals representing the full range of the Draize irritancy scores. When evaluated using the 20 recommended Reference Chemicals of the PS (Table 1), the proposed similar or modified test methods should have reliability and accuracy values which are comparable or better than those derived from the VRM (Table 2 of this Annex) (2) (17). The reliability and accuracy values that should be achieved are provided in paragraphs 8 to 12 of this Annex. Non-classified chemicals (UN GHS No Category) and classified chemicals (UN GHS Category 2) (1), representing different chemical classes are included. The reliability of the test method, as well as its ability to correctly identify UN GHS Category 2 irritant chemicals and, depending on the regulatory framework in member countries, also its ability to correctly identify UN GHS No Category chemicals (for member countries that do not adopt optional UN GHS Category 3), should be determined prior to its use for testing new test chemicals.

4. These PS are based on the EC-ECVAM PS (8), updated according to the UN GHS systems on classification and labelling (1) (2) (9). The original PS (22) were defined upon completion of the validation study (17) and were based on the EU classification system as described in the 28th amendment to the Dangerous Substances Directive (31). Due to the adoption of the UN GHS system for classification and labelling in EU (EU CLP) (3), which took place between the finalisation of the validation study and the completion of this Test Guideline, the PS have been updated (8) (9). This update concerned: *i*) the composition of the PS Reference Chemicals and *ii*) the defined reliability and accuracy values (2) (9) (24).

5. The PS comprises the following three elements (10):

I) Essential Test Method Components
II) Minimum List of Reference Chemicals
III) Defined Reliability and Accuracy Values

I) Essential Test Method Components

6. These consist of essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed, mechanistically and functionally similar or modified test method. These components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to essential test method components will help to assure that a similar or modified proposed test method is based on the same concepts as the validated test methods used to define the PS (10). The essential test method components are described in detail in paragraphs 16 to 21 of the Test Guideline:

The general conditions (paragraph 16)
The functional conditions, which include:
- viability (paragraph 17);
- barrier function (paragraph 18);
- morphology (paragraph 19);
- reproducibility (paragraph 20); and,
- quality control (paragraph 21)

For specific parameters (e.g. for Tables 2 and 3), adequate values should be provided for any new similar or modified test method; these specific values may vary depending on the specific test method.

II) Minimum List of Reference Chemicals

7. Reference Chemicals are used to determine if the performance (reliability and accuracy) of a proposed similar or modified test method is comparable or better than that of the VRM (2) (8) (9) (17) (24). An evaluation on the basis of these reference chemicals can be performed only for methods proven to be structurally and functionally sufficiently similar in reference to element I) of the PS, or representing a minor modification of one of the validated test methods used to define the present PS. The 20 recommended Reference Chemicals listed in Table 1 of this Annex include chemicals representing different chemical classes (*i.e.* chemical categories based on functional groups), and are representative of the full range of Draize irritancy scores (from non-irritant to strong irritant). The chemicals included in this list comprise 10 UN GHS Category 2 chemicals and 10 non-categorised chemicals, of which 3 are optional UN GHS Category 3 chemicals. Under this Test Guideline, the optional Category 3 is considered as No Category. The chemicals listed in Table 1 are selected on the basis of data from the VRM and relate to chemicals used for the prospective validation study (17) as well as chemicals used in the optimisation phases following Pre-validation. Due regard has been given to chemical functionality and physical state when composing this list (15) (19). The Reference Chemicals represent the minimum number of chemicals that should be used to evaluate the accuracy and reliability of a proposed similar or modified test method, but should not be used for the development of new test methods. In situations where a listed chemical is unavailable, other chemicals for which adequate *in vivo* reference data are available could be used, primarily from the chemicals used in the optimisation phase following pre-validation or the validation study of the VRM. If desired, additional chemicals representing other chemical classes and for which adequate *in vivo* reference data are available may be added to the minimum list of Reference Chemicals to further evaluate the accuracy of the proposed test method.

Table 1: Minimum List of Reference Chemicals for Determination of Accuracy and Reliability Values for Similar or Modified RhE Skin Irritation Test Methods[1]

Chemical	CAS Number	Physical state	*In vivo* score	VRM* Cat. based on *in vitro*	UN GHS Cat. based on *in vivo* results
NON-CLASSIFIED CHEMICALS					
1-bromo-4-chlorobutane	6940-78-9	Liquid	0	Cat. 2	No Cat.
diethyl phthalate	84-66-2	Liquid	0	No Cat.	No Cat.
naphthalene acetic acid	86-87-3	Solid	0	No Cat.	No Cat.
allyl phenoxy-acetate	7493-74-5	Liquid	0.3	No Cat.	No Cat.
isopropanol	67-63-0	Liquid	0.3	No Cat.	No Cat.
4-methyl-thio-benzaldehyde	3446-89-7	Liquid	1	Cat. 2	No Cat.
methyl stearate	112-61-8	Solid	1	No Cat.	No Cat.
heptyl butyrate	5870-93-9	Liquid	1.7	No Cat.	No Cat. (*Optional Cat. 3*)
hexyl salicylate	6259-76-3	Liquid	2	No Cat.	No Cat. (*Optional Cat. 3*)
cinnamaldehyde	104-55-2	Liquid	2	Cat. 2	No Cat. (*Optional Cat. 3*)
CLASSIFIED CHEMICALS					
1-decanol[2]	*112-30-1*	*Liquid*	*2.3*	*Cat. 2*	*Cat. 2*
cyclamen aldehyde	103-95-7	Liquid	2.3	Cat. 2	Cat. 2
1-bromohexane	111-25-1	Liquid	2.7	Cat. 2	Cat. 2
2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	86604-75-3	Solid	2.7	Cat. 2	Cat. 2
di-n-propyl disulphide[2]	*629-19-6*	*Liquid*	*3*	*No Cat.*	*Cat. 2*
potassium hydroxide (5% aq.)	1310-58-3	Liquid	3	Cat. 2	Cat. 2
benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	7340-90-1	Liquid	3.3	Cat. 2	Cat. 2
1-methyl-3-phenyl-1-piperazine	5271-27-2	Solid	3.3	Cat. 2	Cat. 2
heptanal	111-71-7	Liquid	3.4	Cat. 2	Cat. 2
tetrachloroethylene	127-18-4	Liquid	4	Cat. 2	Cat. 2

*) VRM = validated reference methods (Annex 2)

[1] The chemical selection is based on the following criteria; (i), the chemicals are commercially available; (ii), they are representative of the full range of Draize irritancy scores (from non-irritant to strong irritant); (iii), they have a well-defined chemical structure; (iv), they are representative of the chemical functionality used in the validation process; and (v), they are not associated with an extremely toxic profile (*e.g.* carcinogenic or toxic to the reproductive system) and they are not associated with prohibitive disposal costs.

[2] Chemicals that are irritant in the rabbit but for which there is reliable evidence that they are non-irritant in humans (32) (33) (34).

III) Defined Reliability and Accuracy Values

8. For purposes of establishing the reliability and relevance of proposed similar or modified test methods to be transferred between laboratories, all 20 Reference Chemicals in Table 1 should be tested in at least three laboratories. However, if the proposed test method is to be used in a single laboratory only, multi-laboratory testing will not be required for validation. It is however essential that such validation studies are independently assessed by internationally recognised validation bodies, in agreement with international guidelines (10). In each laboratory, all 20 Reference Chemicals should be tested in three independent runs performed with different tissue batches and at sufficiently spaced time points. Each run should consist of a minimum of three concurrently tested tissue replicates for each included test chemical, NC and PC.

