新規試験法提案書

ヒトエストロゲン受容体結合による活性化・拮抗作用物質を検出するBG1Luc ER TA法

平成26年１月

国立医薬品食品衛生研究所

新規試験法提案書

平成 26 年 1 月 20 日

No.　2013-02

ヒトエストロゲン受容体結合による活性化・拮抗作用物質を検出する BG1Luc ER TA 法に関する提案

平成 25 年 6 月 11 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：ヒトエストロゲン受容体結合による活性化・拮抗作用物質を検出する BG1Luc ER TA 法は、他の類似試験法と同程度に、行政上利用可能である。

この提案書は、OECD (Organisation for Economic Co-operation and Development) Test Guideline OECD Test Guideline（TG）457 およびICCVAM **(Interagency Coordinating Committee on the Validation of Alternative Methods)** Test Method Evaluation Report, The LUMI-CELL® ER (BG1Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicalsをもとに、内分泌かく乱試験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「ヒトエストロゲン受容体結合による活性化・拮抗作用物質を検出する BG1Luc ER TA 法」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）
一鬼　勉　（日本化学工業協会）*
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小野寺博志　（独立行政法人　医薬品医療機器総合機構）
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）
谷田智子　（独立行政法人　医薬品医療機器総合機構）*
西川秋佳　（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）
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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

西川秋佳　（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）：委員長
川西　徹　（国立医薬品食品衛生研究所）
小川久美子　（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）
菅野　純　（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　毒性部）
倉持憲路　（厚生労働省　医薬食品局　化学物質安全対策室）*
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佐々木正広　（厚生労働省　医薬食品局　化学物質安全対策室）
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高木篤也　（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　毒性部　動物管理室）
広瀬明彦　（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　総合評価研究室）
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）
光岡俊成　（厚生労働省　医薬食品局　審査管理課）
山本順二　（厚生労働省　医薬食品局　化学物質安全対策室）*
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 BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists

At the meeting concerning the above method, held on 11 June 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:

BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists is considered to be useful as a screening of endocrine disrupter substances as well as similar test methods for regulatory use.

Following the review of the results of OECD (Organisation for Economic Co-operation and Development) Test Guideline OECD Test Guideline (TG) 457 and ICCVAM (**Interagency Coordinating Committee on the Validation of Alternative Methods)** Test Method Evaluation Report, The LUMI-CELL® ER (BG1Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals, it is concluded that BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists such as screening of endocrine disrupter substances 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 endocrine disrupter testing.

Takemi Yoshida
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

20 January, 2014

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

ヒトエストロゲン受容体結合による活性化・拮抗作用物質を検出する
BG1Luc ER TA 法の評価会議報告書

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ヒトエストロゲン受容体結合による活性化・拮抗作用物質を検出する BG1Luc ER TA 法の評価会議報告書

JaCVAM 評価会議

平成 25 年 6 月 11 日

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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以上

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内分泌攪乱物質が大きな問題となってから、物質のエストロゲン様作用を測定する *in vitro* 試験法の開発が社会的に求められてきた。BG1LucER TA 法（以下「当該試験法」）は、この社会的要請に応えて開発された試験法の一つで、化学物質が ヒトエストロゲン受容体（ER）に対して活性物質（agonist）あるいは拮抗性物質（antagonist）であることを検出して、エストロゲン活性あるいは拮抗作用を示す内分泌攪乱性の予測に役立つものとして提案されたものである [1)]。当該試験法に対して従来試験法と言えるものは確立していない。しかし、内分泌攪乱性を規定する毒性ないし生物活性についてはまだ十分には特定されていない部分が残されており、ICCVAM の“*In vitro* ER and AR binding and TA reference substances list[2)]”では、複数の根拠を総合して内分泌攪乱性物質を分類している。

米国では 2012 年に ICCVAM が当該試験法の評価結果を報告している [1)]。OECD においても 2012 年 10 月 2 日にこれをガイドライン TG457 に取り入れている [3)]。これに対して日本では、JaCVAM の評価委員会が当該試験法の評価を行い、平成 24 年 11 月に結果を報告している [4)]。以下に示すのは、この報告に基づいた JaCVAM 評価会議の評価結果である。

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。

化学物質のエストロゲン活性を検出しようとする *in vivo* の従来試験法としては、げっ歯類を用いた子宮肥大試験がある。化学物質のエストロゲン活性を検出しようとする *in vitro* の試験法としては、エストロゲン受容体結合性試験と、ヒトエストロゲン-α 受容体安定導入細胞を用いる転写活性化試験 (Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay; ER-α STTA; OECD TG455）がある。当該試験法はこれらと類似の役割が期待されているものである。

2. 当該試験法と従来試験法の間にどのような科学的つながりがあるか。

測定指標は異なるが、エストロゲン活性または拮抗性を基礎とするという意味で、当該試験法の科学的メカニズムは、げっ歯類子宮肥大試験と共通である。

類似の試験法である ER-α STTA 法は、活性物質が ER と結合することによって誘導される DNA 転写活性化をレポーター遺伝子（Luciferase）の活性化による luciferin の発光を指標として測定するものであり、当該試験法の原理はこれと共通である。

類似の試験法であるエストロゲン受容体結合性試験は、被験物質と ER との結合性を測定するものであるが、結合した物質が ER を刺激するのかリガンドに拮抗するのかは判別できないところが当該試験法と異なっている。

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。

EU（ECVAM）と日本（JaCVAM）を併せて ICCVAM が組織した運営委員会の管理の下で、3 試験施設（米国の Xenobiotic Detection Systems, Inc.、ECVAM Joint Research Centre、日本の日吉株式会社）による検証試験が実施された。被験物質は、ICCVAM がエストロゲンおよびアンドロゲンに関する *in vitro* 試験法の評価に用いるべき物質として 2003 年に発表した 78 物質である [2)]。判定に必要な用量反応曲線が得られず、エストロゲン活性・拮抗性の有無が判定困難であった 7 物質が、この検証試験の解析・評価から除外されたことは、当該試験法の利用に当たって留意すべきことである。

この試験における当該試験法の正確性は、ER 活性 35 物質において、感度が 96%（27/28）、特異度が 100%（7/7）で、一致率は 97%（34/35）であった。ER 拮抗性 25 物質においては、感度が 100%（3/3）、特異度も 100%（22/22）で、一致率は 100%（25/25） であった。

この試験結果についての国際的第三者評価において、当該試験法は、ER 活性および ER 拮抗性を検出する *in vitro* 試験法として適正なものであるとされている。

よって、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と言える。

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

代替すべき試験法は確立されていないが、当該試験法の評価対象となるのは、医薬品、動物用医薬品、農薬、産業化学物質等である。ただし、不溶性物質および揮発性物質に対応する試験手順は確立されていない。

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、現時点ではハザード評価に有用である。

なお、当該試験法は、被験物質の濃度に応じた活性の測定が可能であるから、リスク評価に使用できる可能性はあるが、まだ検証が行われていないので、有用性は不明である。

6. 当該試験法は、目的とする物質また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、化学物質の ER 活性および ER 拮抗性を調べることができる。当該試験法は、基本的なプロトコルとして、試験物質を DMSO に溶解して適用しており、DMSO に不溶の物質および揮発性物質に適用できるかどうかは、検討がなされていないので不明である。

7. 当該試験法はプロトコルの微細な変更に対して頑健であるか。

検証試験に参加した 3 施設での施設内再現性は 12 被験物質について 100%であった。ER 活性物質における施設間再現性は 67%（8/12）、ER 拮抗性物質での施設間再現性も 100%（12/12）であったので、ある程度の頑健性は期待できる。

8. 当該試験法の技術習得は、適切な訓練を経ている担当者にとって容易なものであるか。

この試験で必要な技術は、培養細胞を用いる試験法一般の技術および細胞の発光を測定する技術であり、適切な訓練によって容易に習得できるものである。

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。

当該試験法のために必要な機器は、通常の細胞培養に要する装置のほか、細胞発光の測定に用いる光度計であり、高価なものではない。試験に要する時間は、凍結保存された細胞を融解して増殖するのに 48～72 時間、試験用培地に細胞を播種してさらに 48～72 時間、試験物質と接触させて Luciferase 活性化を誘導するのに 19～24 時間を要するが、その後は細胞を融解させて発光を測定し、データを集計解析するのみである。これらの作業に要する時間は他の *in vitro* 類似試験法と変わらない。

10. **当該試験法は、動物福祉の観点及び科学的見地から、目的とする物質また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。**

当該試験法は培養細胞を用いる *in vitro* 試験法であり、他の類似試験法と同じ程度に、行政上利用可能である。

参考文献

1) ICCVAM Test Method Evaluation Report. The LUMI-CELL® ER (BG1Luc ER TA) Test Method: An In Vitro Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals. (NIH Publication No. 11-7814; 2011)
2) ICCVAM Evaluation of *In vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. (NIH Publication No: 03-4503; 2003)
3) OECD TG457：BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists. (2012)
4) BG1LucER TA (LUMI-CELL ER) 法：*in vitro* ヒトエストロゲン受容体活性物質試験法の評価報告（平成 24 年 11 月）

BG1LucER TA (LUMI-CELL ER) 法：*in vitro* ヒトエストロゲン受容体活性物質試験法の評価報告書

平成 24 年 11 月 29 日

内分泌かく乱試験法評価委員会

委員名：

小野　宏（委員長：（一財）食品薬品安全センター秦野研究所）

丸野内棣（藤田保健衛生大学）

井口泰泉（基礎生物学研究所）

中澤憲一（国立医薬品食品衛生研究所）

用語集

Accuracy：正確度

Concordance：一致率

Estoregen receptor（ER）α/β：エストロゲン受容体 α/β

Estrogen responsive element（ERE）：核内のエストロゲン反応部位

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

Federal Register：（米国）連邦官報

Independent International Scientific Peer Review Panel：国際第三者専門技術評価委員会

Interagency Committee on the Validation of Alternative Methods（ICCVAM)：米国代替法評価省庁間連絡委員会

Inter-laboratory validation study：施設間検証

Intra-laboratory validation study：施設内検証

Japanese Center for Validation of Alternative Methods（JaCVAM）：日本代替法評価センター

National Institute of Health（NIH）：米国国立衛生研究所

National Toxicology Program Interagency Center for the Validation of Alternative Methods（NICEATM）：毒性学国家事業代替試験法検証省庁間連絡センター

Reliability：信頼性

Transcription activation（TA）：転写活性化

US Environmental Protection Agency（US EPA）：米国環境保護庁

Uterotrop(h)ic assay：子宮肥大試験

Organisation for Economic Co-operation and Development（OECD）：経済協力開発機構

Xenobiotic Detection Systems, Inc.（XDS）：XDS 社

Validation study：検証試験

Validation study management team：検証試験運営委員会

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

LUMI-Cell ER 試験法すなわち BG1LucER TA 法（詳しくは「BG1Luc4E2 細胞を用いるエストロゲン受容体（ER）転写活性化（TA）試験」）は、物質のエストロゲン活性を測定する *in vitro* 試験法の一つで、内分泌攪乱物質対策のために開発が求められてきたものである。本試験法は、米国 North Carolina 州 Durham にある Xenobiotic Detection Systems, Inc.（XDS）社で開発されたもので、ヒト卵巣癌由来株細胞 BG1 にレポーター遺伝子として Luciferase responsive element を含む plasmid を核内のエストロゲン反応部位（ERE）の下流に安定的に導入した細胞を用い、この細胞に内在する ER の活性化によって起る遺伝子変化を発光で検出しそれを定量的に測定する試験法である。Interagency Committee on the Validation of Alternative Methods（ICCVAM）はこの試験法の検証試験（Validation study）に基づく評価を行い、その有用性を認め、US Environmental Protection Agency（US EPA）および Organisation for Economic Co-operation and Development（OECD）に試験法ガイドラインとして採択するよう提案した。

内分泌機能に影響する生物活性を有する物質は、ホルモン等の天然の生体物質のほか合成化学物質にも多数知られるようになったが、その活性の有無と程度について未調査の物質が多い。こうした物質のうち、大量の曝露によって生体の内分泌機能およびこれと関係した生殖発生等に影響を及ぼすもの（内分泌攪乱物質と呼ばれる）であることが懸念されるものがあり、現在、影響の有無を確認する試験法の開発が求められている。また、この確認の対象となる物質の数が膨大なものであるため、効率的なスクリーニングを行う試験法の開発と実用化が求められてきた。スクリーニングの指標として有望な性質には物質の内分泌活性があり、物質のホルモン受容体との結合性または内分泌機能の活性化を測定する方法が注目されている。エストロゲン活性に関する試験法としては、すでに *in vivo* 試験法として「囓歯類を用いる子宮肥大試験」が確立され、検証試験を行った上で国際的な試験法ガイドライン（OECD TG）となっているが、より簡便で迅速な非動物試験として培養細胞を用いる *in vitro* 試験法の開発が進められている。

物質の ER との結合性を *in vitro* で試験する方法には、生体または培養細胞から抽出した ER に対する化学物質の競合結合反応の測定があり、たとえばラット子宮から抽出した ER を用いる「ラット子宮エストロゲン受容体結合性試験」が挙げられる。これは無細胞系の結合試験であり、詳細な用量反応関係を確認でき、物質の ER 結合性を標準物質、たとえばエストラジオールと比較して定量的に決定できるものである。しかし、ER に対しては受容体刺激物質ばかりでなく拮抗物質も結合性があるので、両者の区別が出来ない。その区別のためには、物質と ER との結合が起こす生物学的効果を確認する必要があり、有意義な指標として生体に誘発されるエストロゲン効果を測定する *in vivo* 試験（たとえばラット子宮肥大試験）が利用されている。*In vitro* でも、ER が活性物質と結合したのちに細胞内で起こる応答が観察できれば、エストロゲン効果を確認できる。活性物質と結合した細胞内 ER は、核内の ERE との結合を通じて関連物質をコードする遺伝子（DNA）の転写を起こす。このような遺伝子を介する応答に必要な機構を保持する細胞において検査することは意義がある。

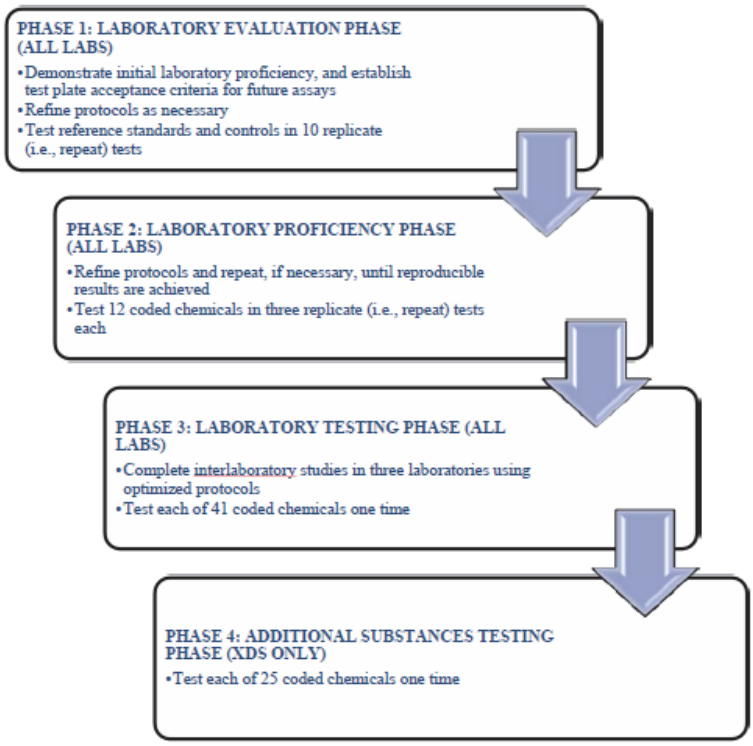
BG1LucER TA 法は、エストロゲン活性物質による ER 結合の次の過程として関連遺伝子の転写が誘導されることを観察する方法である。細胞には転写活性化を調べるために、当該遺伝子と連結した位置（下流）に発光誘発遺伝子を組み込んでおき、転写が起れば細胞が発光するような操作を加えてある。このような細胞としては、すでに HeLa 細胞にヒトエストロゲン受容体 α（hERα）と発光酵素（Luciferase）遺伝子を組み込んだ細胞が開発されており、この細胞を用いた試験法「hERα-HeLa-9903 細胞を用いる安定導入転写活性化試験 Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay（hERα-HeLa STTA）」が、US EPA の試験法として採択され（OPPTS 890.1300, 2009）、また OECD 試験法ガイドラインに収載されている（OECD TG 455, 2009）。

hERα-HeLa STTA 法と本 BG1LucER TA 法の相違は、(1) 前者が hERα を HeLa 細胞に組み込んだものであるのに対し、本法は BG1 細胞に内在する ER を利用する、(2) 前者では組み込まれた受容体は ERα であるが、本法の BG1 細胞は ERα と ERβ の双方を具えている、(3) 前者は専ら ER 刺激物質（アゴニスト）の試験を行うが、本法では、ER 拮抗物質（アンタゴニスト）の活性を測定するプロトコルも用意されている、ことである。

2004 年 ICCVAM は、XDS 社から提案された BG1LucER TA 法を評価し、施設間検証試験に付してその有用性を広く確認するに値するものであると認めた。これを受けて National Toxicology Program Interagency Center for the Validation of Alternative Methods（NICEATM）が国際的施設間検証試験を企画、実行した。Japanese Center~~re~~ for Validation of Alternative Methods（JaCVAM）および European Centre for Validation of Alternative Methods（ECVAM）はこれに協力して検証試験運営委員会を組織した。試験施設として、米国の XDS 社、日本の株式会社日吉、および欧州の ECVAM がこの検証試験に参加し、BG1LucER TA 法によって物質の ER アゴニスト活性および ER アンタゴニスト活性の試験を実施し、そのデータに基づいて本試験法の試験性能の正確性と信頼性を評価するための検証を行った。（Fig.1-1）

検証試験の第 1 段階では、ER アゴニスト、ER アンタゴニスト各 10 物質の試験を行い、参加試験施設の技量の評価を行った。第 2ー4 段階では、プロトコルに評価と改良を加えつつ、*in vitro* ER アゴニスト、アンタゴニスト試験の検証に用いるべき参照物質として ICCVAM が選定していた 78 物質の試験を行った。

Figure 1-1 NICEATM/ECVAM/JaCVAM Validation Study Phases



検証試験の結果、3 試験機関のデータは高い一致率を示した。ICCVAM 参照物質の分類（陽性・陰性）と 3 試験機関のデータの一致率も高いものであった。従って、BG1LucER TA 法は、物質の *in vitro* ER アゴニスト活性を正確に検出する試験であり、既存の ER TA 法（hERα-HeLa STTA）と同等の性能を有し、また物質の *in vitro* ER アンタゴニスト活性を正確に検出できる試験法である、と結論された。試験法の信頼性については、試験法施設内再現性、施設間再現性ともに良好な結果であり、技術移転性、試験法の頑健性に問題はなく、通常の細胞培養実験の施設と発光分析機器が備えられていれば、技術的に特に困難な部分もないので、行政規制試験とする妥当性は認められる。BG1Luc 細胞は California 大学 Davis 校の Research Technology Transfer Services から、あるいは XDS 社から入手することが出来る。

この検証試験の終了後、NICEATM と ICCVAM は、2011 年 1 月、Independent International Scientific Peer Review Panel：以下 Panel）を組織して Peer Review を委嘱した。Panel は複数回の電話会議を行った後、2011 年 3 月 29ー31 日 Maryland 州 Bethesda の National Institute of Health（NIH）で集合会議を開催し、5 月に報告書を提出した。これは、2011 年 5 月 18 日の Federal Register に発表された。

この試験法は、同年 OECD に提案され、TG455 に組み入れるべく加盟各国に意見聴取回覧中である。前述のように、すでに TG455 として hERα-HeLa STTA が収載されているが、原理的および機能的に同様な性能に基づいた試験法として、同じ TG455 に ANNEX 3 として加えることとされている。（ERα-HeLa cell 法は ANNEX 2 となる）。

本試験法の今後の問題点として、細胞に内在する 2 種類のエストロゲン受容体 ERα と ERβ の測定

結果に対する寄与の比率を明らかにすることが課題として残っている。これは、内分泌攪乱性がエストロゲン活性に起因するという考えにおいて、ERα と ERβ の関与の程度を推量するという重要な課題である。この分別測定法の開発ができれば、物質のエストロゲン活性の詳細な把握が可能になり、この分野の研究と化学物質管理に大いに貢献すると考えられる。

２．試験法の妥当性

2-1　試験法の概略

１）目的と原理

ここで取り上げる試験法は内分泌撹乱物質のうちエストロゲンに関連する化学物質を測定し、その人体、自然界に対する弊害を避けることを目的として開発された。本法、BG1LucER TA 法はその一つで生体の ER に結合し、アゴニスト作用、あるいはアンタゴニスト作用を示す化学物質をスクリーニング、測定するために ER を発現する培養細胞に ER 応答性のルシフェラーゼ遺伝子をレポーター遺伝子として導入・安定発現させ、被検化学物質を投与して細胞に反応させた後、ルシフェラーゼ活性の変化を（ルシフェリンの）発光により測定できる様にしたものである。

２）標準測定条件

次の点に留意する必要がある。

a. 培養細胞の維持管理：培養細胞は全て無菌状態で取り扱う必要がある。準備段階の培養から、96 ウェル・プレート中の試験に至るまで、細胞がフラスコやウェル内で均一な密度になる様に維持されなければならない。ICCVAM 報告書に添付されたプロトコル（Appendix B）にある培養処方を忠実に守ること。細胞濃度の調製方法なども含めて、培養細胞の扱い方を前もって特別に訓練を受けておく必要がある。
b. 標準条件の設定：測定値は全て相対値で比較するので 17β-エストラジオール（E2）の適切な濃度を常に同一の 96 ウェル・プレート上で標準として測定し、それに対する相対値として表す。
c. 被験化合物：反応陽性の化学物質と同時に陰性の化学物質も試験すること、さらに ER アンタゴニスト作用のある化学物質の効果も同時に試験することが重要である。

2-2　妥当性の検討

１）総合的検討：BG1LucER TA 法の基となる細胞、BG1 は天然の ER を発現しており、ER アゴニストおよび ER アンタゴニストの両者が測定可能な *in vitro* 系である。溶媒に溶解可能（DMSO を用いる場合もある）な広範な被験化学物質に適用できる点で非常に価値が高い。ただし ERα 型／β 型の化学物質との結合やそれぞれの生理的作用効果およびその識別が十分に解明されていないのでその点は早期に識別可能な系との比較検討が望まれる。

理論的視点から：

ER アゴニスト活性および ER アンタゴニスト作用のある化学物質による ERα 型／β 型に対する単

なる結合能ではなく活性を測定できる。*in vitro* 系ではあるが、バイオアッセイであるため反応系、培養細胞の維持管理が結果に影響を及ぼす可能性は否定できない。このことは表面上には現れ難い試験結果の変動につながる可能性が高いので十分に注意が必要である。

測定系構築上の視点から：

a. 培養細胞：BG1LucER TA 法ではヒト卵巣癌由来株細胞、BG1 細胞が ER を発現していることを基にアッセイ系を構築している。一般にヒトの培養細胞では染色体の形態および数の異常を高頻度に有することが知られている。その影響は ER の発現にもある程度及ぶことが予想される。現在のところこうした染色体異常を防ぐ手段は知られていない。従って、ER の発現のチェックを標準参照物質の使用によって常に実行する必要がある。

b. レポーター遺伝子：このアッセイ系ではレポーター遺伝子を導入し、ER によりレポーターを発現させる。ヒトの ER にはα型とβ型が存在し、その両者へのアゴニスト／アンタゴニスト結合やその活性化による生理的作用の違いは十分には解明されていない。しかしヒト ERαと ERβの cDNA は既に単離されているのでその機能解明は近い将来明らかとなることが期待される。これらの cDNA を同一または別々の細胞に導入したレポーター・アッセイ系（HeLa-9903）も既に報告されている。本法でもその様な細胞を用いた系の測定結果との比較検討をすべきである。

c. 内分泌撹乱物質の影響はヒトに対するエストロゲン様物質に限ったことではなく、種々のホルモン様物質がヒトのみならず地球上の生態系にも広範な影響を及ぼしていることはよく知られている。その観点からヒト ER アッセイ系の外にヒトアンドロゲン受容体（AR）を用いたアンドロゲン様物質や関連するホルモン様物質のスクリーニングおよび他の生物種に対する同様の測定法の開発やそれらと比較検討を視野に入れる必要がある。

試験法操作上の視点から：

a. 準備段階を含めた培養容器内の細胞密度の調整。特に細胞を均一に播種できているかどうかのチェックが必要である。

b. 生細胞を倒立顕微鏡でチェックする際に容器全体をチェックする必要がある。

2-3　BG1LucER TA 法の問題点

1）　アッセイシステム

以下のような問題がある。

a. 使用培養細胞について：BG1 細胞はヒト ERα と ERβ を発現している。しかしその発現強度がどの程度であるか、また強度比を一定に維持する管理方法の記載が無い。ヒト ERαと ERβの cDNA は既に入手可能な段階にある。他の試験結果によるとアゴニスト、アンタゴニストの種類によって、ERα と ERβ とで反応性の異なる化学物質が複数知られている。近い将来 ERα と ERβ の生理機能や発現細胞の違いなどが明らかになる可能性が大きい。従って早

期に両受容体を介する細胞応答を別々に測定できる系を確立すべきである。

b. 本法ではアッセイ培養に入る前に細胞はフェノールレッド不含培地で培養され、しかもルシフェリン反応に対する影響を除外するため、培地の種類もアッセイ前に交換される。こうした煩雑さを緩和するため、今日では同じ薬品会社からフェノールレッドやその他の薬品による影響の出ないフッ化ルシフェリンが発売されているので、ルシフェラーゼ・アッセイ系をルシフェリンからこれに変えるのがよいと思われる。培地は DMEM に統一すべきである。

c. 被験化合物のうち揮発性あるいは DMSO 不溶性の物質については適切な溶解補助剤（可溶解剤）を使用するか、別の試験法を今後検討すべきである。

d. 96 ウェル・プレートのウェル中の細胞の顕微鏡写真をデータに添付する方が良い。測定の信頼度のチェックに利用できる。

３．検証試験に用いた物質の分類と妥当性

Figure 1-1 に示すように、検証試験の 2〜4 段階で、それぞれ 12、41、25 物質、合計 78 物質が試験に供された。それらの一覧を Table 1 に示す。これらは ER アゴニスト、アンタゴニストの両者の評価に用いられた。ステロイド、有機酸、炭化水素など広範な種類かつ様々な反応強度の物質が利用されている。試験法の感度を評価するため、陰性物質は約 25%を占めており、選ばれた物質は妥当であると判断されている。

４．試験法のデータと結果の有用性

ICCVAM により選ばれた 78 物質の結果から、陽性、陰性と確実に分類される物質のみが正確性の評価に使われた。ER アゴニストの評価では、下記 Table 2 に示す 42 物質（陽性 33 物質、陰性 9 物質）、ER アンタゴニストの評価では Table 3 に示す 25 物質（陽性 3 物質、陰性 22 物質）である。さらに、ER アゴニストに関しては 42 物質のうち下に示す 7 物質がアッセイの正確性を求める評価から除外された。この理由は、データが正確な判断には不十分であったため、陽性か陰性かの判断には不適切とされたことによる。ただし、評価に用いられた物質を含め、*in vivo* 試験（子宮肥大試験）の結果が得られている物質は少ない。

・Clomiphene citrate
・*p,p'*-DDE
・5α-Dihydrotestosterone
・Flutamide
・Procymidone
・Resveratrol
・Tamoxifen

Table 2 では、陽性 33/42、陰性 9/42 であるが、結果を詳しく見ると、3 機関の結果がそろって陽性なのは 22/33 で、11/33 は 1〜2 の機関で陽性ではないと報告されている。さらに陰性のうち 2/9 は 3 機関の結果がそろっていない。このデータはスクリーニング用の参考として相応しいとは言えない。

次に Table 3 について見ると、ER アンタゴニスト作用のあるもの（陽性；POS）3/25 のうち 1 つは 3 機関の結果がそろっていない。陰性（NEG）19/25 のうちの 1 つでは陽性（POS）の結果が得られている。これらは ER アンタゴニスト作用を明らかにする試験法として十分ではないことを示している。

化学物質の構造による分類（ステロイド類、ベンゼン単環等）も示した方が良いと考えられる。また、バリデーションとして実施すべき化学物質として、ER アンタゴニストを増やすべきである。指標とする化学物質のヒト ERα と ERβ の反応性も示した方が良いであろう。

５．試験法の正確性

BG1LucER TA 法の正確性を評価するため、ICCVAM 参照分類と比較した。ER アゴニストの評価は 35 物質を用いて実施された。Table 4 に示すように、3 施設の正確性の一致率は 97%（34/35)であり、そのうち感度は 96%（27/28）、特異度は 100%（7/7）であった。偽陽性はなく、偽陰性率は 4%（1/28）であった。この結果は、各施設の結果から個別に計算した場合でも同様であった。

ER アンタゴニストの評価については、25 物質を用いて実施された。Table 5 に示すように、3 施設の正確性の一致率は 100%（25/25）であり、そのうち感度は 100%（3/3）、特異度は 100%（22/22）であった。偽陽性も偽陰性もなかった。この結果は、各施設ごとの結果でも同様であった。

ER 結合試験データと BG1LucER TA 法の比較では、一致率は 97%（33/34）であり、medroxyprogesterone acetate が BG1LucER TA では陽性となり、一致しなかった。同様に、*in vivo* 子宮肥大試験との比較では、一致率は 92%（12/13）であり、butylbenzyl phthalate が BG1LucER TA 法では陽性となり、一致しなかった。

６．試験方法の信頼性

施設内再現性は、検証試験での各施設のすべてのプレートの標準物質および対照実験の結果、ならびに、12 物質の 3 施設における 3 回の実験で求められた第 2 段階の結果から求めた。Table 6 に示すように ER アゴニスト試験では、標準物質である 17β-estradiol（E2）の EC50 値は 8～11×10^{-12}M であった。各施設の 3 回の結果は 100%一致したが、12 物質のうちいくつかの種類は施設間で一致しなかった。

Table 7 に示すように ER アンタゴニスト試験では、標準物質である raloxifene の IC50 値は 1.1～1.3×10^{-9}M であった。各施設の 3 回の結果は 100%一致したが、12 物質のうちいくつかの種類は施設間で一致しなかった。