9. The calculation of the reliability and accuracy values of the proposed test method should be done considering all four criteria below together, ensuring that the values for reliability and relevance are calculated in a predefined and consistent manner:

1. Only the data of runs from complete run sequences qualify for the calculation of the test method within, and between-laboratory variability and predictive capacity (accuracy).
2. The final classification for each Reference Chemicals in each participating laboratory should be obtained by using the mean value of viability over the different runs of a complete run sequence.
3. Only the data obtained for chemicals that have complete run sequences in all participating laboratories qualify for the calculation of the test method between-laboratory variability.
4. The calculation of the accuracy values should be done on the basis of the individual laboratory predictions obtained for the 20 Reference Chemicals by the different participating laboratories.

In this context, a **run sequence** consists of three independent runs from one laboratory for one test chemical. A **complete run sequence** is a run sequence from one laboratory for one test chemical where all three runs are valid. This means that any single invalid run invalidates an entire run sequence of three runs.

Within-laboratory reproducibility

10. An assessment of within-laboratory reproducibility should show a concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of the 20 Reference Chemicals within one single laboratory equal or higher (≥) than 90%.

Between-laboratory reproducibility

11. An assessment of between-laboratory reproducibility is not essential if the proposed test method is to be used in a single laboratory only. For methods to be transferred between laboratories, the concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of the 20 Reference Chemicals between preferentially a minimum of three laboratories should be equal or higher (≥) than 80%.

Predictive capacity

12. The predictive capacity (sensitivity, specificity and accuracy) of the proposed similar or modified test method should be comparable or better to that of the VRM, taking into consideration additional information relating to relevance in the species of interest (Table 2 of this Annex). The sensitivity should be equal or higher (≥) than 80% (2) (8) (9) (24). However, a further specific restriction applies to the

sensitivity of the proposed *in vitro* test method in as much as only two *in vivo* Category 2 reference chemicals, *1-decanol* and *di-n-propyl disulphide,* may be misclassified as No Category by more than one participating laboratory. The specificity should be equal or higher (≥) than 70% (2) (8) (9) (24). There is no further restriction with regard to the specificity of the proposed *in vitro* test method, *i.e.* any participating laboratory may misclassify any *in vivo* No Category chemical as long as the final specificity of the test method is within the acceptable range. The accuracy should be equal or higher (≥) than 75% (2) (8) (9) (24). Although the sensitivity of the VRM calculated for the 20 Reference Chemicals listed in Table 1 is equal to 90%, the defined minimum sensitivity value required for any similar or modified test method to be considered valid is set at 80% since both *1-decanol* (a borderline chemical) and *di-n-propyl disulphide* (a false negative of the VRM) are known to be non-irritant in humans (32) (33) (34), although being identified as irritants in the rabbit test. Since RhE models are based on cells of human origin, they may predict these chemicals as non-irritant (UN GHS No Category).

Table 2: Required predictive values for sensitivity, specificity and accuracy for any similar or modified test method to be considered valid

Sensitivity	Specificity	Accuracy
≥ 80%	≥ 70%	≥ 75%

Study Acceptance Criteria

13. It is possible that one or several tests pertaining to one or more test chemicals does/do not meet the test acceptance criteria for the test and control chemicals or is/are not acceptable for other reasons. To complement missing data, for each test chemical a maximum number of two additional runs are admissible ("retesting"). More precisely, since in case of retesting also PC and NC have to be concurrently tested, a maximum number of two additional runs may be conducted for each test chemical.

14. It is conceivable that even after retesting, the minimum number of three valid runs required for each tested chemical is not obtained for every Reference Chemical in every participating laboratory, leading to an incomplete data matrix. In such cases the following three criteria should all be met in order to consider the datasets acceptable:

1. All 20 Reference Chemicals should have at least one complete run sequence;

2. In each of at least three participating laboratories, a minimum of 85% of the run sequences need to be complete (for 20 chemicals; *i.e.* 3 invalid run sequences are allowed in a single laboratory);

3. A minimum of 90% of all possible run sequences from at least three laboratories need to be complete (for 20 chemicals tested in 3 laboratories; *i.e.* 6 invalid run sequences are allowed in total).

-JSAAE Skin Irritation Test Validation Study-

SKIN IRRITATION TEST USING THE RECONSTRUCTED HUMAN MODEL "LABCYTE EPI-MODEL 24"

Ver. 8.3

LabCyte EPI-MODEL24 SKIN IRRITATION TEST-42 HOURS

S.O.P.

TABLE OF CONTENTS

1. RATIONAL AND BACKGROUND

1.1 SKIN IRRITATION TEST using LabCyte EPI-MODEL 24 (SIT using LabCyte EPI-MODEL 24)

The SIT using LabCyte EPI-MODEL24 is designed for the prediction of acute skin irritation of chemicals by measurement of its cytotoxic effect, as reflected in the MTT assay, on the Reconstructed Human Epidermis (RHE) model. The SIT using LabCyte EPI-MODEL24 is not a kit; LabCyte EPI-MODEL24 tissues are commercially available per tissues item (with a minimum of 24 LabCyte EPI-MODEL24 tissues per order).

1.2 BACKGROUND OF SIT using LabCyte EPI-MODEL24

Performance standards for applying human skin models to in vitro skin irritation testing were also defined based on the validated test EpiSkin™ test method (ECVAM SIVS, 2007). These performance standards can be then used to evaluate the accuracy and reliability of other analogous test methods (also referred to as “me-too” tests) that are based on similar scientific principles and measure or predict the same biological or toxic effect.

Based on the GHS-EU classification, 12 irritants and 13 non-irritants in the draft performance standards (ECVAM 2007) and the statement by ESAC (ESAC2009) were performed the validation study through the 7 labs SIT using LabCyte EPI-MODEL24. Results were summarized at JSAAE 1st report and 2nd report on this validation study.

1.3 BASIS OF THE METHOD

Chemical-induced skin irritation, manifested by erythema and oedema, is the results of a cascade of events beginning with penetration of the stratum corneum and damage to the underlying layers of keratinocytes. The dying keratinocytes release mediators that begin the inflammatory cascade which acts on the cells in the dermis, particularly the stromal and endothelial cells. It is the dilation and increased permeability of the endothelial cells that produce the observed erythema and oedema. The RhE-based test methods measure the initiating events in the cascade.

The relative viability of the treated tissues was measured at the end of the treatment exposure (15 minutes) followed by a post-exposure period (42 hours) using MTT [(3-4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazoliumbromide] assay. A cutoff value of 50% viability of the negative control value was considered and used to classify test substances as irritant (I) or non irritant (NI). The culture environment might allow the detection of very small quantities of cytokines secreted by the epidermis in response to topical application of test substances.

1.3.1 TEST SYSTEM DESCRIPTION

LabCyte EPI-MODEL24 is a new, commercially available RhE model produced by Japan Tissue Engineering Co. Ltd. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand human keratinocytes while maintaining their phenotype, they were cultured with 3T3-J2 cells as a feeder layer (Rheinwald and Green, 1975; Green, 1978).

Reconstruction of human cultured epidermis is achieved by cultivating and proliferating keratinocytes on an inert filter substrate (surface 0.3 cm_2) at the air-liquid interface for 13 days with an optimized medium containing 5% fetal bovine serum. It constructs a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum. LabCyte EPI-MODEL24 is embedded in an agarose gel containing nutrient solution and shipped in 24-well plates at around 18°C.

1.3.1.1 Quality control of the test system

The LabCyte EPI-MODEL24 is manufactured according to defined quality assurance procedures. Each batch production was provided with quality controls such as storage conditions, RHE instructions for use, lot number and origin, histology (demonstration of human epidermis-like structure with multilayered stratum corneum), cell viability, barrier function integrity ($0.14 \leq IC50 \leq 0.4$).

1.3.1.2 Precautions

The epidermal cells are taken from healthy donor negative to HIV, and Hepatitis. Nevertheless, handling procedures for biological materials should be followed:

a) It is recommended to wear gloves during handling with the skin and kit components.
b) After use, the epidermis, the material and all media in contact with it should be decontaminated prior to disposal (e.g. using special containers or autoclaving).

1.3.2 ASSAY QUALITY CONTROL

1.3.2.1 Assay Acceptance Criterion 1: Negative Control

The absolute OD of the negative control (NC) tissues (treated with sterile DPBS) in the MTT assay is an indicator of tissue viability obtained in the testing laboratory after shipping and storing procedures and under specific conditions of use.

$0.7 \leq$ Mean OD (A570/650) measured value ≤ 2.5

1.3.2.2 Assay Acceptance Criterion 2: Positive Control

A 5% SDS (in H_2O) solution (see 7.6.3) is used as positive control (PC) and tested concurrently with the test chemicals. Concurrent means here the PC has to be tested in each assay, but not more than one PC is required per testing day. Viability of positive control should be within 95±1 % confidence interval of the historical data.

Mean tissue viability ≤ 40%

1.3.2.3 Assay Acceptance Criterion 3: Standard Deviation (SD)

Since in each test skin irritancy potential is predicted from the mean viability determined on 3 single tissues, the variability of tissue replicates should be acceptably low.

Standard Deviation (SD) of tissue viability of 3 identically treated replicates for negative control and positive control ≤ 18 %

1.4 LIMITATION OF THE METHOD

One limitation of this assay method is a possible interference of the test substance with the MTT endpoint. A colored test substance or one that directly reduces MTT (and thereby mimics dehydrogenase activity of the cellular mitochondria) may interfere with the MTT endpoint. However, these test substance are a problem only if at the time of the MTT test (i.e. 42 hours after test substance exposure) sufficient amounts of the test substance are still present on (or in) the tissues. In case of this unlikely event, the (true) metabolic MTT reduction and the contribution by a colored test material or (false) direct MTT reduction by the test material can be quantified by a procedure described in Section 3.2.

The method is not designed for testing of highly volatile test substances, gases and aerosols.

1.5 BRIEF BASIC PROCEDURE

On the day of receipt, LabCyte EPI-MODEL24 tissues are conditioned by incubation to release transportstress related compounds and debris overnight. After pre-incubation, tissues are topically exposed to the test chemicals for 15 minutes. Preferably, three tissues are used per test chemical (TC) and for the positive control (PC) and negative control (NC). Tissues are then thoroughly rinsed, blotted to remove the test substances, and transferred to fresh medium. After 42 hr incubation period, the MTT assay is performed by transferring the tissues to the well containing MTT medium (0.5 mg/ml). After 3 hr MTT incubation, the blue formazan salt formed by cellular mitochondria is extracted with 0.3 mL/tissue of isopropanol and the optical density of the extracted formazan is determined using a spectrophotometer at 570 nm and 650 nm as reference. Relative cell viability is calculated for each tissue as % of the mean of the negative control tissues. Skin irritation potential of the test material is predicted if the remaining relative cell viability is below 50%.

1.6 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)

According to the GHS classification (Category 2 or no label), an irritant is predicted if the mean relative tissue viability of three individual tissues exposed to the test substance is reduced below 50% of the mean viability of the negative controls.

In vitro results	In vivo prediction
Tissue viability is ≤ 50%	Irritant
Tissue viability is > 50%	Non Irritant

2. MATERIALS

2.1 LabCyte EPI-MODEL 24

2.1.1 LabCyte EPI-MODEL 24 KIT COMPONENTS

LabCyte EPI-MODEL 24 kit components are shown in Table 1.

Table 1 - LabCyte EPI-MODEL24 Kit Components

Component	Qty	Description
LabCyte EPI-MODEL 24 plate	1 plate	Contains 24 culture inserts with tissues fixed in nutritive agar medium for transport (usable area: 0.3cm^2).
Assay Medium	1 bottle	Basic medium for incubation (30mL). Store at refrigeration temperature.
24-well plate	1 plate	Blank plate for use in assay. Store at room-temperature.

2.1.2 SHIPMENT OF LabCyte EPI-MODEL24

LabCyte EPI-MODEL 24 is packed in a special container (Icompo/NIPPON EXPRESS CO., LTD) and delivered by NIPPON EXPRESS CO., LTD. After the Icompo is delivered, examine the contents and make sure that all kit components (LabCyte EPI-MODEL24 plate, assay medium, and 24-well assay plate) are included in the package. Confirm lot numbers and expiration dates also. Record details in the Methods Documentation Sheet (MDS) 1.

NIPPON EXPRESS will pick up the Icompo at a later date (generally, the day after the date of delivery), and we ask that you return it with a slip documenting receipt, as well as the insulating materials.

2.1.3 INSTRUCTIONS FOR USE OF LabCyte EPI-MODEL 24

Begin incubating all of the culture inserts after opening the package. Do not store the culture inserts again after opening.

The human epidermis cells used in LabCyte EPI-MODEL 24 originate from a normal donor and are HIV-, HBV-, HCV-, and HPV-negative. However, handle them with enough care and in accordance with the laboratory biosafety guidelines since they contain raw materials of human origin.

2.2 TEST CHEMICALS

Coded test chemicals are delivered to each laboratory.

2.3 CONSUMABLES

The following consumables are required.

* The described quantities are necessary so that 1 to 6 samples can be assayed once.

- Assay Medium, 100mL (J-TEC: 402250) 1 bottle
- MTT, 25mg (J-TEC: 403026) 1 bottle
- Wide orifice cell saver tips for micro-pipettes (sterile) 96 tips 1 box
- 24-well assay plate (Becton,Dickinson and Company: 353047) 7 plates
- 96-well plate (Becton,Dickinson and Company: 353072) 1 plates
- Phosphate buffered saline (PBS) 500mL (Invitrogen: 14190-144) 2 bottles
- Isopropanol 500mL (Wako Pure Chemical Industries: 164-08335) 1 bottle
- SLS 25g (SIGMA:L4390) 1 bottle
- Sterile distilled water 20mL (Otsuka Pharmaceutical: 36A1X00001) 1 bottles
- Sterile cotton buds (JAPAN COTTON BUDS: 10A754D) 1 box

2.4 OTHERS

2.4.1 EQUIPMENT / INSTRUMENTS

- Safety cabinet (or clean bench)
- Water bath (37 °C)
- CO_2 incubator (37 °C, 5%CO_2, capable of maintaining high humidity)
- Autoclave
- 96-well multi-plate reader (required filters: 450nm, 570nm, 650nm)
- Precision balance (0.1mg)
- Aspirator
- Stop-watches
- Adjustable micro-pipette (10-200µL, 200-1000µL)
- Sharp-edged forceps (sterile)
- Micro spatula (sterile)
- Beaker (1~2L: sterile)
- Sterilizable poly wash bottle (500~1000mL: sterile)

2.4.2 CONSUMMABLE ITEMS

- Micro-pipette tips (sterile: 10~200µL, 200~1000µL)
- Microtubes (1.5mL)
- Scalpel (KEISEI MEDICAL INDUSTRIAL: Keisei Scalpel 11A)

3. TEST METHOD

*Perform operations in Section 3.1.1~3.1.4 and Section 3.3.1~3.3.2 aseptically in a safety cabinet (or clean bench).

*Operations other than above do not need to be performed with an aseptic technique. For these operations, refer to **Section 2.1.3 INSTRUCTIONS FOR USE OF LabCyte EPI-MODEL 24**

3.1 PREPARATIONS

3.1.1 MTT SOLUTION

(1) Dissolve MTT in the assay medium to prepare the MTT medium (final concentration: 0.5mg/mL)
Use ultrasonic cleaning equipment or a vortex mixer as necessary in order to completely dissolve the MTT.
*Store in a dark, cold place and use it within 24 hours.