施設間再現性については、施設内再現性と同様に 12 物質の 3 施設のおける 3 回の実験で求められた第 2 段階の結果から求めた。Table 8 に示すように、ER アゴニストが陽性と示されたのは 67%（8/12）であり、ER アンタゴニストでは 100%（12/12）であった。Table 9 に示すように、41 物質を用いた第 3 段階の結果では、このうちの 5 物質の ER アゴニストは不適切なデータで評価できなかった。少なくとも 2 施設において、残り 36 物質の結果は 100%一致した。ER アンタゴニスト活性については、93%（38/41）が一致した。

７．データの質

評価報告書では記載がないが、背景評価報告書によると、XDS 社と ECVAM は GLP ガイドラインに準拠して実験を実施した。株式会社日吉には検証試験に先立ち、OECD の GLP 基準に則ったガイダンス文書を渡した。株式会社日吉の QC（quality control）と保証方法は ISO9000 及び ISO2000 にも準じた。QA（quality assurance）はすべての施設で適切に実施された。

８．試験法の有用性、限界および提言

1) ER アンタゴニストによる結果：BG1LucER TA 法は原理の項でも述べた様に単にアンタゴニストと ER との結合に止まらずアゴニストの活性抑制効果を検出できる点が優れている。他の同様の試験法の結果との一致度、BG1LucER TA 法の感受性、特異性、偽陽性・偽陰性の識別は良好な結果を示した。

2) 試験方法から見た結果：他の試験法に比しアゴニスト、アンタゴニストの濃度が 1/100 で検出できるのでより正確であり、偽陽性・偽陰性の生じる可能性は少ない。

3) BG1LucER TA 法による化学物質の ER アゴニスト活性・ER アンタゴニスト活性のスクリーニング結果は、エストロゲン活性の試験に用いられる既存の細胞系（内在性のヒト ER を利用する MCF-7 細胞、 ヒト ERα を安定的に組み込んだ HeLa 細胞）などと比較しても、偽陽性や偽陰性の識別は良好である。また、化学物質のエストロゲン活性をルシフェリン発光により定量化するが、MCF-7 細胞に比べて増殖が早いため、試験時間が短縮され、多数の化学物質のスクリーニングに向いている。

4) ERα と ERβ の両方を内在的に発現しているため化学物質のエストロゲン作用を総体として検出可能である。ER を介した化学物質のエストロゲン活性を一次スクリーニングするには便利な試験系である。一方、選択した物質が ERα あるいは ERβ に特異的に結合して作用するのかを調べるためには、どちらかの ER サブタイプを発現させた細胞系を用いた二次的なスクリーニングが必要となる。

5) BG1 細胞の内在的な ERα と ERβ の存在比が細胞の継代によって変化しないことを保証しておくことが必要である。それぞれの ER に対するアゴニスト、アンタゴニストは市販されているので、これらの物質を用いた応答性を調べておくことも必要であろう。

6) 溶解補助剤（可溶化剤）として DMSO が用いられているが、DMSO に溶けにくい物質については、他の有用な補助剤（可溶化剤）を検討し、それが BG1 細胞に影響しないことを検証する必要がある。

7) 化学物質の複合影響の可能性が指摘されてきているが、BG1LucER TA 法を用いて複数の物質を同時に曝露した場合の複合作用の検討は行われていない。今後、複合曝露の検討が必要である。

8) 現時点では揮発性物質の取り扱いについて明確な指針が無いと思われる。今後の検討が期待される。

9) 代謝されてからエストロゲン作用を示す物質の評価についても、BG1LucER TA 法を用いてどのよ

うにスクリーニングするかの検討が必要である。

10) BG1LucER TA 法を国内の化学物質評価にどのように利用するかについての検討も今後の課題である。

11）本法ではアッセイ培養に入る前に細胞はフェノールレッド不含培地で培養され、しかも培地の種類もアッセイ前に交換される。今日では同じ薬品会社からフェノールレッドやその他の薬品による影響の出ない試薬（フッ化ルシフェリン）が発売されているためこれを利用すればフェノールレッド含有の有無は問題にならない。しかし、培地は DMEM に統一すべきである。

12) 本試験法の実用上の懸念として、試験系の安定性の問題が指摘されよう。一般に遺伝子を導入した細胞は、安定的導入とは言うものの、継代ごとに遺伝子発現には多少の変化が起るため、長期にわたって同じ反応性を保つことは期待できない。BG1 細胞についても、継代早期の反応性の明らかな細胞を多量に分割凍結保存して、逐次利用するような措置が望ましく、細胞を含む試験材料の供給に関する配慮が必要である。少なくとも、使用する試験系の標準対照物質に対する反応を試験の度ごとに確認することが必要である。

13) 基礎的な試験操作の正確性を保証することが必要で、試験施設がこの細胞系の使用に習熟することが、当然ながら求められる。試験準備の際に、培養液中の細胞の濃度を確認し、ウェルごとの細胞数が均等であることを保証するような記録、たとえば顕微鏡での確認を励行するような配慮が望ましい。

９．その他の試験方法の科学的な報告

内分泌撹乱作用につながると考えられる ER に関しての試験法としては、問題とする物質の受容体への結合実験がまず想起される。結合実験では化学物質との相互作用が容易となるよう、受容体が水相に露出していることが望ましいが、この目的には細胞を破壊した非細胞系（cell-free）が有利であり、実際、この系での結合実験は以前より行われている。受容体と相互作用を示す物質としては、アゴニストとアンタゴニストがあり、単純な結合実験では両者の区別が困難である。さらに、非細胞系での結果は細胞系と一致しないという報告も多く、細胞内に特有の結合を制御する因子の存在が示唆されている。よって、結合を含めた受容体との相互作用については細胞系の利用が望ましい。

細胞系での物質の ER への結合は、内在的な転写活性の増加を目安とすることにより、アゴニスト作用の有無を判定できる。このようなレポーター・アッセイは、導入の一過型／安定型を問わず、酵母、ゼブラフィッシュ肝細胞株、HepG2、HeLa、CV-1 など種々の細胞で行われている。

以上のことは主として先に発見されたα型の受容体（ERα）についての記述であるが、ER にはこれとは別にβ型（ERβ）が存在する。ERα、ERβ の両者は塩基配列に相同性があるが、コードする遺伝子は異なっており、生体内での発現の様相にも差異がある。また、内分泌撹乱化学物質が ER を介して影響を及ぼすとしてもその全てが ERE 下流遺伝子の転写の修飾に直接起因すると断じるには問題があり、ジェネティック（genetic；遺伝子的）な転写の修飾ではなく、影響のエピジェネティック（epigenetic；後成的）な側面についても考察が加えられている。いずれにしても、ERα、ERβ の両受

容体が発現しており、エピジェネティックな影響も観察しうる細胞系が利用に適している。

ERα、ERβ はともに結晶化され X 線により構造が解析されている。この結果より、ERα、ERβ のエストロゲンおよび関連物質の結合についての検討や考察がなされている。これに加え、化学物質の ER に対する定量的構造-活性相関（QSAR）も試みられているが、現在のところ結果の集積とその解析の段階に止まっており、動物実験の代替に達してはいない。

上述の試験のうち、HeLa 細胞でルシフェラーゼをレポーターとした ER 活性化アッセイは、hERα-HeLa STTA 法として OECD の試験法ガイドラインにも収載されているが、これに対する BG1LucER TA 法の優位性については、1 の“本試験法の科学的妥当性と規制試験法としての妥当性”に記されている。他の試験法については、代替法としての定義、あるいは、普遍性等の要件を充たすとは言えず、当然検証試験は行われていない。よって、ERα、ERβ の両受容体を介する生体への影響という点で、BG1LucER TA 試験法の今後の研究の発展が望まれる。

１０．結論

BG1LucER TA 法は、化学物質の *in vitro* での ER アゴニスト活性および ER アンタゴニスト活性を検出するスクリーニング試験法であり、その検査性能の有用性を確認する検証試験は国際的に 3 試験施設共同試験によって完了している。本試験法は、化学物質の ER に対する結合性を見る試験法とは異なり、その結合の結果による DNA の転写活性化まで知ることが出来、さらに、ER アゴニストとアンタゴニストの活性を分けて検査することが出来る。その試験法としての科学的妥当性と規制試験法としての妥当性について、ICCVAM の選定した 78 種の参照物質を用いて検証試験が行われた。検証の結果、ICCVAM の評価報告書ではこの試験法の正確性と信頼性を高く評価しており、行政規制の試験法としても、既存の ER TA 法（hERα-HeLa STTA : OECD TG455）と同等であると認めている。

ただし、本試験法では検証試験において、試験施設間にある程度の結果の相違が見られている。そのような場合、再試験を行うなどして、試験施設における試験操作上の問題の有無について明らかにすべきであった。また、参照物質として全 78 物質が規定されていたが、その全てについて全参加試験施設のデータが比較検討されたわけではなかった。一部の物質は、反応が不明確であるとして評価から除外されている。その理由について検討すべきであった。

本試験法は、ヒト卵巣癌由来細胞株 BG1 細胞を起源としており、ER は細胞に内在するものを利用しているので、ヒト ER の反応が細胞内にあるがままで観察できることが利点とされている。しかし、この細胞には ERα と ERβ の双方が存在し、それらが試験による TA 反応のどの程度ずつを担っているかが不明である。これは、ICCVAM 報告書にも述べてあるとおり、今後の、しかし至急の研究課題である。

Table 1. Reference Substaces Tested for ER TA Activity

Substance	CASRN	MeSH Chemical Class[a]	Product Class[b]	Purity (%)	Manufacturer
12-*O*-Tetradecanoylphorbol-13-acetate	16561-29-8	Hydrocarbon (Cyclic)	Laboratory Chemical	>99.5	LC Laboratories
17α-Estradiol	57-91-0	Steroid	Pharmaceutical, Veterinary Agent	99.5	Sigma-Aldrich Corporation
17α-Ethinyl estradiol	57-63-6	Steroid	Pharmaceutical, Veterinary Agent	≥98.0	Sigma-Aldrich Corporation
17ß-Estradiol	50-28-2	Steroid	Pharmaceutical, Veterinary Agent	98.0	Sigma-Aldrich Corporation
17ß-Trenbolone	10161-33-8	Steroid	Pharmaceutical	96.6	Spectrum Chemicals & Laboratory Products
19-Nortestosterone	434-22-0	Steroid	Pharmaceutical, Veterinary Agent	98.0	Toronto Research Chemicals, Inc. (TRC)
2-*sec*-Butylphenol	89-72-5	Phenol	Chemical Intermediate, Pesticide Intermediate	98.0	Sigma-Aldrich Corporation

Substance	CASRN	MeSH Chemical Class[a]	Product Class[b]	Purity (%)	Manufacturer
2,4,5-Trichlorophenoxyacetic acid	93-76-5	Carboxylic Acid	Herbicide	99.3	Sigma-Aldrich Corporation
4-Androstenedione	63-05-8	Steroid	Pharmaceutical	98.6	Sigma-Aldrich Corporation/ Hiyoshi-International Laboratory USA
4-Cumylphenol	599-64-4	Phenol	Chemical Intermediate	99.9	Sigma-Aldrich Corporation
4-Hydroxytamoxifen	68047-06-3	Hydrocarbon (Cyclic)	Pharmaceutical	99.5	Sigma-Aldrich Corporation
4-Hydroxyandrostenedione	566-48-3	Steroid	Pharmaceutical	99.6	Sigma-Aldrich Corporation
4-*tert*-Octylphenol	140-66-9	Phenol	Chemical Intermediate, Pharmaceutical Intermediate	99.3	Chem Service, Inc.
5α-Dihydrotestosterone	521-18-6	Steroid	Pharmaceutical	≥97.5	Sigma-Aldrich Corporation
Actinomycin D	50-76-0	Heterocyclic Compound, Polycyclic Compound	Laboratory Chemical, Pharmaceutical, Veterinary Agent	99.7	USB Corporation
Ammonium perchlorate	7790-98-9	Amine, Onium Compound	Industrial Chemical, Laboratory Chemical, Pharmaceutical	100.0	Sigma-Aldrich Corporation
Apigenin	520-36-5	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate	>99.0	Sigma-Aldrich Corporation
Apomorphine	58-00-4	Heterocyclic Compound	Pharmaceutical, Veterinary Agent	99.8	Sigma-Aldrich Corporation
Atrazine	1912-24-9	Heterocyclic Compound	Herbicide	98.0	Chem Service, Inc.
Bicalutamide	90357-06-5	Amide	Pharmaceutical	>99.5	LKT Laboratories, Inc.
Bisphenol A	80-05-7	Phenol	Chemical Intermediate, Flame Retardant, Fungicide	97.0	Sigma-Aldrich Corporation
Bisphenol B	77-40-7	Phenol	Chemical Intermediate, Flame Retardant, Fungicide	97.4	City Chemical LLC

Substance	CASRN	MeSH Chemical Class[a]	Product Class[b]	Purity (%)	Manufacturer
Butylbenzyl phthalate	85-68-7	Carboxylic Acid, Ester, Phthalic Acid	Plasticizer, Industrial Chemical	98.0	Sigma-Aldrich Corporation
Chrysin	480-40-0	Flavonoid, Heterocyclic Compound	Natural Product	99.8	Sigma-Aldrich Corporation
Clomiphene citrate	50-41-9	Amine, Carboxylic Acid, Heterocyclic Compound	Pharmaceutical	100.0	Sigma-Aldrich Corporation
Corticosterone	50-22-6	Steroid	Pharmaceutical	99.0	Sigma-Aldrich Corporation
Coumestrol	479-13-0	Heterocyclic Compound	Natural Product	98.0	BIOMOL International, Inc.
Cycloheximide	66-81-9	Heterocyclic Compound	Fungicide, Pharmaceutical, Veterinary Agent	99.0	Sigma-Aldrich Corporation
Cyproterone acetate	427-51-0	Steroid	Pharmaceutical	99.6	Sigma-Aldrich Corporation
Daidzein	486-66-8	Flavonoid, Heterocyclic Compound	Natural Product	≥97.5	Alfa Aesar GmbH
Dexamethasone	50-02-2	Steroid	Pharmaceutical, Veterinary Agent	99.0	Sigma-Aldrich Corporation
Di-*n*-butyl phthalate	84-74-2	Ester, Phthalic Acid	Cosmetic Ingredient, Industrial Chemical, Plasticizer	≥98.0	City Chemical LLC
Dibenzo[*a*,*h*]anthracene	53-70-3	Polycyclic Compound	Laboratory Chemical, Natural Product	99.9	Supelco Analytical
Dicofol	115-32-2	Hydrocarbon (Cyclic), Hydrocarbon (Halogenated)	Pesticide	98.0	Chem Service, Inc.
Diethylhexyl phthalate	117-81-7	Phthalic Acid	Pesticide Intermediate, Plasticizer	98.0	Alfa Aesar GmbH
Diethylstilbestrol	56-53-1	Hydrocarbon (Cyclic)	Pharmaceutical, Veterinary Agent	≥99.0	Sigma-Aldrich Corporation
Estrone	53-16-7	Steroid	Pharmaceutical, Veterinary Agent	99.0	Sigma-Aldrich Corporation
Ethyl paraben	120-47-8	Carboxylic Acid, Phenol	Pharmaceutical, Preservative	99.0	Sigma-Aldrich Corporation