(2) Record details of step (1) above in the MDS 4.

3.1.2 POSITIVE CONTROL SUBSTANCE

(1) Weigh 500mg of SLS precisely.

(2) To prepare a positive control solution, put the SLS into a graduated cylinder or measuring flask and dilute to 10mL with distilled water (final concentration: 5% w/v)]
* Store in a dark, cold place and use it within 24 hours.

(3) Record details of steps (1) and (2) above in the MDS 3.

3.1.3 NEGATIVE CONTROL SUBSTANCE

(1) Use distilled water.

3.1.4 POLY WASH BOTTLE FOR PBS

(1) Sterilize poly wash bottle using an autoclave.

(2) Fill the sterilized poly wash bottle aseptically with sterile PBS.

3.2 TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH MTT ENDPOINT

There are two kinds of test chemicals that interfere with the MTT assay as follows.

(a) Chemical that stains epidermis tissues.

(b) Chemical that is able to directly reduce MTT.

Test chemical that stains the epidermis tissues has a possibility to transfer from the epidermis tissues to the extraction solution and to affect the optical density (OD) measurements.

Test chemical that is able to directly reduce MTT can affect the optical density (OD) measurements, if the test chemical is present in the epidermis tissues when the MTT viability test is performed. Detection procedure of these test chemicals is described below.

3.2.1 DETECTION OF THE CHEMICALS THAT STAIN THE TISSUE

3.2.1.1 STEP1 (PRELIMINARY TEST)

(1) Add 25μL (Liquid) or 25mg (Solid) of the test chemical into wells of 24-well assay plate preliminarily filled with 0.5mL of distilled water. Untreated distilled water is used as control.

(2) Close the lid of 24-well assay plate and incubate the mixture in CO_2 incubator for 15 minutes.

(3) After incubation, shake the mixture gently and evaluate the staining of the distilled water macroscopically.

(4) When the color of the solution changes significantly, the test chemical is presumed to have the potential to stain the tissue and a functional check on viable tissues (Step2) should be performed. When the color of the solution does not change significantly, the test chemical is determined not to have a potential to stain the tissue.

3.2.1.2 STEP2 (FUNCTIONAL CHECK ON VIABLE TISSUE)

(1) Add 25μL (Liquid) or 25mg (Solid) of the test chemical, which clearly changed the color of the distilled water (Step1), onto the surface of the epidermis tissues. Distilled water is used as negative control.

(2) Follow all procedures described in this SOP Section 3.3 EXECUTION OF THE TEST. However, incubate the tissue for 3 hours in culture media without MTT instead of incubating in media containing MTT to evaluate the staining of the epidermis tissues.

(3) Calculate ratio of staining by test chemical from the following formula.

$$\text{Ratio of staining by test chemical (\%)} = \frac{OD_{\text{test chemical}} - OD_{\text{negative control}}}{OD_{\text{negative control}}} \times 100$$

(4) When the ratio of staining by test chemical is <5%, correction of the results is not necessary.

When the ratio is between 5% and 30%, the corrected MTT OD is calculated using the following formula.test chemical

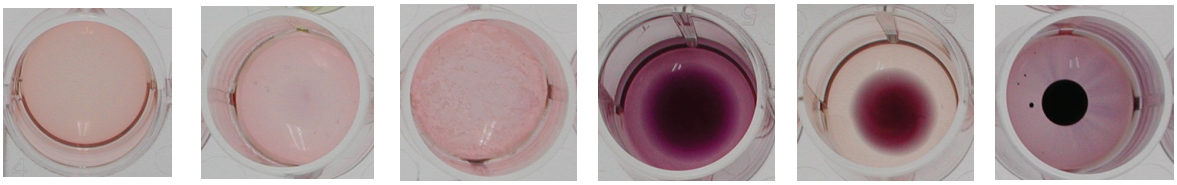
Corrected MTT OD = OD stained tissue (MTT assay)-OD stained tissue (no MTT assay)

When the ratio of staining by test chemical is >30%, the test chemical must be considered incompatible with the test. However, when the Cell viability (%), which is calculated according to the procedures described in this SOP Section 3.3.5.2, is <50%, the test chemical is determined as irritant. Therefore correction of the results or determination of incompatibility of the test chemical is not necessary.

3.2.2 DETECTION OF CHEMICALS THAT DIRECTLY REDUCE MTT

3.2.2.1 STEP3 (PRELIMINAY TEST)

(1) Add 25μL (Liquid) or 25mg (Solid) of the test chemical into wells of 24-well assay plate preliminarily filled with 0.5mL of MTT medium. Untreated MTT medium is used as control.
(2) Close the lid of 24-well assay plate and incubate the mixture in CO_2 incubator for 1 hour.
(3) After incubation, shake the mixture gently and evaluate the staining of the MTT medium macroscopically.
(4) When the MTT medium turns blue/purple significantly, the test chemical can reduce MTT and additional functional check (Step4) must be performed.



5% SLS | 1-bromo hexane | 1,1,1-tetrachloroethane | 5-(1,1-dimethylethyl)-2-methyl | cinnamaldehyde | eugenol

Photo 1 – Example of test for direct MTT reduction ability (STEP 3). Test substances 5-(1,1-dimethylethyl)-2-methyl, cinnamaldehyde and eugenol have directly reduced MTT. In these cases, Step 4 must be performed.

3.2.2.2 STEP4 (FUNCTIONAL CHECK ON VIABLE TISSUE)

(1) Add 25μL (Liquid) or 25mg (Solid) of the test chemical, which clearly changed the color of the MTT medium into blue/purple (Step3), onto the surface of the epidermis tissues.

Distilled water is used as negative control.

(2) Follow all procedures described in this SOP Section 3.3 EXECUTION OF THE TEST. However, use the epidermis tissues that has been freeze-killed at -20 °C or lower for more than 24 hours instead of viable epidermis tissues.

(3) Calculate ratio of staining by test chemical from the following formula.

$$\text{Ratio of staining by test chemical (\%)} = \frac{OD_{\text{test chemical}} - OD_{\text{negative control}}}{OD_{\text{negative control}}} \times 100$$

(4) When the ratio of staining by test chemical is <30%, correct OD data using the following formula.

$$\text{Corrected OD} = OD(\text{viable tissue})_{\text{test chemical}} - [OD(\text{freeze-killed tissue})_{\text{test chemical}} - OD(\text{freeze-killed tissue})_{\text{negative control}}]$$

When the ratio of staining by test chemical is >30%, the test chemical must be considered incompatible with the test. However, When the Cell viability (%), which is calculated according to the procedures described in this SOP Section 3.3.5.2, is <50%, the test chemical is determined as irritant. Therefore correction of the results or determination of incompatibility of the test chemical is not necessary.

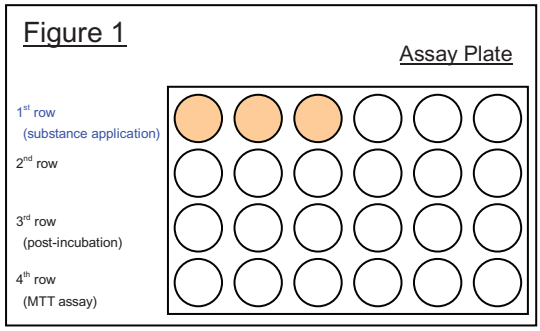
3.3 EXECUTION OF THE TEST

3.3.1 PREPARATION OF LabCyte EPI-MODEL 24 (DAY -1)

(1) Pre-warm the assay medium for 30 minutes to 37 °C using a water bath.