Substance	CASRN	MeSH Chemical Class[a]	Product Class[b]	Purity (%)	Manufacturer
Fenarimol	60168-88-9	Heterocyclic Compound, Pyrimidine	Fungicide	99.5	Chem Service, Inc.
Finasteride	98319-26-7	Steroid	Pharmaceutical	>99.0	Sigma-Aldrich Corporation
Flavone	525-82-6	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical	99.7	Sigma-Aldrich Corporation
Fluoranthene	206-44-0	Polycyclic Compound	Industrial Chemical, Laboratory Chemical, Pharmaceutical Intermediate	99.6	Sigma-Aldrich Corporation
Fluoxymestrone	76-43-7	Steroid	Pharmaceutical	>99.0	Sigma-Aldrich Corporation
Flutamide	13311-84-7	Amide	Pharmaceutical, Veterinary Agent	100.0	Sigma-Aldrich Corporation
Genistein	446-72-0	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical	98.8	Sigma-Aldrich Corporation
Haloperidol	52-86-8	Ketone	Pharmaceutical, Veterinary Agent	>99.0	Sigma-Aldrich Corporation
Hydroxyflutamide	52806-53-8	Amide	Pharmaceutical	99.4	LKT Laboratories, Inc.
Kaempferol	520-18-3	Flavonoid, Heterocyclic Compound	Natural Product	99.0	INDOFINE Chemical Company, Inc.
Kepone	143-50-0	Hydrocarbon (Halogenated)	Pesticide	>99.9	Supelco Analytical
Ketoconazole	65277-42-1	Heterocyclic Compound	Pharmaceutical	>99.0	Sigma-Aldrich Corporation
L-Thyroxine	51-48-9	Amino Acid	Pharmaceutical, Veterinary Agent	98.0	Sigma-Aldrich Corporation
Linuron	330-55-2	Urea	Herbicide	99.5	Chem Service, Inc.
Medroxyprogesterone acetate	71-58-9	Steroid	Pharmaceutical	99.0	Sigma-Aldrich Corporation
meso-Hexestrol	84-16-2	Steroid	Pharmaceutical, Veterinary Agent	99.3	City Chemical LLC
Methyl testosterone	58-18-4	Steroid	Pharmaceutical, Veterinary Agent	99.0	Sigma-Aldrich Corporation
Mifepristone	84371-65-3	Steroid	Pharmaceutical	99.1	Sigma-Aldrich Corporation

Substance	CASRN	MeSH Chemical Class[a]	Product Class[b]	Purity (%)	Manufacturer
Morin	480-16-0	Flavonoid, Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate	95.3	TCI America
Nilutamide	63612-50-0	Heterocyclic Compound, Imidazole	Pharmaceutical	100.0	Sigma-Aldrich Corporation
Norethynodrel	68-23-5	Steroid	Pharmaceutical	≥95.0	Research Plus Inc.
o,p'-DDT	789-02-6	Hydrocarbon (Halogenated)	Pesticide	98.9	Chem Service, Inc.
Oxazepam	604-75-1	Heterocyclic Compound	Pharmaceutical, Veterinary Agent	99.5	Sigma-Aldrich Corporation
p-n-Nonylphenol	104-40-5	Phenol	Chemical Intermediate	99.6	Alfa Aesar GmbH
p,p' - Methoxychlor	72-43-5	Hydrocarbon (Halogenated)	Pesticide, Veterinary Agent	99.1	Chem Service, Inc.
p,p'-DDE	72-55-9	Hydrocarbon (Halogenated)	Pesticide Intermediate	99.0	Sigma-Aldrich Corporation
Phenobarbital	50-06-6	Heterocyclic Compound, Pyrimidine	Pharmaceutical, Veterinary Agent	100.0	Spectrum Chemical Manufacturing Corp.
Phenolpthalin	81-90-3	Carboxylic Acid, Phenol	Dye, Laboratory Chemical	95.0	Sigma-Aldrich Corporation
Pimozide	2062-78-4	Heterocyclic Compound	Pharmaceutical	>99.0	Sigma-Aldrich Corporation
Procymidone	32809-16-8	Polycyclic Compound	Fungicide	99.0	Chem Service, Inc.
Progesterone	57-83-0	Steroid	Pharmaceutical, Veterinary Agent	≥99.0	Sigma-Aldrich Corporation
Propylthiouracil	51-52-5	Heterocyclic Compound, Pyrimidine	Pharmaceutical, Veterinary Agent	100.0	Sigma-Aldrich Corporation
Raloxifene HCl	82640-04-8	Hydrocarbon (Cyclic)	Pharmaceutical	100.0	Sigma-Aldrich Corporation
Reserpine	50-55-5	Heterocyclic Compound, Indole	Pharmaceutical, Veterinary Agent	98.0	Sigma-Aldrich Corporation
Resveratrol	501-36-0	Hydrocarbon (Cyclic)	Natural Product	≥99.0	Sigma-Aldrich Corporation
Sodium azide	26628-22-8	Azide, Salt (Inorganic)	Chemical Intermediate, Fungicide, Herbicide	99.7	Sigma-Aldrich Corporation

Substance	CASRN	MeSH Chemical Class[a]	Product Class[b]	Purity (%)	Manufacturer
Spironolactone	52-01-7	Lactone, Steroid	Pharmaceutical	99.7	Sigma-Aldrich Corporation
Tamoxifen	10540-29-1	Hydrocarbon (Cyclic)	Pharmaceutical	≥99.0	Sigma-Aldrich Corporation
Testosterone	58-22-0	Steroid	Pharmaceutical, Veterinary Agent	>99.0	Sigma-Aldrich Corporation
Vinclozolin	50471-44-8	Heterocyclic Compound	Fungicide	99.5	Chem Service, Inc.

Abbreviations: CASRN = CAS Registry Number (American Chemical Society); MeSH = Medical Subject Headings (U.S. National Library of Medicine).

[a] Substances were assigned to one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at http://www.nlm.nih.gov/mesh).

[b] Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

Table 2. 42 ICCVAM-Recommended Substances Used to Evaluate ER Agonist Accuracy

Substance	CASRN	Classification[a]				
		ICCVAM Consensus	BG1Luc ER TA Consensus[b]	XDS	ECVAM	Hiyoshi
17∝-Estradiol	57-91-0	POS	POS	POS (1/1)	POS (3/3)	POS (2/2)
17∝-Ethinyl estradiol	57-63-6	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
17ß-Estradiol	50-28-2	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
19-Nortestosterone	434-22-0	POS	POS	POS (1/1)	NT	NT
4-Cumylphenol	599-64-4	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
4-*tert*-Octylphenol	140-66-9	POS	POS	I (1/1)	POS (1/1)	POS (2/2)
5∝-Dihydrotestosterone	521-18-6	POS	I	I (1/1)	I (1/1)	POS (1/1)
Apigenin	520-36-5	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Atrazine	1912-24-9	NEG	NEG	NEG (3/3)	POS (3/3)	NEG (3/3)
Bicalutamide	90357-06-5	NEG	NEG	NEG (1/1)	NT	NT
Bisphenol A	80-05-7	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Bisphenol B	77-40-7	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Butylbenzyl phthalate	85-68-7	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Chrysin	480-40-0	POS	POS	POS (2/2)	NT	NT
Clomiphene citrate	50-41-9	POS	I	I (1/1)	NEG (1/1)	POS (1/1)
Corticosterone	50-22-6	NEG	NEG	NEG (3/3)	POS (3/3)	NEG (4/4)
Coumestrol	479-13-0	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Daidzein	486-66-8	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Dicofol	115-32-2	POS	POS	POS (1/1)	NEG (1/1)	POS (1/1)
Diethylstilbestrol	56-53-1	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Estrone	53-16-7	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Ethyl paraben	120-47-8	POS	POS	I (1)	POS (1/1)	POS (1/1)
Fenarimol	60168-88-9	POS	POS	POS (1/1)	NT	NT
Flutamide	13311-84-7	NEG	I	I (1)	NT	NT
Genistein	446-72-0	POS	POS	POS (3/3)	POS (3/3)	POS (4/4)
Hydroxyflutamide	52806-53-8	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Kaempferol	520-18-3	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Kepone	143-50-0	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
L-Thyroxine	51-48-9	POS	NEG	NEG (1/1)	NT	NT
Linuron	330-55-2	NEG	NEG	NEG (1/1)	NT	NT
meso-Hexestrol	84-16-2	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Methyl testosterone	58-18-4	POS	POS	POS (3/3)	POS (1/1)	POS (2/2)

Substance	CASRN	Classification[a]				
		ICCVAM Consensus	BG1Luc ER TA Consensus[b]	XDS	ECVAM	Hiyoshi
Norethynodrel	68-23-5	POS	POS	POS (2/2)	POS (1/1)	POS (2/2)
o,p'-DDT	789-02-6	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
p-n-Nonylphenol	104-40-5	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
p,p'-DDE	72-55-9	POS	I	I (1/1)	I (1/1)	NEG (1/1)
p,p'- Methoxychlor	72-43-5	POS	POS	POS (1/1)	POS (1/1)	POS (2/2)
Phenobarbital	50-06-6	NEG	NEG	NEG (1/1)	NEG (1/1)	NT
Procymidone	32809-16-8	NEG	I	I (1/1)	NT	NT
Resveratrol	501-36-0	POS	I	POS (1/1)	I (1/1)	NEG (2/3)
Spironolactone	52-01-7	NEG	NEG	NEG (1/1)	NT	NT
Tamoxifen	10540-29-1	POS	I	I (1/1)	I (1/1)	POS (1/1)

Abbreviations: BG1Luc ER TA = LUMI-CELL BG1Luc4E2 ER TA test method; CASRN = CAS Registry Number (American Chemical Society); ECVAM = European Centre for the Validation of Alternative Methods; I = inadequate (positive or negative classification could not be determined because of poor-quality data); NEG = negative; NT = not tested; POS = positive; XDS = Xenobiotic Detection Systems, Inc.

[a] Number in parentheses represents test results (POS, NEG, or I) over the total number of trials that met test plate acceptance criteria.

[b] BG1Luc ER TA consensus classification represents the majority classification among the three validation laboratories.

Table 3. 25 ICCVAM-Recommended Substances Used to Evaluate ER Antagonist Accuracy

Substance	CASRN	Classification[a]				
		ICCVAM Consensus	BG1Luc ER TA Consensus[b]	XDS	ECVAM	Hiyoshi
17α–Ethinyl estradiol	57-63-6	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
4-Hydroxytamoxifen	68047-06-3	POS	POS	POS (1/1)	I (2/2)	POS (1/1)
5α-Dihydrotestosterone	521-18-6	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Apigenin	520-36-5	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (4/4)
Bisphenol A	80-05-7	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Butylbenzyl phthalate	85-68-7	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (4/4)
Chrysin	480-40-0	NEG	NEG	NEG (1/1)	NT	NT
Coumestrol	479-13-0	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Daidzein	486-66-8	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Di-*n*-butyl phthalate	84-74-2	NEG	NEG	NEG (2/2)	NEG (1/1)	NEG (1/1)
Dicofol	115-32-2	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Diethylhexyl phthalate	117-81-7	NEG	NEG	NEG (1/1)	NEG (2/2)	NEG (1/1)
Diethylstilbestrol	56-53-1	NEG	NEG	NEG (1/1)	NEG (1/1)	POS (1/1)
Genistein	446-72-0	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
Kaempferol	520-18-3	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Kepone	143-50-0	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Mifepristone	84371-65-3	NEG	NEG	NEG (1/1)	NT	NT
Norethynodrel	68-23-5	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
o,p'-DDT	789-02-6	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (4/4)
p-n-Nonylphenol	104-40-5	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
p,p'-DDE	72-55-9	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Progesterone	57-83-0	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
Raloxifene HCl	82640-04-8	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Resveratrol	501-36-0	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
Tamoxifen	10540-29-1	POS	POS	POS (4/4)	POS (3/3)	POS (3/3)

Abbreviations: BG1Luc ER TA = LUMI-CELL BG1Luc4E2 ER TA test method; CASRN = CAS Registry Number (American Chemical Society); ECVAM = European Centre for the Validation of Alternative Methods; I = inadequate (positive or negative classification could not be determined because of poor-quality data); NEG = negative; NT = not tested; POS = positive; XDS = Xenobiotic Detection Systems, Inc.

[a] Number in parentheses represents test results (POS, NEG, or I) over the total number of trials that met test plate acceptance criteria.

[b] BG1Luc ER TA consensus classification represents the majority classification among the three validation laboratories.

Table 4. Accuracy of the BG1LucER TA agonist Data

Laboratory	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
Combined	35[a]	97% (34/35)	96% (27/28)	100% (7/7)	0% (0/7)	4% (1/28)
XDS	34	97% (33/34)	96% (27/28)	100% (6/6)	0% (0/6)	4% (1/28)
ECVAM	29	86% (25/29)	92% (23/25)	50% (2/4)	50% (2/4)	8% (2/25)
Hiyoshi	32	94% (30/32)	93% (27/29)	100% (3/3)	0% (0/3)	7% (2/29)

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; N = number; XDS = Xenobiotic Detection Systems, Inc.

[a] A total of 42 substances were evaluated in the BG1Luc ER TA agonist test method. Seven substances did not produce a consensus classification and were omitted, leaving 35 substances for analysis.

Table 5. Accuracy of the BG1LucER TA antagonist Data

Laboratory	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
Combined	25	100% (25/25)	100% (3/3)	100% (22/22)	0% (0/22)	0% (0/3)
XDS	25	100% (25/25)	100% (3/3)	100% (22/22)	0% (0/22)	0% (0/3)
ECVAM	23	100% (23/23)	100% (3/3)	100% (20/20)	0% (0/20)	0% (0/3)
Hiyoshi	23	96% (22/23)	100% (3/3)	95% (19/20)	5% (1/20)	0% (0/3)

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; N = number; XDS = Xenobiotic Detection Systems, Inc.

Table 6. Agonist E2 EC50 Control Values

Laboratory	Mean	SD	N
E2 Reference Standard EC_{50} (M)			
XDS	1.1×10^{-11}	6.7×10^{-12}	93
ECVAM	1.1×10^{-11}	1.9×10^{-11}	60
Hiyoshi	8.0×10^{-12}	2.8×10^{-12}	65

Table 7. Antagonist Raloxifene EC50 Control Values

Laboratory	Mean	SD	N
Raloxifene Reference Standard IC_{50} (M)			
XDS	1.1×10^{-9}	5.6×10^{-10}	79
ECVAM	1.3×10^{-9}	5.6×10^{-10}	62
Hiyoshi	1.2×10^{-9}	2.9×10^{-10}	53

Table 8. Interlaboratory Agreement for phase 2 Test Substances

Results Among Laboratories	Agonist Testing	Antagonist Testing
Agreement Among Laboratories	8/12 (67%)	12/12 (100%)
+++	8/12	2/12
−−−	0/12	10/12
Discordance Among Laboratories	4/12 (33%)	0/12 (0%)
++−	1/12	0/12
+−−	3/12	0/12

+ denotes a positive test result.

- denotes a negative test result.

+++ indicates that the substance was classified as positive at all three laboratories.

--- indicates that the substance was classified as negative at all three laboratories.

++- indicates that a test substance was classified as positive in two of three laboratories. The substance was classified as negative in the third laboratory.

+-- indicates that the test substance was classified as positive in one of three laboratories.

Table 9. Interlaboratory Agreement for phase 3 substances Tested Once at Each Laboratory

Results Among Laboratories	Agonist Testing	Antagonist Testing
Agreement Among Laboratories	30/36 (83%)	38/41 (93%)
+++	18/36	2/41
---[a]	4/36	33/41
++I	2/36	1/41
--I	6/36	2/41
Discordance Among Laboratories	6/36 (17%)	3/41 (7%)
++-	3/36	0/41
+--	0/36	1/41
+-I	3/36	2/41

Abbreviations: I = inadequate data.

Only those substances that produced a definitive result in at least two of the three laboratories were used in this evaluation. Five substances that produced an inadequate result in two laboratories during agonist testing were not included in this table.

+ denotes a positive test result.

- denotes a negative test result.

+++ indicates that the substance was classified as positive at all three laboratories.

--- indicates that the substance was classified as negative at all three laboratories.

Adopted:
2 October 2012

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists

INTRODUCTION

1. In 1998, the Organisation for Economic Co-operation and Development (OECD) initiated the revision of existing and the development of new Test Guidelines for the screening and testing of Endocrine Disrupting Chemicals. Since that time, several potential assays have been developed into Test Guidelines (TG), with additional assays still under development. These assays are contained within the "OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters" (CF), which was revised in 2012. The original and revised CFs are included as Annexes in the Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (1). The revised CF comprises five levels, each level corresponding to a difference level of biological complexity (1). The BG1Luc Estrogen Receptor Transactivation (BG1Luc ER TA) Test Method for Identifying Estrogen Receptor Agonists and Antagonists is included in level 2 for "*in vitro assays providing data about selected endocrine mechanism(s)/pathway(s) (Mammalian and non mammalian methods)"* (1).

2. *In vitro* TA assays are based upon the production of a reporter gene product induced by a chemical, following binding of the chemical to a specific receptor and subsequent downstream transactivation. TA assays using activation of reporter genes are screening assays that have long been used to evaluate the specific gene expression regulated by specific nuclear receptors, such as the estrogen receptors (ERs) (2) (3) (4) (5). They have been proposed for detection of estrogenic transactivation regulated by the ER (6) (7) (8).

3. In vertebrate species, there are at least two major subtypes of nuclear ERs, α and β, which are encoded by distinct genes. The respective proteins have different biological functions as well as different tissue distributions and ligand binding affinities (9) (10) (11). Nuclear ERα mediates the classic estrogenic response (12) (13) (14) (15), and therefore, most models currently being developed to measure ER activation are specific to ERα. The BG1Luc cell lines predominantly express endogenous ERα and a minor amount of endogenous ERβ (27) (28) (29). This method is being proposed for screening and prioritisation purposes, but can also provide mechanistic information that can be used in a weight of evidence approach.

4. The BG1Luc ER TA test method has been validated by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (16). It utilizes a stably transfected ER responsive luciferase reporter gene in the human ovarian adenocarcinoma cell line, BG-1, to provide concentration-response data for substances with *in vitro* ER agonist or antagonist activity (17). Performance Standards are available to facilitate the development and validation

of similar test methods [(31) for the agonist part, and (32) for the antagonist part]. The Mutual Acceptance of Data will only be guaranteed for test methods, developed according to the Performance Standards, if they have been reviewed and adopted by OECD.

5. Definitions and abbreviations used in this TG are described in Appendix 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

6. The interaction of estrogens with ERs can affect transcription of estrogen-controlled genes, which could lead to the initiation or inhibition of cellular processes, including those necessary for cell proliferation, normal fetal development, and adult homeostasis (18) (19) (20). Perturbation of normal estrogenic systems may have the potential to trigger adverse health effects.

7. This TG describes an assay that uses the BG1Luc4E2 cell line to evaluate TA mediated by both ERα and ERß. TA mediated by the ERs is considered one of the key mechanisms of endocrine disruption (ED), although there are other mechanisms through which ED can occur, including (i) interactions of other receptor and enzymatic systems with the endocrine system, (ii) metabolic activation and/or inactivation of hormones, (iii) distribution of hormones to tissues, and (iv) clearance of hormones from the body. This test method addresses TA induced by chemical binding to the ERs as indicated by the production of luciferase in an *in vitro* system. Thus, results should not be directly extrapolated to the complex signalling and regulation of the intact endocrine system *in vivo*.

8. This TG is applicable to a wide range of substances, provided they can be dissolved in dimethyl sulfoxide (DMSO; CASRN 67-68-5*)*, do not react with DMSO or the cell culture medium, and are not cytotoxic at the concentrations being tested. If use of DMSO is not possible, another vehicle such as ethanol or water may be used (see paragraph 20). The demonstrated performance of the BG1Luc ER TA (ant)agonist test method suggests that data generated with this test method may inform upon ER mediated mechanisms of action, and could be considered for prioritization of substances for further testing.

9. This test method is specifically designed to detect hERα and hERß-mediated TA by measuring chemiluminescence as the endpoint. Chemiluminescence use in bioassays is widespread because luminescence has a high signal-to-background ratio (21). However, the activity of firefly luciferase in cell-based assays can be confounded by compounds that inhibit the luciferase enzyme, causing both apparent inhibition or increased luminescence due to protein stabilization (21). In addition, in some luciferase-based ER reporter gene assays, non-receptor-mediated luminescence signals have been reported at phytoestrogen concentrations higher than 1 μM due to the over-activation of the luciferase reporter gene (2) (22). While the dose-response curve indicates that true activation of the ER system occurs at lower concentrations, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like over-activation of the luciferase reporter gene needs to be examined carefully in stably transfected ER TA assay systems (23).

PRINCIPLE OF THE TEST

10. *In vitro* TA assays using a reporter gene provide mechanistic data. The assay is used to indicate ER ligand binding, followed by translocation of the receptor-ligand complex to the nucleus. In the nucleus, the receptor-ligand complex binds to specific DNA response elements and transactivates the reporter gene (*luc*), resulting in the production of luciferase and the subsequent emission of light, which can be quantified using a luminometer. Luciferase activity can be quickly and inexpensively evaluated with a number of commercially available kits.

11. The BG1Luc ER TA utilizes an ER responsive human ovarian adenocarcinoma cell line, BG-1, which has been stably transfected with a firefly *luc* reporter construct under control of four estrogen response elements placed upstream of the mouse mammary tumor virus promoter (MMTV), to detect substances with *in vitro* ER agonist or antagonist activity. This MMTV promoter exhibits only minor cross-reactivity with other steroid and non-steroid hormones (17). The protocols (agonist and antagonist) for this TG incorporate essential test method components for *in vitro* ER TA assays that were recommended by ICCVAM (8).

12. Criteria for data interpretation are described in detail in paragraphs 51 through 53. Briefly, a positive response is identified by a concentration-response curve containing at least three points with nonoverlapping error bars (mean ± SD), as well as a change in amplitude (normalized relative light unit [RLU]) of at least 20% of the maximal value for the reference substance (17β-estradiol [E2; CASRN 50-28-2] for the agonist assay, raloxifene HCl [Ral; CASRN 84449-90-1]/E2 for the antagonist assay).

PROCEDURE

Cell Line

13. The stably transfected BG1Luc4E2 cell line is used for the assay. The cell line is available with a technical licensing agreement from the University of California, Davis, California, USA[1], and from Xenobiotic Detection Systems Inc., Durham, North Carolina, USA[2].

Stability of the Cell Line

14. To maintain the stability and integrity of the cell line, the cells should be grown for more than one passage from the frozen stock in cell maintenance media (paragraph 16). Cells should not be cultured for more than 30 passages. For the BG1Luc4E2 cell line, 30 passages will be approximately three months.

Cell Culture and Plating Conditions

15. Procedures specified in the Guidance on Good Cell Culture Practice (24) (25) should be followed to assure the quality of all materials and methods in order to maintain the integrity, validity, and reproducibility of any work conducted.

16. BG1Luc4E2 cells are maintained in RPMI 1640 medium supplemented with 0.9% Pen-Strep and 8.0% fetal bovine serum (FBS) in a dedicated tissue culture incubator at 37ºC ± 1ºC, 90% ± 5% humidity, and 5.0% ± 1% CO_2/air.

17. Upon reaching ~80% confluence, BG1Luc4E2 cells are subcultured and conditioned to an estrogen-free environment for 48 hours prior to plating the cells in 96-well plates for exposure to test substances and analysis of estrogen dependent induction of luciferase activity. The estrogen-free medium (EFM) contains Dulbecco's Modification of Eagle's Medium (DMEM) without phenol red, supplemented

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with 4.5% charcoal/dextran-treated FBS, 1.9% L-glutamine, and 0.9% Pen-Strep. All plasticware should be free of estrogenic activity.

Acceptability Criteria

18. Acceptance or rejection of a test is based on the evaluation of reference standard and control results from each experiment conducted on a 96-well plate. Each reference standard is tested in multiple concentrations and there are multiple samples of each reference and control concentration. Results are compared to quality controls (QC) for these parameters that were derived from the agonist and antagonist historical databases generated by each laboratory during the demonstration of proficiency. The historical databases are updated with reference standard and control values on a continuous basis. Changes in equipment or laboratory conditions may necessitate generation of updated historical databases.

Agonist Test

Range Finder Test

- Induction: Plate induction is measured by dividing the average highest E2 reference standard relative light unit (RLU) value by the average DMSO control RLU value. Five-fold induction is usually achieved, but for the purposes of acceptance, induction should be greater than or equal to four-fold.
- DMSO control results: Solvent control RLU values should be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- An experiment that fails either acceptance criterion will be discarded and repeated.

Comprehensive Test

It includes acceptance criteria from the agonist range finder test and the following:

- Reference standard results: The E2 reference standard concentration-response curve should be sigmoidal in shape and have at least three values within the linear portion of the concentration-response curve.
- Positive control results: Methoxychlor control RLU values should be greater than the DMSO mean plus three times the standard deviation from the DMSO mean.
- An experiment that fails any single acceptance criterion will be discarded and repeated.

Antagonist Test

Range Finder Test

- Reduction: Plate reduction is measured by dividing the average highest Ral/E2 reference standard RLU value by the average DMSO control RLU value. Five-fold reduction is usually achieved, but for the purposes of acceptance, reduction should be greater than or equal to three-fold.
- E2 control results: E2 control RLU values should be within 2.5 times the standard deviation of the historical E2 control mean RLU value.
- DMSO control results: DMSO control RLU values should be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- An experiment that fails any single acceptance criterion will be discarded and repeated.

Comprehensive Test

It includes acceptance criteria from the antagonist range finder test and the following:

- Reference standard results: The Ral/E2 reference standard concentration-response curve should be sigmoidal in shape and have at least three values within the linear portion of the concentration-response curve.
- Positive control results: Tamoxifen/E2 control RLU values should be less than the E2 control mean minus three times the standard deviation from the E2 control mean.
- An experiment that fails any single acceptance criterion will be discarded and repeated.

Reference Standards, Positive, and Vehicle Controls

19. Reference standards and controls are listed in paragraphs 20 through 29.

Vehicle Control (Agonist and Antagonist Assays)

20. The vehicle that is used to dissolve the test substances should be tested as a vehicle control. The vehicle used during the validation of the BG1Luc method was 1% v/v dimethyl sulfoxide (DMSO, (CASRN 67-68-5)) (see paragraph 33). If a vehicle other than DMSO is used, all reference standards, controls, and test substances should be tested in the same vehicle, if appropriate.

Reference Standard (Agonist Range Finder)

21. The reference standard is E2 (CASRN 50-28-2). For range finder testing the reference standard is comprised of a serial dilution of four concentrations of E2 (1.84×10^{-10}, 4.59×10^{-11}, 1.15×10^{-11}, and 2.87×10^{-12}M), with each concentration tested in duplicate wells.

Reference Standard (Agonist Comprehensive)

22. E2 for comprehensive testing is comprised of a 1:2 serial dilution consisting of 11 concentrations (ranging from 3.67×10^{-10} to 3.59×10^{-13}M) of E2 in duplicate wells.

Reference Standard (Antagonist Range Finder)

23. The reference standard is a combination of Ral (CASRN 84449-90-1) and E2 (CASRN 50-28-2). Ral/E2 for range finder testing is comprised of a serial dilution of three concentrations of Ral (3.06×10^{-9}, 7.67×10^{-10}, and 1.92×10^{-10}M) plus a fixed concentration (9.18×10^{-11} M) of E2 in duplicate wells.

Reference Standard (Antagonist Comprehensive)

24. Ral/E2 for comprehensive testing is comprised of a 1:2 serial dilution of Ral (ranging from 2.45×10^{-8} to 9.57×10^{-11}M) plus a fixed concentration (9.18×10^{-11} M) of E2 consisting of nine concentrations of Ral/E2 in duplicate wells.

Weak Positive Control (Agonist)

25. The weak positive control is 9.06×10^{-6} M *p,p'*-methoxychlor (methoxychlor; CASRN 72-43-5) in EFM.

Weak Positive Control (Antagonist)

26. The weak positive control consists of tamoxifen (CASRN 10540-29-1) 3.36×10^{-6} M with 9.18×10^{-11} M E2 in EFM.

E2 Control (Antagonist Assay Only)

27. The E2 control is 9.18×10^{-11} M E2 in EFM and used as a base line negative control.

Fold-Induction (Agonist)

28. The induction of luciferase activity of the reference standard (E2) is measured by dividing the average highest E2 reference standard RLU value by the average DMSO control RLU value, and the result should be greater than four-fold.

Fold-Reduction (Antagonist)

29. The mean luciferase activity of the reference standard (Ral/E2) is measured by dividing the average highest Ral/E2 reference standard RLU value by the average DMSO control RLU value and should be greater than three-fold.

Demonstration of Laboratory Proficiency

30. To demonstrate proficiency with the BG1Luc ER TA test method, a laboratory should compile agonist and antagonist historical databases with reference standard and control data generated from at least 10 independent agonist and 10 independent antagonist experiments, conducted on different days. These experiments are the foundation for reference standards and the historical controls. Future acceptable results should be added to enlarge the database. A successful demonstration of proficiency will be achieved by producing values that are no more than 2.5 standard deviations of the historical controls (see paragraph 18).

31. Once the historical databases are compiled, the agonist and antagonist proficiency substances listed in Tables 1 and 2, respectively, should be tested. EC_{50} and IC_{50} values reported in Tables 1 and 2 are provided for information. Laboratories should obtain EC_{50} and IC_{50} values approximating those reported here.