(2) Fill 3 wells of the 1st row of each 24-well assay plate with the pre-warmed assay medium (0.5mL/well).
→ Figure 1

Figure 1

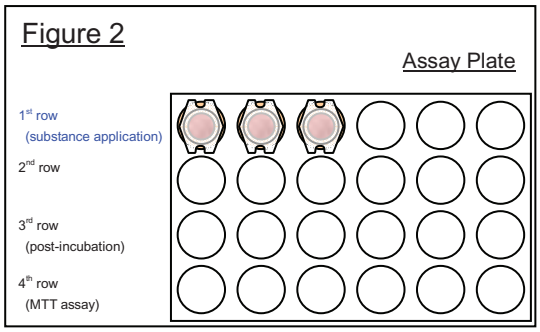


(3) Open the LabCyte EPI-MODEL 24 aluminum package.

(4) Open the LabCyte EPI-MODEL 24 plate lid and pick up the culture inserts using sterile forceps.
*Do not touch the epidermis surface of culture inserts.
*Use forceps to remove agar medium sticking to the outside of the culture inserts.

(5) Transfer the culture inserts into assay medium filled wells of the 1st row using sterile forceps.
→ Figure 2
*Avoid air bubble formation under the tissue inserts.

Figure 2



(6) Place the plate (lid on) in a CO_2 incubator.

(7) Incubate overnight (15~30 hours) until Section 3.3.2 "APPLICATION OF TEST CHEMICALS AND RINSING."

(8) Record details of steps (1) - (7) above in the MDS 2.

3.3.2 APPLICATION OF TEST CHEMICALS AND RINSING (DAY 0)

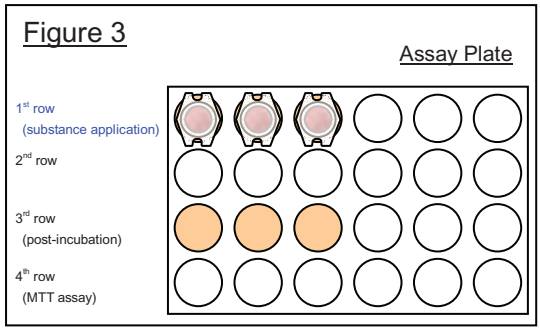
3.3.2.1 PREPARATION OF WELLS FOR POST-INCUBATION (3RD ROW)

(1) Pre-warm the assay medium for 30 minutes to 37 °C using a water bath.

(2) Remove the assay plate from the CO_2 incubator.

(3) Open the lid of the assay plate, and fill 3 wells of the 3rd row with the pre-warmed assay medium (1.0mL/well) using a micropipette.
→ Figure 3

Figure 3



(4) Place the plate (lid on) in a CO_2 incubator.

(5) Incubate until application of test chemicals (0~12 hours).

(6) Record details of steps (1) – (5) above in the MDS 3.

3.3.2.2 APPLICATION OF TEST CHEMICALS

(1) Remove the assay plate from the CO_2 incubator.

(2) Apply test chemicals onto the surface of epidermis tissues in the 1st row of the assay plate. Use 3 wells per test chemical (N=3).

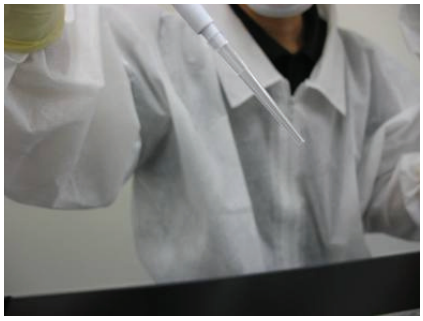
FOR LIQUIDs: Carefully apply 25µL of the test chemical onto the central part of each epidermis using a micropipette. After applied, close the lid of the assay plate and tap the side of the plate outside the safe cabinet (or clean bench) in order for the liquid to spread out over the entire epidermis surface. If necessary, use a micro spatula to coat the unapplied surface with liquids. Do not push the epidermis surface too hard with the spatula.

*Use wide orifice cell saver tips for viscous liquids.

→ Photo 2

Use a pipette, etc. to familiarize yourself with the nature of the test chemicals in advance.

Photo 2 - Pipette tips for viscous liquids



FOR SOLIDs: Weigh out 25mg (±1mg) of the solid chemical with a precision balance in advance. Apply first 25µL of distilled water and then the weighed test chemical onto the epidermis surface. Use a micro spatula if necessary to gently spread the test chemical.

→ Photo 3

*One 24-well assay plate should be used to assay only one test chemical.

→ Figure 4

(1 samples x 3(n) = 3 (culture inserts))

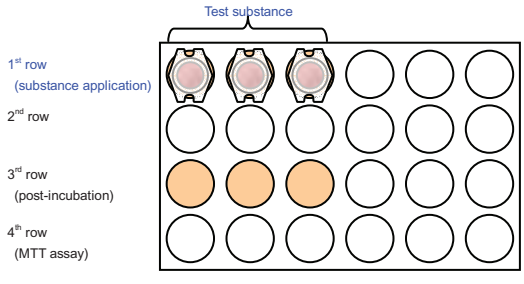
Photo 3 – Applying a solid substance

(3) Apply test chemical onto each well at 1~3-minute intervals.

(4) Incubate each well for 15 minutes in the cabinet (lid on between the intervals).

*Close the lid of the assay plate at all times except when applying samples. It might affect the amount of test sample if the lid is kept open, due to air circulation in the safe cabinet (or clean bench).

Figure 4 Assay Plate



(5) Record details of steps (1) - (4) above in the MDS 3.

3.3.2.3 REMOVAL OF THE TEST CHEMICALS

(1) 15 minutes (±30 seconds) after applying a chemical, open the assay plate and pick up a culture insert with sterile forceps. Discard test chemicals on the tissue by tilting and then tapping the insert on the beaker.

(2) Fill and overflow the culture insert with PBS using a PBS filled poly wash bottle. Hit the PBS stream from the washing bottle on the side-wall of the culture insert and wash on the tissue surface by the PBS current.

→ Photo 4

Attention: Must not to hit the PBS stream on the tissue surface directly. Be careful not to damage the tissue surface.

(3) Discard the PBS into a beaker by tilting the insert. If necessary, remove the PBS inside the culture insert by tapping it on the beaker only once.

→ Photo 5

(4) Repeat steps (2) and (3) at least 15 times or more as much as possible, and remove all residual test chemical on the tissue surface almost completely. Must not do discarding by tapping at only the last washing operation.

(5) Gently remove the leftover PBS outside the culture insert with a sterile cotton bud. But don't touch inside the culture insert by a cotton bud.

→ Photo 6

Attention: Even if residues of washing PBS remain on the tissue surface, don't do at all because it is not necessary to remove them.

(6) If test material remains on the epidermis surface, repeat steps (2) ~ (5) again.

(7) Transfer the blotted culture insert to a well in the 3rd row of the same column (for post-incubation).