Table 1: Agonist Substances for Demonstration of Laboratory Proficiency

Substance	CASRN	Expected Response[a]	BG1Luc ER TA Mean EC_{50} (M)[b,c]	MeSH Chemical Class[d]	Product Class[e]
Ethyl paraben	120-47-8	POS	2.48×10^{-5}	Carboxylic Acid, Phenol	Pharmaceutical, Preservative
Kaempferol	520-18-3	POS	3.99×10^{-6}	Flavonoid, Heterocyclic Compound	Natural Product
Butylbenzyl phthalate	85-68-7	POS	1.98×10^{-6}	Carboxylic Acid, Ester, Phthalic Acid	Plasticizer, Industrial Chemical
Apigenin	520-36-5	POS	1.60×10^{-6}	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
Daidzein	486-66-8	POS	7.95×10^{-7}	Flavonoid, Heterocyclic Compound	Natural Product
Bisphenol A	80-05-7	POS	5.33×10^{-7}	Phenol	Chemical Intermediate, Flame Retardant, Fungicide
Genistein	446-72-0	POS	2.71×10^{-7}	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
Coumestrol	479-13-0	POS	1.32×10^{-7}	Heterocyclic Compound	Natural Product

Substance	CASRN	Expected Response[a]	BG1Luc ER TA Mean EC_{50} (M)[b,c]	MeSH Chemical Class[d]	Product Class[e]
17∝-Estradiol	57-91-0	POS	1.40×10^{-9}	Steroid	Pharmaceutical, Veterinary Agent
Estrone	53-16-7	POS	2.34×10^{-10}	Steroid	Pharmaceutical, Veterinary Agent
Diethylstilbestrol	56-53-1	POS	3.34×10^{-11}	Hydrocarbon (Cyclic)	Pharmaceutical, Veterinary Agent
17∝-Ethinyl estradiol	57-63-6	POS	7.31×10^{-12}	Steroid	Pharmaceutical, Veterinary Agent
Atrazine	1912-24-9	NEG	-	Heterocyclic Compound	Herbicide
Corticosterone	50-22-6	NEG	-	Steroid	Pharmaceutical
Linuron	330-55-2	NEG	-	Urea	Herbicide
Spironolactone	52-01-7	NEG	-	Lactone, Steroid	Pharmaceutical

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; EC_{50} = half maximal effective concentration of a test substance; MeSH = U.S. National Library of Medicine's Medical Subject Headings; NEG = negative; POS = positive.

[a]ICCVAM consensus data compiled and reported in Independent Scientific Peer Review Panel Report: Evaluation of the LUMI-CELL® ER (BG1Luc ER TA) Test Method (16).

[b]Mean EC_{50} calculated from values reported by the laboratories of the BG1Luc ER TA validation study (26).

[c]Table is sorted in the order of expected EC_{50} (M) of response in the BG1Luc assay.

[d]Substances were assigned into one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at: http://www.nlm.nih.gov/mesh).

[e]Substances were assigned into one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Database (available at: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB)

Table 2: Antagonist Substances for Demonstration of Laboratory Proficiency

Substance	CASRN	Expected Response[a]	BG1Luc ER TA Mean IC_{50} (M)[b,c]	MeSH Chemical Class[d]	Product Class[e]
Tamoxifen	10540-29-1	POS	8.17×10^{-7}	Hydrocarbon (Cyclic)	Pharmaceutical

Substance	CASRN	Expected Response[a]	BG1Luc ER TA Mean IC_{50} (M)[b,c]	MeSH Chemical Class[d]	Product Class[e]
4-Hydroxytamoxifen	68047-06-3	POS	2.08×10^{-7}	Hydrocarbon (Cyclic)	Pharmaceutical
Raloxifene HCl	82640-04-8	POS	1.19×10^{-9}	Hydrocarbon (Cyclic)	Pharmaceutical
17∝- Ethinyl estradiol	57-63-6	NEG	-	Steroid	Pharmaceutical, Veterinary Agent
Apigenin	520-36-5	NEG	-	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
Chrysin	480-40-0	NEG	-	Flavonoid, Heterocyclic Compound	Natural Product
Coumestrol	479-13-0	NEG	-	Heterocyclic Compound	Natural Product
Genistein	446-72-0	NEG	-	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
Kaempferol	520-18-3	NEG	-	Flavonoid, Heterocyclic Compound	Natural Product
Resveratrol	501-36-0	NEG	-	Hydrocarbon (Cyclic)	Natural Product

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; IC_{50} = half maximal inhibitory concentration; MeSH = U.S. National Library of Medicine's Medical Subject Headings; NEG = negative; POS = positive.

[a]ICCVAM consensus data compiled and reported in Independent Scientific Peer Review Panel Report: Evaluation of the LUMI-CELL® ER (BG1Luc ER TA) Test Method (16).

[b]Mean IC_{50} calculated from values reported by the laboratories of the BG1Luc ER TA validation study.

[c]Table is sorted in the order of expected IC_{50} (M) of response in the BG1Luc assay.

[d]Substances were assigned into one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at: http://www.nlm.nih.gov/mesh).

[e]Substances were assigned into one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Database (available at: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB)

32. For each proficiency substance, starting concentrations should first be selected based on range finder test results (paragraphs 42 and 43), and then at least two comprehensive tests conducted. Each

comprehensive test should be conducted on a separate experimental day. If the results of the tests contradict each other (e.g., one test is positive, the other negative), or if one of the tests is inadequate, a third additional test should be conducted. Proficiency is demonstrated by correct classification (positive/negative) of each proficiency substance (see Tables 1, 2, and 3). Proficiency testing should be repeated by each technician learning the test methods.

Vehicle

33. Test substances should be dissolved in a solvent that solubilises that test substance and is miscible with the cell medium. Water, ethanol (95% to 100% purity) and DMSO are suitable vehicles. If DMSO is used, the level should not exceed 1.0% (v/v). For any vehicle, it should be demonstrated that the maximum volume used is not cytotoxic and does not interfere with assay performance. Reference standards and controls are dissolved in 100% solvent and then diluted down to appropriate concentrations in EFM.

Preparation of Test Substances

34. Test substances are dissolved in 100% DMSO (or appropriate solvent), and then diluted down to appropriate concentrations in EFM. All test substances should be allowed to equilibrate to room temperature before being dissolved and diluted. Test substance solutions should be prepared fresh for each experiment. Solutions should not have noticeable precipitate or cloudiness. Reference standard and control stocks may be prepared in bulk however, final reference standard, control dilutions and test substances should be freshly prepared for each experiment and used within 24 hours of preparation.

Solubility and Cytotoxicity: Considerations for Range Finding

35. Range finder testing consists of seven point, 1:10 serial dilutions run in duplicate. Initially, test substances are tested up to the maximum concentration of 1 mg/ml (~1 mM) for agonist testing and 20 μg/mL (~10 μM) for antagonist testing.

36. Range finder experiments are used to determine the following:

- Test substance starting concentrations to be used during comprehensive testing
- Test substance dilutions (1:2 or 1:5) to be used during comprehensive testing

37. An assessment of cell viability/cytotoxicity is included in the agonist and antagonist test method protocols and is incorporated into range finder and comprehensive testing. The cytotoxicity method that was used to assess cell viability during the validation of the BG1Luc ER TA (16) was a scaled qualitative visual observation method, however, a quantitative method for the determination of cytotoxicity can be used (see protocol (30)). Data from test substance concentrations that cause more than 20% reduction in viability cannot be used.

Test Substance Exposure and Assay Plate Organization

38. Cells are counted and plated into 96-well tissue culture plates (2×10^5 cells per well) in EFM and incubated for 24 hours to allow the cells to attach to the plate. The EFM is removed and replaced with test and reference chemicals in EFM and incubated for 19-24 hours.

39. Special considerations will need to be applied to those compounds that are highly volatile since nearby control wells may generate false positive results. In such cases, "plate sealers" may help to effectively isolate individual wells during testing, and is therefore recommended in such cases.

Range Finder Tests

40. Range finder testing uses all wells of the 96-well plate to test up to six substances as seven point 1:10 serial dilutions in duplicate (see Figures 1 and 2).

- *Agonist* range finder testing uses four concentrations of E2 in duplicate as the reference standard and four replicate wells for the DMSO control.
- *Antagonist* range finder testing uses three concentrations of Ral/E2 with 9.18×10^{-11} M E2 in duplicate as the reference standard, with three replicate wells for the E2 and DMSO controls.

Figure 1: Agonist Range Finder Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS1-1	TS1-1	TS2-1	TS2-1	TS3-1	TS3-1	TS4-1	TS4-1	TS5-1	TS5-1	TS6-1	TS6-1
B	TS1-2	TS1-2	TS2-2	TS2-2	TS3-2	TS3-2	TS4-2	TS4-2	TS5-2	TS5-2	TS6-2	TS6-2
C	TS1-3	TS1-3	TS2-3	TS2-3	TS3-3	TS3-3	TS4-3	TS4-3	TS5-3	TS5-3	TS6-3	TS6-3
D	TS1-4	TS1-4	TS2-4	TS2-4	TS3-4	TS3-4	TS4-4	TS4-4	TS5-4	TS5-4	TS6-4	TS6-4
E	TS1-5	TS1-5	TS2-5	TS2-5	TS3-5	TS3-5	TS4-5	TS4-5	TS5-5	TS5-5	TS6-5	TS6-5
F	TS1-6	TS1-6	TS2-6	TS2-6	TS3-6	TS3-6	TS4-6	TS4-6	TS5-6	TS5-6	TS6-6	TS6-6
G	TS1-7	TS1-7	TS2-7	TS2-7	TS3-7	TS3-7	TS4-7	TS4-7	TS5-7	TS5-7	TS6-7	TS6-7
H	E2-1	E2-2	E2-3	E2-4	VC	VC	VC	VC	E2-1	E2-2	E2-3	E2-4

Abbreviations: E2-1 to E2-4 = concentrations of the E2 reference standard (from high to low); TS1-1 to TS1-7 = concentrations (from high to low) of test substance 1 (TS1); TS2-1 to TS2-7 = concentrations (from high to low) of test substance 2 (TS2); TS3-1 to TS3-7 = concentrations (from high to low) of test substance 3 (TS3); TS4-1 to TS4-7 = concentrations (from high to low) of test substance 4 (TS4); TS5-1 to TS5-7 = concentrations (from high to low) of test substance 5 (TS5); TS6-1 to TS6-7 = concentrations (from high to low) of test substance 6 (TS6); VC = vehicle control (DMSO [1% v/v EFM.]).

Figure 2: Antagonist Range Finder Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS1-1	TS1-1	TS2-1	TS2-1	TS3-1	TS3-1	TS4-1	TS4-1	TS5-1	TS5-1	TS6-1	TS6-1
B	TS1-2	TS1-2	TS2-2	TS2-2	TS3-2	TS3-2	TS4-2	TS4-2	TS5-2	TS5-2	TS6-2	TS6-2

C	TS1-3	TS1-3	TS2-3	TS2-3	TS3-3	TS3-3	TS4-3	TS4-3	TS5-3	TS5-3	TS6-3	TS6-3
D	TS1-4	TS1-4	TS2-4	TS2-4	TS3-4	TS3-4	TS4-4	TS4-4	TS5-4	TS5-4	TS6-4	TS6-4
E	TS1-5	TS1-5	TS2-5	TS2-5	TS3-5	TS3-5	TS4-5	TS4-5	TS5-5	TS5-5	TS6-5	TS6-5
F	TS1-6	TS1-6	TS2-6	TS2-6	TS3-6	TS3-6	TS4-6	TS4-6	TS5-6	TS5-6	TS6-6	TS6-6
G	TS1-7	TS1-7	TS2-7	TS2-7	TS3-7	TS3-7	TS4-7	TS4-7	TS5-7	TS5-7	TS6-7	TS6-7
H	Ral-1	Ral-2	Ral-3	VC	VC	VC	E2	E2	E2	Ral-1	Ral-2	Ral-3

Abbreviations: E2 = E2 control; Ral-1 to Ral-3 = concentrations of the Raloxifene/E2 reference standard (from high to low); TS1-1 to TS1-7 = concentrations (from high to low) of test substance 1 (TS1); TS2-1 to TS2-7 = concentrations (from high to low) of test substance 2 (TS2); TS3-1 to TS3-7 = concentrations (from high to low) of test substance 3 (TS3); TS4-1 to TS4-7 = concentrations (from high to low) of test substance 4 (TS4); TS5-1 to TS5-7 = concentrations (from high to low) of test substance 5 (TS5); TS6-1 to TS6-7 = concentrations (from high to low) of test substance 6 (TS6); VC = vehicle control (DMSO [1% v/v EFM.]).
Note: All test compounds are tested in the presence of 9.18×10^{-11} M E2.

41. The recommended final volume of media required for each well is 200 µL. Only use test plates in which the cells in all wells give a viability of 80% and above.

42. Determination of starting concentrations for comprehensive ***agonist*** testing is described in depth in the agonist protocol (30). Briefly, the following criteria are used:

- If there are no points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control, comprehensive testing will be conducted using an 11-point 1:2 serial dilution starting at the maximum soluble concentration.
- If there are points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control, the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one log higher than the concentration giving the highest adjusted RLU value in the range finder. The 11-point dilution scheme will be based on either 1:2 or 1:5 dilutions according to the following criteria:

  An 11-point 1:2 serial dilution should be used if the resulting concentration range will encompass the full range of responses based on the concentration response curve generated in the range finder test. Otherwise 1:5 dilution should be used.

- If a substance exhibits a biphasic concentration response curve in the range finder test, both phases should also be resolved in comprehensive testing.

43. Determination of starting concentrations for comprehensive ***antagonist*** testing is described in depth in the antagonist protocol (30). Briefly, the following criteria are used:

- If there are no points on the test substance concentration curve that are less than the mean minus three times the standard deviation of the E2, control comprehensive testing will be

conducted using an 11-point 1:2 serial dilution starting at the maximum soluble concentration.

- If there are points on the test substance concentration curve that are less than the mean minus three times the standard deviation of the E2 control, the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one of the following:
  - The concentration giving the lowest adjusted RLU value in the range finder
  - The maximum soluble concentration (See antagonist protocol (30), Figure 14-2)
  - The lowest cytotoxic concentration (See antagonist protocol (30), Figure 14-3 for a related example).
- The 11-point dilution scheme will be based on either a 1:2 or 1:5 serial or dilution according to the following criteria:

  An 11-point 1:2 serial dilution should be used if the resulting concentration range will encompass the full range of responses based on the concentration response curve generated in the range finder test. Otherwise a 1:5 dilution should be used.

Comprehensive Tests

44. Comprehensive testing consists of 11-point serial dilutions (either 1:2 or 1:5 serial dilutions based on the starting concentration for comprehensive testing criteria) with each concentration tested in triplicate wells of the 96-well plate (see Figures 3 and 4).

- *Agonist* comprehensive testing uses 11 concentrations of E2 in duplicate as the reference standard. Four replicate wells for the DMSO control and four replicate wells for the methoxychlor control (9.06×10^{-6} M) are included on each plate.
- *Antagonist* comprehensive testing uses nine concentrations of Ral/E2 with 9.18×10^{-11} M E2 in duplicate as the reference standard, with four replicate wells for the E2 9.18×10^{-11} M control, four replicate wells for DMSO controls, and four replicate wells for tamoxifen 3.36×10^{-6}M.

Repeat comprehensive tests for the same chemical should be conducted on different days, to ensure independence. At least two comprehensive tests should be conducted. If the results of the tests contradict each other (e.g., one test is positive, the other negative), or if one of the tests is inadequate, a third additional test should be conducted.

Figure 3: Agonist Comprehensive Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
B	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
C	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
D	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	VC

E	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Meth
F	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Meth
G	E2-1	E2-2	E2-3	E2-4	E2-5	E2-6	E2-7	E2-8	E2-9	E2-10	E2-11	Meth
H	E2-1	E2-2	E2-3	E2-4	E2-5	E2-6	E2-7	E2-8	E2-9	E2-10	E2-11	Meth

Abbreviations: TS11-1 to TS1-11 = concentrations (from high to low) of test substance 1; TS2-1 to TS2-11 = concentrations (from high to low) of test substance 2; E2-1 to E2-11 = concentrations of the E2 reference standard (from high to low); Meth = p,p' methoxychlor weak positive control; VC = DMSO (1% v/v) EFM vehicle control

Figure 4: Antagonist Comprehensive Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
B	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
C	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
D	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	VC
E	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Tam
F	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Tam
G	Ral-1	Ral-2	Ral-3	Ral-4	Ral-5	Ral-6	Ral-7	Ral-8	Ral-9	E2	E2	Tam
H	Ral-1	Ral-2	Ral-3	Ral-4	Ral-5	Ral-6	Ral-7	Ral-8	Ral-9	E2	E2	Tam

Abbreviations: E2 = E2 control; Ral-1 to Ral-9 = concentrations of the Raloxifene/E2 reference standard (from high to low); Tam = Tamoxifen/E2 weak positive control; TS1-1 to TS1-11 = concentrations (from high to low) of test substance 1 (TS1); TS2-1 to TS2-11 = concentrations (from high to low) of test substance 2 (TS2); VC = vehicle control (DMSO [1% v/v EFM.]).

Note: As noted, all reference and test wells contain a fixed concentration of E2 (9.18×10^{-11}M)

Measure of Luminescence

45. Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and with software that controls the injection volume and measurement interval (30). Light emission from each well is expressed as RLU per well.

ANALYSIS OF DATA

EC_{50}/IC_{50} Determination

46. The EC_{50} value (half maximal effective concentration of a test substance [agonists]) and the IC_{50} value (half maximal inhibitory concentration of a test substance [antagonists]) are determined from the concentration-response data. For substances that are positive at one or more concentrations, the concentration of test substance that causes a half-maximal response (IC_{50} or EC_{50}) is calculated using a Hill function analysis or an appropriate alternative. The Hill function is a four-parameter logistic mathematical model relating the substance concentration to the response (typically following a sigmoidal curve) using the equation below:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\lg EC50 - X)\text{HillSlope}}}$$

where Y = response (i.e., RLUs); X = the logarithm of concentration; Bottom = the minimum response; Top = the maximum response; lg EC_{50} (or lg IC_{50}) = the logarithm of X as the response midway between Top and Bottom; and Hillslope describes the steepness of the curve. The model calculates the best fit for the Top, Bottom, Hillslope, and IC_{50} and EC_{50} parameters. For the calculation of EC_{50} and IC_{50} values, appropriate statistical software should be used (e.g. Graphpad Prism® statistical software).

Determination of Outliers

47. Good statistical judgment could be facilitated by including (but not limited to) the Q-test (see agonist and antagonist protocols (30)), for determining "unusable" wells that will be excluded from the data analysis.

48. For E2 reference standard replicates (sample size of two), any adjusted RLU value for a replicate at a given concentration of E2 is considered an outlier if its value is more than 20% above or below the adjusted RLU value for that concentration in the historical database.

Collection and Adjustment of Luminometer Data for Range Finder Testing

49. Raw data from the luminometer are transferred to a spreadsheet template designed for the test method. It should be determined whether there are outlier data points that need to be removed. (See Test Acceptance Criteria for parameters that are determined in the analyses). The following calculations are performed:

Agonist

Step 1 Calculate the mean value for the DMSO vehicle control (VC).
Step 2 Subtract the mean value of the DMSO VC from each well value to normalize the data.
Step 3 Calculate the mean fold induction for the reference standard (E2).
Step 4 Calculate the mean EC_{50} value for the test substances.

Antagonist

Step 1 Calculate the mean value for the DMSO VC.
Step 2 Subtract the mean value of the DMSO VC from each well value to normalize the data.
Step 3 Calculate the mean fold reduction for the reference standard (Ral/E2).
Step 4 Calculate the mean value for the E2 reference standard.
Step 5 Calculate the mean IC_{50} value for the test substances.

Collection and Adjustment of Luminometer Data for Comprehensive Testing

50. Raw data from the luminometer are transferred to a spreadsheet template designed for the test method. Determine whether there are outlier data points that need to be removed. (See Test Acceptance Criteria for parameters that are determined in the analyses). The following calculations are performed:

Agonist

Step 1 Calculate the mean value for the DMSO VC.
Step 2 Subtract the mean value of the DMSO VC from each well value to normalize the data.
Step 3 Calculate the mean fold induction for the reference standard (E2).
Step 4 Calculate the mean EC_{50} value for E2 and the test substances.
Step 5 Calculate the mean adjusted RLU value for methoxychlor.

Antagonist

Step 1 Calculate the mean value for the DMSO VC.
Step 2 Subtract the mean value of the DMSO VC from each well value to normalize the data.
Step 3 Calculate the mean fold induction for the reference standard (Ral/E2).
Step 4 Calculate the mean IC_{50} value for Ral/E2 and the test substances.
Step 5 Calculate the mean adjusted RLU value for tamoxifen.
Step 6 Calculate the mean value for the E2 reference standard.

Data Interpretation Criteria

51. The BG1Luc ER TA is intended as part of a weight of evidence approach to help prioritize substances for ED testing in vivo. Part of this prioritization procedure will be the classification of the test substance as positive or negative for either ER agonist or antagonist activity. The positive and negative decision criteria used in the BG1Luc ER TA validation study are described in Table 3.

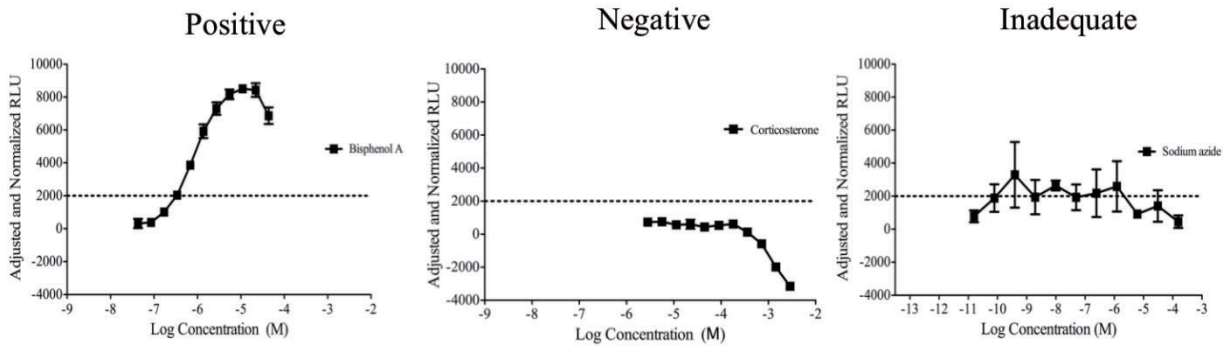
Table 3: Positive and Negative Decision Criteria

AGONIST ACTIVITY	
Positive	– All test substances classified as *positive for* ER agonist activity should have a concentration–response curve consisting of a baseline, followed by a positive slope, and concluding in a plateau or peak. In some cases, only two of these characteristics (baseline–slope or slope–peak) may be defined. – The line defining the positive slope should contain at least three points with non-overlapping error bars (mean ± SD). Points forming the baseline are excluded, but the linear portion of the curve may include the peak or first point of the plateau. – A positive classification requires a response amplitude, the difference between baseline and peak, of at least 20% of the maximal value for the reference substance, E2 (i.e., 2000 RLUs or more when the maximal response value of the reference substance [E2] is adjusted to 10,000 RLUs). – If possible, an EC_{50} value should be calculated for each positive substance.
Negative	The average adjusted RLU for a given concentration is at or below the mean DMSO control RLU value plus three times the standard deviation of the DMSO RLU.
Inadequate	Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test substance is positive or negative. Substance should be retested.

ANTAGONIST ACTIVITY	
Positive	– Test substance data produce a concentration-response curve consisting of a baseline, which is followed by a negative slope. – The line defining the negative slope should contain at least three points with non-overlapping error bars; points forming the baseline are excluded but the linear portion of the curve may include the first point of the plateau. – There should be at least a 20% reduction in activity from the maximal value for the reference substance, Ral/E2 (i.e., 8000 RLU or less when the maximal response value of the reference substance [Ral/E2] is adjusted to 10,000 RLUs). – The highest non-cytotoxic concentrations of the test substance should be less than or equal to $1x10^{-5}$ M. – If possible, an IC_{50} value should be calculated for each positive substance.
Negative	All data points are above the ED_{80} value (80% of the E2 response, or 8000 RLUs), at concentrations less than 1.0×10^{-5} M.
Inadequate	Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test substance is positive or negative. Substance should be retested.

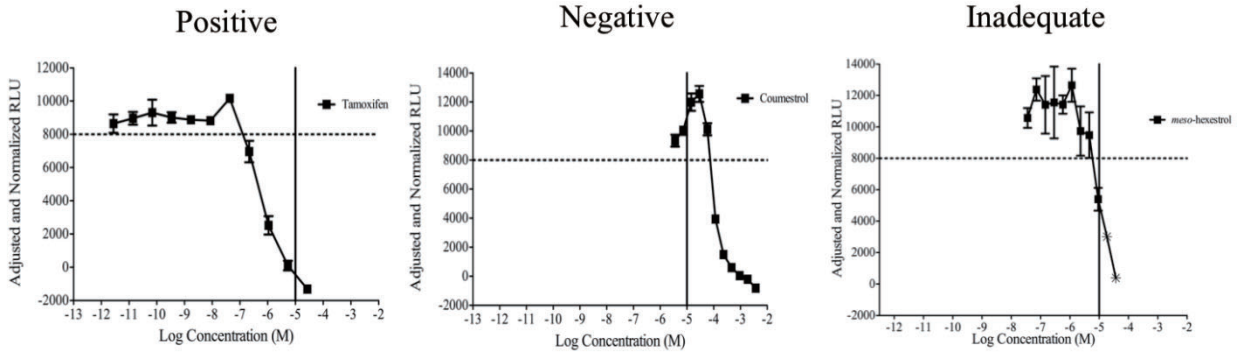
52. Positive results will be characterized by both the magnitude of the effect and the concentration at which the effect occurs, where possible. Examples of positive, negative, and inadequate data are shown in Figures 5 and 6.

Figure 5: Agonist Examples of Positive, Negative and Inadequate Data



Dashed line indicates 20% of E2 response, 2000 adjusted and normalized RLUs.

Figure 6: Antagonist Examples of Positive, Negative, and Inadequate Data



Dashed line indicates 80% of Ral/E2 response, 8000 adjusted and normalized RLUs.

Solid line indicates 1.00×10^{-5} M. For a response to be considered positive, it should be below the 8000 RLU line, and at concentrations less than 1.00×10^{-5}M.

Asterixed concentrations in the meso-hexestrol graph indicate viability scores of "2" or greater.

The test results for *meso*-hexestrol are considered inadequate data because the only response that is below 8,000 RLU occurs at 1.00×10^{-5}M.

53. The calculations of EC_{50} and IC_{50} can be made using a four-parameter Hill Function (See agonist protocol and antagonist protocol (30) for more details). Meeting the acceptability criteria indicates the assay system is operating properly, but it does not ensure that any particular run will produce accurate data. Duplicating the results of the first run is the best assurance that accurate data were produced.

Test Report

54. The test report should contain the following information:

Test substance and control test substances:

- identification data (*e.g.* CAS number, if available; source; purity; known impurities; lot number);
- physical nature and physicochemical properties (*e.g.* volatility, stability, solubility);
- if mixture, composition and relative percentages of components.

Cells:

- source of cells;
- passage number of cells at thawing;
- number of cell passages (from thawing);
- methods for maintenance of cell cultures.

Test conditions:

- cytotoxicity data and solubility limitations;
- concentration of test substance;
- volume of vehicle and test substance added;

- incubation temperature, humidity, and CO_2 concentration;
- duration of treatment;
- cell density during treatment.

Acceptability check (See agonist protocol and antagonist protocol (30) for more details):

For range finder tests:
- DMSO control RLU values (mean, SD, CV);
- fold inductions or reductions for each assay plate;
- E2 control values (antagonist assay only);
- did experiment pass or fail acceptance; if fail, what criteria were failed;

For comprehensive experiments:
- DMSO control RLU values (mean, SD, CV);
- fold inductions or reductions for each assay plate;
- positive control results;
- reference standard results;
- E2 control results (antagonist assay only)
- did experiment pass or fail acceptance; if fail, what criteria were failed;

Results:

- raw and normalised data of luminescent signals;
- dilution (1:2 or 1:5) used for each test substance;
- were test substance results positive, negative, or inadequate;
- IC_{50}/EC_{50} values, if appropriate;
- statistical analyses, if any, together with a measures of error and confidence (e.g., SEM, SD, CV or 95% CI) and a description of how these values were obtained.

Discussion of results:

Conclusion:

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http://iccvam.niehs.nih.gov/docs/endo_docs/ERTA-TMER/AppxB1-AgonProtocol.pdf
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APPENDIX 1

DEFINITIONS AND ABBREVIATIONS

Acceptability criteria: Minimum standards for the performance of experimental controls and reference standards. All acceptability criteria should be met for an experiment to be considered valid.

Accuracy: (a) The closeness of agreement between a test method result and an accepted reference value. (b) The proportion of correct outcomes of a test method.

Agonist: A substance that produces a response, e.g., transcription, when it binds to a specific receptor.

Antagonist: A substance that inhibits a response, e.g., transcription, when it binds to a specific receptor.

BG-1: Immortalized human ovarian adenocarcinoma cells that endogenously express estrogen receptors alpha and beta.

BG-1Luc4E2: The BG-1Luc4E2 cell line was derived from BG-1 immortalized adenocarcinoma cells that endogenously express both forms of the estrogen receptor (ERα and ERβ) and have been stably transfected with the plasmid pGudLucERE. This plasmid contains four copies of a synthetic oligonucleotide containing the estrogen response element upstream of the mouse mammary tumor viral (MMTV) promoter and the firefly luciferase gene.

Cell morphology: The shape and appearance of cells grown in a monolayer in a single well of a tissue culture plate. Cells that are dying often exhibit abnormal cell morphology.

CF: The OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupters.

Charcoal/dextran treatment: Treatment of serum used in cell culture. Treatment with charcoal/dextran (often referred to as "stripping") removes endogenous hormones and hormone-binding proteins.

Cytotoxicity: The adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function. For most substances, toxicity is a consequence of non-specific alterations in "basal cell functions" (i.e., via mitochondria, plasma membrane integrity, etc.).

DMEM: Dulbecco's Modification of Eagle's Medium

DMSO: Dimethyl sulfoxide

E2: 17β-estradiol

EC_{50}: The half maximal effective concentration of a test substance.

ED: Endocrine disruption

EE: 17α-ethynyl estradiol

EFM: Estrogen-free medium. Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 4.5% charcoal/dextran-treated FBS, 1.9% L-glutamine, and 0.9% Pen-Strep.

ER: Estrogen receptor

ERE: Estrogen response element

FBS: Fetal bovine serum

hERα: Human estrogen receptor alpha

hERß: Human estrogen receptor beta

IC_{50}: The half maximal effective concentration of an inhibitory test substance.

ICCVAM: The Interagency Coordinating Committee on the Validation of Alternative Methods

MMTV: Mouse Mammary Tumor Virus

Proficiency: The demonstrated ability to properly conduct a test method prior to testing unknown substances.