→ Figure 5

Photo 4 - Rinse 1

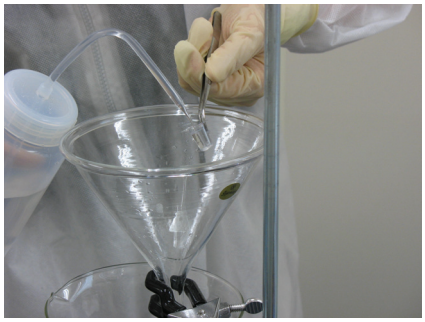


Photo 5 - Rinse 2

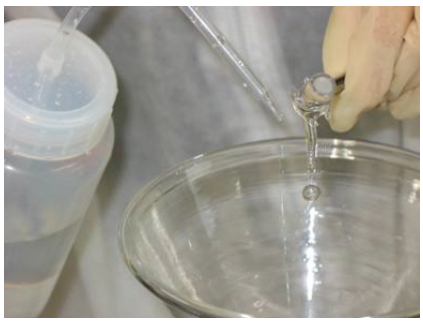


Photo 6 - Rinse 3

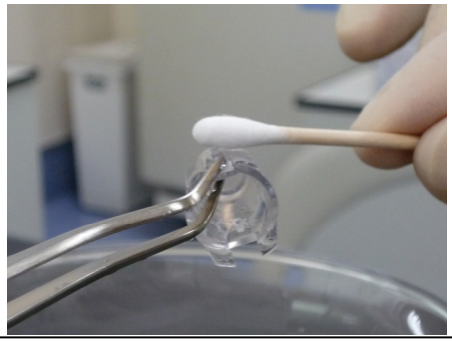
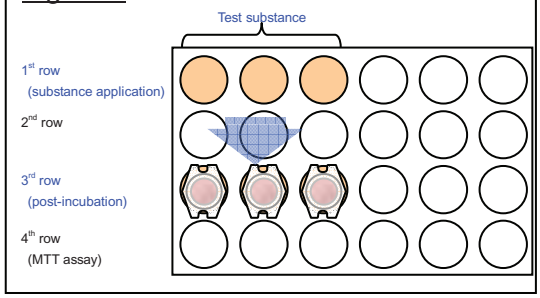


Figure 5 Assay Plate



*Avoid air bubble formation under the culture inserts.

(8) Repeat steps (1) ~ (7) for all the culture inserts at 1~3-minute intervals.

(9) Record details of steps (1) – (8) above in the MDS 3.

3.3.3 POST TREATMENT INCUBATION (DAY 0~2)

(1) Close the lid of the assay plate and place it in a CO_2 incubator.

(2) Incubate for 42 hours.

3.3.4 MTT ASSAY (DAY 2)

3.3.4.1 PREPARATION OF WELLS FOR MTT ASSAY

(1) Pre-warm MTT medium for 30 minutes to 37°C using a water bath.

(2) Remove the assay plate from the CO_2 incubator.

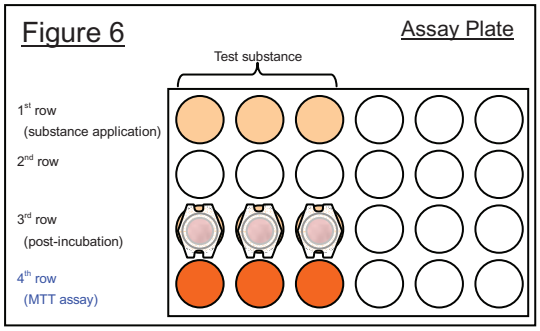
(3) Open the lid of the assay plate, and fill each well of the 4th row with the pre-warmed MTT medium (0.5mL/well) using a micropipette.
→ Figure 6

(4) Close the lid of the assay plate and place it in the CO_2 incubator.

(5) Incubate until starting MTT assay (about 0 ~ 12 hours).

(6) Record details of steps (1) – (5) above in the MDS 4.

Figure 6 Assay Plate



3.3.4.2 MTT ASSAY

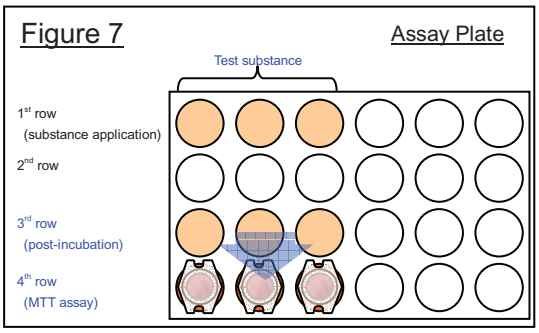
(1) Remove the assay plate from the CO_2 incubator after 42 hours (±1 hour) of post-incubation.

(2) Transfer each culture insert from the 3rd row to the 4th row of the corresponding column.
→ Figure 7
*Avoid dripping from the base end surface of the culture insert into other wells.
*Avoid air bubble formation under the culture inserts.

(3) Close the lid of the assay plate and place it in the CO_2 incubator.

(4) Incubate for 3 hours.

Figure 7 Assay Plate



(5) Record details of steps (1) – (4) above in the MDS 4.

3.3.5 FORMAZAN EXTRACTION AND MEASUREMENT (DAY 2~3)

3.3.5.1 FORMAZAN EXTRACTION

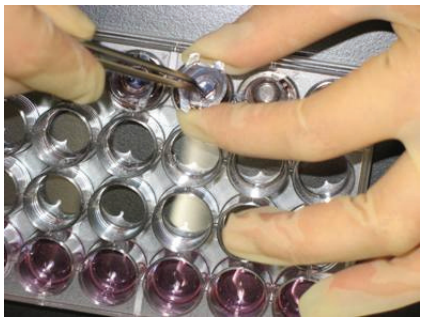
(1) Remove the assay plate(s) from the CO_2 incubator 3 hours (±5 minutes) after the MTT assay.

(2) Open the lid of the assay plate and pinch the cultured epidermis from each culture insert of the 4th row with forceps.

→ Photo 7

*Use a micro spatula to scratch up the epidermis or a scalpel to cut the membrane filter on the base of the culture insert if the cultured epidermis cannot be pinched due to damage from a test chemical.

Photo 7 - Detachment of epidermis



(3) Transfer the epidermis tissue into a 1.5mL micro tube.

(4) Add 300µL of isopropanol to the micro tubes and soak the entire epidermis tissue in the isopropanol.

(5) Incubate the micro tubes in a dark cold place (or refrigerator) overnight (more than 15 hours) in order to completely extract pigments.

*Tighten the micro tube seal.

*Periodically shaking the micro tubes will contribute to a more efficient extraction.

(6) Shake the micro tubes to mix the solution.

*If split epidermis tissues are suspended, wait until they sink or gently centrifuge them (if a centrifuge is available).

(7) Transfer 200µL of the solution in each micro tube into each well on a 96-well plate.

*One well of 200µL of isopropanol should be set as a blank.

*Figure 8 shows an example of allocation in a 96-well plate.

Figure 8 – Allocation for a 96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank											
B	DW-1	Sample 1-1	Sample 3-1	Sample 5-1	Sample 7-1	Sample 9-1	Sample 11-1	Sample 13-1	Sample 15-1	Sample 17-1	Sample 19-1	
C	DW-2	Sample 1-2	Sample 3-2	Sample 5-2	Sample 7-2	Sample 9-2	Sample 11-2	Sample 13-2	Sample 15-2	Sample 17-2	Sample 19-2	
D	DW-3	Sample 1-3	Sample 3-3	Sample 5-3	Sample 7-3	Sample 9-3	Sample 11-3	Sample 13-3	Sample 15-3	Sample 17-3	Sample 19-3	
E	5% SLS-1	Sample 2-1	Sample 4-1	Sample 6-1	Sample 8-1	Sample 10-1	Sample 12-1	Sample 14-1	Sample 16-1	Sample 18-1	Sample 20-1	
F	5% SLS-2	Sample 2-2	Sample 4-2	Sample 6-2	Sample 8-2	Sample 10-2	Sample 12-2	Sample 14-2	Sample 16-2	Sample 18-2	Sample 20-2	
G	5% SLS-3	Sample 2-3	Sample 4-3	Sample 6-3	Sample 8-3	Sample 10-3	Sample 12-3	Sample 14-3	Sample 16-3	Sample 18-3	Sample 20-3	
H												

(8) Record details of steps (1) – (7) above in the MDS 5.

3.3.5.2 OPTICAL DENSITY MEASUREMENTS OF THE EXTRACTS

(1) Using a 96-well plate reader, measure optical densities (OD) at 570nm and 650nm and determine the measured OD by subtracting the 570nm OD from the 650nm OD.
The equation is shown below:

Measured OD = [570nm OD_{sample} – 570nm OD_{blank}] – [650nm OD_{sample} – 650nm OD_{blank}]
*Set the plate reader-calculated value as the measured OD if the 96-well plate reader performs automatic calculations.

(2) Calculate the cell viability of a sample using the equation below. Furthermore, calculate the variability (SD) of tissue replicates.

(3) Record details of steps (1) and (2) above in the MDS 5.

$$\text{Cell Viability (\%)} = \frac{\text{Measured } OD_{sample}}{\text{Mean Measured } OD_{NC}} \times 100$$

4. ASSESSMENT

4.1 CONDITIONS FOR A SUCCESSFUL STUDY

The skin irritation test should be considered successful if both of the following criteria have been met.

- Tissue viability: 0.7 ≤ mean OD (A570/650) measured value for negative control ≤ 2.5.
- Positive control: mean tissue viability for 5% SLS (positive control) ≤ 40%.
- SD: SD (negative control and positive control) of tissue viability of 3 identically treated replicates ≤ 18 %

4.2 ASSAY CRITERIA

The criteria for in vitro interpretation are shown below.

The test must be performed 3 times per a sample in total. Sort the tissue viabilities obtained from the repeated tests in ascending order, and classify the irritancy based on the median of those tissue viabilities.

Tissue Viability (primary)	Classification
Tissue viability is ≤ 50%	Irritant
Tissue viability is > 50%	Non Irritant

[FLOWCHART] ASSESSMENT FLOWCHART

(1) Tissue viability in negative control → (either criterion is not met) → Assay Failure

0.7 ≤ Mean OD measured value ≤ 2.5

Positive control (5% SLS) result should be “irritant”

Mean tissue viability ≤ 40%

SD”

SD (negative control and positive control) of tissue viability of 3 identically treated replicates ≤ 18 %

↓

(All criteria are met)

↓

(2) Assessment of test samples (3-time repeated tests: all tests satisfy (1))

The median of the 3 tissue viabilities (%) ≤ 50% → (Yes) → Classified as irritant

↓

(No)

↓

Classified as non irritant

MDS 1:
RECEIPT OF LABCYTE EPI-MODEL 24

Laboratory name: _____ Test name: _____ Test No. : _____

1. LabCyte EPI-MODEL 24

Date received : _____

Lot No. : _____

Exouration date : _____
(MM/DD/YYYY)

Accessories : Assay medium, 30mL ☐ (Lot No. : _____ Expiration date : _____)
(MM/DD/YYYY)

24 well assay plate ☐

Note

2. Assay medium

Date received : _____

Lot No. : _____

Expiration date : _____
(MM/DD/YYYY)

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:_____ Name:_____
(MM/DD/YYYY)

MDS 2:
PRE-INCUBATION OF LABCYTE EPI-MODEL 24 (Section 3.3.1)

Laboratory name: _____ Test name: _____ Test No. : _____

1. Warm up the assay medium and add 0.5mL of the assay medium to the wells of the 1st row on the 24-well assay plate.

   Assay medium : (Lot No. : _____ Expiration date : _____)
   (MM/DD/YYYY)

   Warm for 30 min. ☐

   Add 0.5mL of assay medium to each well ☐

   Number of plates : _____

2. Transfer culture inserts to wells in the 1st row on the 24-well assay plate.

   LabCyte EPI-MODEL 24 : (Lot No. : _____ Expiration date : _____)
   (MM/DD/YYYY)

   Time/date executed : _____
   (MM/DD/YYYY HH:MM)

   Confirm that there are no bubbles under the cell culture insert. ☐

3. LabCyte EPI-MODEL 24 is cultured in CO_2 incubator overnight.

   Time/date of culture start : _____
   (MM/DD/YYYY HH:MM)

   Planned time/date of exposure to test chemical : _____
   (MM/DD/YYYY HH:MM)

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:_____ Name:_____
(MM/DD/YYYY)

MDS 3-1:
APPLICATION OF TEST CHEMICALS, RINSING AND POST-INCUBATION (Section 3.1.2, 3.3.2 ~ 3.3.3)

Laboratory name: ________________ Test name: ________________ Test No.: ____________

1. Preparation of positive control.

Weight of SLS ________ mg Preparation vol. ____________ mL Operation date：__________________
(MM/DD/YYYY HH/MM)

2. Warm up the assay medium and add 1.0mL of the assay medium to the wells of the 3rd row on the 24-well assay plate.

Assay medium：（Lot No.：__________________________ Expiration date：______________________）
(MM/DD/YYYY)

Warm for 30 min. □ Add 1.0mL of assay medium. ☐ Time/date executed：____________________
(MM/DD/YYYY HH/MM)

3. Apply test chemicals to the LabCyte EPI-MODEL 24.

Time/date execution started：__________________ Time/date completed：______________________________
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

4. After exposure to test chemical for 15 min., wash out the LabCyte EPI-MODEL 24 and transfer the culture inserts to the 3rd row on the 24-well assay plate.

PBS：（Lot No.：__________________________ Expiration date：______________________）
(MM/DD/YYYY)

Time/date execution started：__________________ Time/date completed：______________________________
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

Confirm that there are no bubbles under the cell culture insert. ☐

5. Test chemical information

Test chemical code No.	Lot No.	Physical state	Test chemical vol.(weight) (measured weight)	Time of application	Exposure time（15min.）
Distilled Water (Negative control)		Liquid	25μL	:	☐
5%SLS (Positive control)		Liquid	25μL	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐

Date:__________________ Operator:__________________ Check date:____________ Study director:________________
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:________________________ Name:____
(MM/DD/YYYY)

MDS 3-2:
APPLICATION OF TEST CHEMICALS, RINSING AND POST-INCUBATION
(Section 3.3.2~3.3.3)

Laboratory name: _______________ Test name: _______________ Test No. : ___________

5. Test chemical information (continued)

Test chemical code No.	Lot No.	Physical state	Test chemical vol.(weight) (measured weight)	Time of application	Exposure time (15min.)
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐
		Liquid, viscous, solid	25μL, (mg, mg, mg)	:	☐

6. Culture LabCyte EPI-MODEL 24 in CO_2 incubator for 42 hrs.

Time/date post-incubation started：_______________________________
(MM/DD/YYYY HH:MM)

Time/date post-incubation completed：_______________________________
(MM/DD/YYYY HH:MM)

Note

Date:__________________ Operator:__________________ Check date:____________ Study director:________________
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:________________________ Name:____
(MM/DD/YYYY)

MDS 4:
MTT ASSAY (Section 3.3.4)

Laboratory name: _____ Test name: _____ Test No. : _____

1. Preparation of MTT medium
   Preparation vol. _____mL Lot No. _____ Time/date executed : _____
   (MM/DD/YYYY HH/MM)

2. Warm up the MTT medium and add 0.5mL of the MTT medium to the wells in the 4th row on the 24-well assay plate.

   MTT medium. :（Lot No. : _____ Expiration date : _____）
   (MM/DD/YYYY)
   Warm for 30 min. ☐ Add 0.5mL of the MTT medium. ☐ Time/date executed : _____
   (MM/DD/YYYY HH/MM)

3. After post-incubation, the LabCyte EPI-MODEL24 transfer to wells of 4th row of 24-well assay plate.

   Time/date started : _____ Time/date completed : _____
   (MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

   Confirm that there are no bubbles under the cell culture insert. ☐

4. Store LabCyte EPI-MODEL 24 culture overnight in CO_2 incubator for 42 hrs.

Information on MTT reaction time

Test chemical code No.	Lot No.	MTT reaction start time	Time when MTT reaction ends	Test chemical code No..	Lot No.	MTT reaction start time	Time when MTT reaction ends
Distilled Water (Negative control)		:	:			:	:
5%SLS (Positive control)		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:_____ Name:_____
(MM/DD/YYYY)

MDS 5:
FORMAZAN EXTRACTION AND MEASUREMENT (Section 3.3.5)

Laboratory name: _____ Test name: _____ Test No. : _____

1. After MTT reaction, use forceps to pick up the cultured epidermis from the cell culture insert and put it in a 1.5mL microtube.

Did you use a scalpel to cut out the cultured epidermis? ☐
Date of execution : _____
(MM/DD/YYYY)

2. Add isopropanol (300µL) to microtube so that the cultured epidermis is completely immersed in isopropanol.

Isopropanol Lot No._____ To add isopropanol (300µL) ☐
Immersion of the cultured epidermis in isopropanol. ☐
Date of execution : _____
(MM/DD/YYYY)

3. For MTT formazan extraction, allow micro tube to stand in a cold and dark space.
Place micro tube in a cold and dark space. ☐

4. Extract solution (200mL) is transferred to each well on the 96-well plate.

Transfer to the 96-well plate. ☐
Time/date executed : _____
(MM/DD/YYYY HH/MM)

Sample location on 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank											
B	Distilled Water-1											
C	Distilled Water-2											
D	Distilled Water-3											
E	5% SLS-1											
F	5% SLS-2											
G	5% SLS-3											
H												

5. Analyze extract OD at 570nm and 650nm, and calculate the OD(570nm-650nm).

Analyze OD at 570nm and 650nm. ☐
Calculate the OD(570nm-650nm). ☐
Calculate cell viability and SD. ☐
Cell viability and SD are recorded on a separate data sheet. ☐
The data sheet is attached to the back of this sheet. ☐
Check for input errors. ☐
Time/date executed : _____
(MM/DD/YYYY HH/MM)

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:_____ Name:_____
(MM/DD/YYYY)

REVISION HISTORY

Rev.	Content	Date Revised
Ver.1	1) First version	27/02/2008
Ver.2	1) Revised clerical error.	28/02/2008
Ver.3	1) Revised the post-incubation time and assessment criteria in compliance with the EpiSkin method described in “Performance Standards for Applying Human Skin Models to in vitro Skin Irritation Testing” 2) Added photos and figures for instruction.	17/03/2008
Ver.4	1) Added MDS 1~6. 2) Added instruction and operational steps regarding the IL-1α ELISA kit. 3) Added subsections “Delivery of LabCyte EPI-MODEL24” and “Instruction For Use of LabCyte EPI-MODEL24” to Section 2. 4) Added the description regarding test chemicals to Section 2. 5) To Section 2, added the description of materials provided by J-TEC separately from other materials. 6) Stated the specific calculation procedures in Section 3.2.5.2 “OPTICAL DENSITY MEASUREMENTS OF EXTRACTS”.	15/05/2008
Ver.4.1	1) Moved scalpel from Section 2.4 “MATERIALS PROVIDED BY J-TEC” to Section 2.5 “MATERIALS NOT PROVIDED WITH THE J-TEC KITS”. 2) Removed the description regarding how to execute procedures alone. 3) Moved IL-1α ELISA reagents from Section 3.1 “PREPARATIONS” to Section 3.2 “TEST METHOD”. 4) Added a flowchart for the IL-1α ELISA procedures. 5) Changed from “in a cold dark place” to “in a cold dark place (or refrigerator)” regarding formazan extraction. 6) Added the description ~~of~~ “ultrasonic cleaning equipment or vortex mixer” as an example of an MTT dissolution method. 7) Changed the exposure time column from entering actual time to checkboxes on the MDS 3.	21/05/2008
Ver.5.0	1) Corrected typing errors in the section number for IL-1α ELISA reagents.	27/08/2008

	2) Removed the space for SLS lot numbers on the MDS 3. 3) Removed the space for PBS lot numbers on the MDS 3. 4) Added the space for isopropanol lot numbers on the MDS 5. 5) Added a checkbox about using a scalpel when removing tissues in the MDS 5. 6) Added the space for IL-1α ELISA kit lot numbers on the MDS 6. 7) Changed the applicable parts of product codes and kit components in Section 2.2, with the change of IL-1α ELISA kit types to a 96 well test only. 8) Decreased the volume by half to 10mL and changed the storage condition from within 1 month to within 24 hours in Section 3.1.2 “POSITIVE CONTROL SUBSTANCE”. 9) Added the manufacturers and product codes of the 24-well plate and 96-well plate in Section 2.4 “MATERIALS PROVIDED BY J-TEC”. 10) Added specific time frames for incubation or culturing. 11) Added the conditions for a successful study in Section 4 “ASSESSMENT” 12) Changed the specific method of applying liquids in Section 3.2.2.2 “APPLICATION OF TEST CHEMICALS”. 13) Added descriptions in English on the MDS Sheets. 14) Changed the application time interval from 1 minute to 1~3minute(s). 15) Numbered figures and flowcharts. 16) Increased the size of spaces for lot numbers on the MDS Sheets. 17) Changed spaces for dates from MM/DD to MM/DD/YYYY. 18) Added director check date, study director, secretariat check date and name at the end of each MDS. 19) Changed the size of matrixes for sample allocation to a 96-well plate in the MDS 5 & 6. 20) Changed the test chemical name to test chemical code in the MDS 3 & 4. 21) Divided the MDS 3 into MDS 3-1 and 3-2, and added spaces for date, operator, check date, study director at the end of the MDS 3-1, and spaces for laboratory name, test name and test no. at the beginning of the MDS 3-2.	
Ver. 6.0	1) Removed the descriptions regarding the measurement of IL-1α	27/02/2009

	production, since the validation committee decided to use cell viabilities only as an index for the skin irritancy test at the meeting in 2009. 2) Revised the expression “the materials provided by J-TEC” for the validation study to that for a standard skin irritancy test preparation. 3) Clearly stated the cell viability equation to use the mean of measured values. 4) Clearly stated to use the median of cell viabilities from the three-time repeated tests as assay criteria.	
Ver. 6.01	1) In order to avoid the possible influence of volatile test chemicals on the results of other test chemicals, the types of test chemicals per plate was changed from 2 chemicals to just 1 chemical.	23/03/2009
Ver. 7.01	1) Test for detecting chemicals that interfere with MTT endpoint was added to Section 3.2.	03/07/2009
Ver. 7.2	1) Revised clerical error.	30/09/2009
Ver. 8.1	1) Added the description about rational and background as following chapters in Section 1. LabCyte EPI-MODEL 24 SKIN IRRITATION TEST (SIT using LabCyte EPI-MODEL24) BACKGROUND OF SIT using LabCyte EPI-MODEL24 BASIS OF THE METOD LIMITATION OF THE METHOD BRIEF BASIC PROCEDURE DATA INTERPRETATION PROCEDURE (PREDICTION MODEL) 2) Added photo about chemicals that directly reduce MTT in Section 3. 3) Added the washing protocol more detail in Section 3. 4) Added assessment about SD.	30/06/2010
Ver.8.2	1) Changed description about the washing protocol in Section 3. 2) Changed unit of consumable reagents and vessels from per a validation study to per a test.	17/08/2010
Ver.8.3	1) Changed description more briefly about the washing protocol in Section 3.	14/06/2011