Proficiency Chemicals: A list of substances that can be used by laboratories to demonstrate technical competence with a standardized test method. Selection criteria for these substances typically include that they represent the range of responses, are commercially available, and have high quality reference data available.

Ral: raloxifene HCl

Ral/E2: The antagonist reference standard, which is a combination of raloxifene HCl (Ral) and 17β-estradiol (E2).

Reference standard: a reference substance used to demonstrate the adequacy of a test method. 17β-estradiol is the estrogenic reference standard and Raloxifene HCl the anti-estrogenic reference standard for the BG1Luc ER TA.

Reliability: A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time.

RLU: Relative Light Units

RNA: Ribonucleic Acid

RPMI: RPMI 1640 medium supplemented with 0.9% Pen-Strep and 8.0% fetal bovine serum (FBS)

SD: Standard deviation

Stable transfection: When DNA is transfected into cultured cells in such a way that it is stably integrated into the cells genome, resulting in the stable expression of transfected genes. Clones of stably transfected cells are selected by stable markers (e.g., resistance to G418).

TA: Transactivation

TG: Test Guideline

Transcription: mRNA synthesis

Transactivation: The initiation of mRNA synthesis in response to a specific chemical signal, such as a binding of an estrogen to the estrogen receptor.

Validation: The process by which the reliability and accuracy of a procedure are established for a specific purpose.

VC: The vehicle (DMSO) that is used to dissolve test and control chemicals is tested solely as vehicle without dissolved chemical.

ICCVAM Test Method Evaluation Report
The LUMI-CELL® ER (BG1Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals

Interagency Coordinating Committee on the Validation of Alternative Methods

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Table of Contents

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List of Abbreviations and Acronyms

AR	Androgen receptor
BRD	Background review document
CASRN	CAS Registry Number® (a trademark of the American Chemical Society)
CERI	Chemicals Evaluation and Research Institute, Japan
CV	Coefficient of variation
DMSO	Dimethyl sulfoxide
EAC	Endocrine-active compound
EC_{50}	Half–maximal effective concentration
ECVAM	European Centre for the Validation of Alternative Methods
ED	Endocrine disruptor
EDSP	Endocrine Disruptor Screening Program (U.S. EPA)
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee (U.S. EPA)
EDWG	ICCVAM Interagency Endocrine Disruptor Working Group
EPA	U.S. Environmental Protection Agency
ER	Estrogen receptor
FDA	U.S. Food and Drug Administration
FR	*Federal Register*
FW	Formula weight
GLP	Good Laboratory Practice
I	Inadequate
IC_{50}	Half-maximal inhibitory concentration
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems, Inc.
ISO	International Organization for Standardization
JaCVAM	Japanese Center for the Validation of Alternative Methods
KoCVAM	Korean Center for the Validation of Alternative Methods
M	Molar
Max	Maximum
MeSH®	Medical Subject Headings (U.S. National Library of Medicine)
N	Number; negative
NEG	Negative
NICEATM	National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods

NIEHS	U.S. National Institute of Environmental Health Sciences
NIH	U.S. National Institutes of Health
NT	Not tested
NTP	U.S. National Toxicology Program (U.S. NIH)
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances (U.S. EPA)
PN	Presumed negative
POS	Positive
PP	Presumed positive
RLU	Relative light unit
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SD	Standard deviation
SMT	Study Management Team
STTA	Stably transfected human estrogen receptor-α transcriptional activation
TA	Transcriptional activation
TG	Test Guideline
U.S.C.	United States Code
XDS	Xenobiotic Detection Systems, Inc.

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Preface

Endocrine-active compounds (EACs) are both naturally occurring and synthetic substances. Some may, depending on the dose, interfere with the normal function of hormones in the endocrine system. Public health concerns have resulted largely from studies indicating that animal populations exposed to high levels of these substances, sometimes referred to as endocrine disruptors (EDs), have an increased incidence of reproductive and developmental abnormalities (EPA 1997; NRC 1999). In response to growing concerns about possible adverse health effects in humans exposed to such substances, the U.S. Congress enacted relevant provisions to safeguard public health in the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 301 et seq.); the Food Quality Protection Act (7 U.S.C. 136); and the 1996 Amendments to the Safe Drinking Water Act (110 Stat 1613). The U.S. Environmental Protection Agency (EPA) was required to develop and validate a screening and testing program to identify substances with endocrine-disrupting activity. The EPA subsequently established the Endocrine Disruptor Screening Program (EDSP) and initiated efforts to standardize and validate test methods for inclusion in the EDSP (66 FR 23022). Validation is necessary to assess the usefulness and limitations of a test method for a specific proposed purpose and to characterize the extent to which test methods are sufficiently accurate and reproducible for their intended use (ICCVAM 1997).

In April 2000, the EPA nominated four types of *in vitro* test methods for detecting substances with potential endocrine-disrupting activity for review by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). These included *in vitro* estrogen receptor (ER) and androgen receptor (AR) binding and ER and AR transcriptional activation (TA) test methods. The EPA also asked ICCVAM to develop performance standards that could be used to define acceptable *in vitro* ER and AR binding and TA assays. It was envisioned that these standards would be based on the performance of adequately validated *in vitro* ER- and AR-based assays.

In 2002, the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) prepared background review documents (BRDs) that included all available information on each of the four types of test methods (ICCVAM 2002d, 2002a, 2002c, 2002b). In a public meeting, an independent international expert panel (Panel) reviewed the information on the 137 assays described in the BRDs and concluded that there were no adequately validated *in vitro* ER- or AR-based test methods (ICCVAM 2002e). Based on recommendations from the Panel, ICCVAM published the *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors*, which included a list of reference substances that should be used to validate each of the four types of *in vitro* test methods (ICCVAM 2003a). It also identified essential test method components that should be included in each of the standardized test method protocols used for future validation studies. ICCVAM recommended that future performance standards for these methods be based on test methods that have undergone adequate validation studies using the recommended accuracy chemicals and essential test method components.

In January 2004, Xenobiotic Detection Systems, Inc. (XDS; Durham, NC), nominated the LUMI-CELL® BG1Luc4E2 ER TA test method (BG1Luc ER TA test method) for an interlaboratory validation study. ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) recommended that the BG1Luc ER TA test method be considered a high priority for interlaboratory validation studies due to the lack of adequately validated test methods and the regulatory and public health need for such test methods. NICEATM subsequently led and coordinated an international validation study with its counterparts in Japan (JaCVAM) and Europe (ECVAM), using laboratories sponsored by each validation organization. NICEATM organized a validation Study Management Team (SMT) to oversee the scientific aspects of the

validation study and coordinate the day-to-day activities among the participating laboratories. A representative from the recently established Korean Center for the Validation of Alternative Methods (KoCVAM) joined the SMT in 2010.

ICCVAM reviewed the validation status of the BG1Luc ER TA test method for identification of substances with ER agonist or antagonist activity. NICEATM and the ICCVAM Interagency Endocrine Disruptor Working Group (EDWG) prepared a draft BRD that provided a comprehensive description and the data from the validation study used to assess the accuracy and reliability of the BG1Luc ER TA test method.

NICEATM convened an independent international scientific peer review panel (Panel) that met in public on March 29–30, 2011. The Panel was charged with reviewing the draft BRD for completeness, assessing the extent that established validation and acceptance criteria were adequately addressed, and determining the extent to which the data and information supported draft ICCVAM test method recommendations on the usefulness and limitations of the BG1Luc ER TA test method. The Panel also evaluated the proposed performance standards. The Panel included expert scientists nominated by ECVAM, JaCVAM, and KoCVAM.

ICCVAM considered the conclusions and recommendations of the Panel, along with comments from the public and SACATM, and then finalized the BRD and test method recommendations, which are provided in this test method evaluation report. As required by the ICCVAM Authorization Act (42 U.S.C. 285*l*-3), ICCVAM forwarded this report and recommendations to Federal agencies for their consideration and acceptance decisions where appropriate. The BG1Luc ER TA test method protocol and performance standards were also forwarded to the Organisation for Economic Co-operation and Development (OECD) Test Guidelines Programme for consideration and adoption as international testing guidelines.

We gratefully acknowledge the organizations and scientists who generated and provided data and information for this document, especially the staff at the participating validation laboratories: XDS, Inc., in Durham, North Carolina; Hiyoshi Corporation in Japan; and the In Vitro Methods Unit at ECVAM in Italy. We would also like to recognize the efforts of the individuals who contributed to its preparation, review, and revision. We thank Dr. David Hattan (U.S. Food and Drug Administration) for serving as Chair of the EDWG, as well as the members of the EDWG and ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this test method evaluation report. We also want to thank Dr. Warren Casey, Deputy Director of NICEATM, for his excellent leadership and extensive efforts on this project.

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Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has completed its evaluation of the validation status of the LUMI-CELL® BG1Luc4E2 estrogen receptor (ER) transcriptional activation (TA) test method (hereafter BG1Luc ER TA test method) as a screening test to identify substances with *in vitro* ER agonist and antagonist activity. The BG1Luc ER TA test method uses BG-1 cells, a human ovarian adenocarcinoma cell line that is stably transfected with an estrogen-responsive luminescence (luciferase reporter) gene, to measure whether and how much a substance induces (agonist) or inhibits (antagonist) TA activity via ER-mediated pathways. Such substances could interfere with the normal function of hormones in the endocrine system (i.e., endocrine disruptors), which may lead to abnormal growth, development, or reproduction.

This test method evaluation report provides ICCVAM's recommendations for the BG1Luc ER TA test method based on the results of an international validation study and the demonstrated validity (usefulness and limitations). The report also includes (1) recommendations for future studies, (2) performance standards to evaluate functionally and mechanistically similar test methods, (3) protocols recommended by ICCVAM for future data collection and evaluation of the BG1Luc ER TA test method, and (4) a final background review document (BRD) describing the validation status of this test method.

In 2004, Xenobiotic Detection Systems, Inc. (XDS; Durham, NC), nominated the LUMI-CELL ER test method to ICCVAM for an interlaboratory validation study. ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) recommended that the BG1Luc ER TA test method be considered a high priority for interlaboratory validation studies based on the lack of adequately validated test methods and the regulatory and public health need for such test methods.

When the BG1Luc ER TA validation study was initiated, no *in vitro* ER TA test methods were considered adequately valid for regulatory use. Today, only one *in vitro* ER TA test method is considered adequately validated by national and international agencies, the Organisation for Economic Co-operation and Development (OECD) Stably Transfected Human Estrogen Receptor-α Transcriptional Activation (STTA) Assay for the Detection of Estrogenic Agonist-Activity, described in OECD Chemicals Test Guideline (TG) 455 (OECD 2009). Validated by the Chemicals Evaluation and Research Institute (CERI, Japan), this method has been adopted by the U.S. Environmental Protection Agency (EPA) as OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line [HeLa-9903]) (EPA 2009).

After recommendation by ICCVAM and SACATM, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) led and coordinated an international validation study with its counterparts in Europe (the European Centre for the Validation of Alternative Methods [ECVAM]) and Japan (the Japanese Center for the Evaluation of Alternative Methods [JaCVAM]) to assess the accuracy and reliability of the BG1Luc ER TA test method for the qualitative detection of substances with *in vitro* ER agonist or antagonist activity. The BG1Luc ER TA test method was evaluated using laboratories in the United States (XDS), Europe (ECVAM), and Japan (Hiyoshi Corporation).

The validation study proceeded in four phases. During Phase 1, each of the three participating centers (NICEATM, ECVAM, and JaCVAM) selected validation laboratories. The protocols were reviewed, and the laboratories demonstrated proficiency with the test method by successfully completing 10 replicate agonist and 10 replicate antagonist tests. In Phases 2 through 4, the protocols were evaluated and refined, and 78 ICCVAM reference substances that

should be used to standardize and validate *in vitro* ER and androgen receptor binding and TA test methods were tested.

After this study was completed, NICEATM, ICCVAM, and the ICCVAM Interagency Endocrine Disruptor Working Group (EDWG) prepared a draft BRD and draft test method recommendations. The drafts were provided to an independent international scientific peer review panel (hereafter Panel) and to the public for comment. The Panel met in public session on March 29–30, 2011, to discuss its peer review of the ICCVAM draft BRD and to provide conclusions and recommendations regarding the validation status of the BG1Luc ER TA test method. The Panel also reviewed how well the information contained in the draft BRD supported ICCVAM's draft test method recommendations.

In finalizing this test method evaluation report and the BRD, which is included here as an appendix, ICCVAM considered (1) the conclusions and recommendations of the Panel, (2) comments from SACATM, and (3) public comments.

ICCVAM Recommendations: Test Method Usefulness and Limitations
ICCVAM concludes that the accuracy and reliability of the BG1Luc ER TA test method support its use to screen substances for *in vitro* ER agonist and/or antagonist activity. This determination is based on an evaluation of data from the validation study and the corresponding accuracy and reliability. ICCVAM concludes that the accuracy of this assay is at least equivalent to that of the current ER TA test method included in regulatory testing guidance (EPA OPPTS 890.1300) (EPA 2009).

ICCVAM Recommendations: BG1Luc ER TA Test Method Protocol
For use of the BG1Luc ER TA test method to screen substances for *in vitro* ER agonist and/or antagonist activity, ICCVAM recommends using the ICCVAM BG1Luc ER TA protocols (included here as **Appendices B1** and **B2**). All future studies intended to further characterize the usefulness and limitations of the BG1Luc ER TA test method should use these protocols.

ICCVAM Recommendations: Future Studies
ICCVAM considers the BG1Luc ER TA test method to be valid as described. However, ICCVAM recommends the following for interested parties to further characterize and potentially improve the usefulness and applicability of the BG1Luc ER TA test method:

- Additional validation studies may be performed to determine whether the BG1Luc ER TA test method or other similar assays could replace the rat uterine cytosol ER binding assay.
- Further work may be carried out to determine if the BG1Luc ER TA test method could be combined with other methods (to include *in vitro* metabolic activation) in a weight-of-evidence approach to replace the uterotrophic bioassay.
- Additional studies/evaluations may be conducted to more completely characterize the ratio of ERα and ERβ in the BG-1 cell line and the extent to which these receptor subtypes contribute to the overall performance of the BG1Luc ER TA test method.
- Additional studies/evaluations may be conducted to determine the feasibility of testing volatile substances using CO_2-permeable plastic film or other methods to seal the test plates.
- Additional studies/evaluations may be conducted to determine if substances that are not soluble in dimethyl sulfoxide (DMSO) could be tested in another vehicle that would more adequately dissolve the substance in culture media.
- Additional studies may be conducted to account for metabolic activation that could expand the utility of this and other ER TA test methods.
- As ER antagonists are identified, additional studies/evaluations may be conducted to expand the database of positive substances tested and thereby better characterize the

usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with ER antagonist activity.

ICCVAM encourages users to provide to ICCVAM all data that are generated from future studies. These data could be used to further characterize the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with ER agonist or antagonist activity.

Validation Status of the BG1Luc ER TA Test Method

ICCVAM evaluated the BG1Luc ER TA test method for its ability to correctly identify *in vitro* ER agonists and antagonists. For this analysis, test substance classification (positive or negative for ER agonist/antagonist activity) obtained during the validation study was compared to the ICCVAM reference classification of the same substance, which was based on a preponderance of available data.

The BG1Luc ER TA test method accuracy was evaluated based on several different analyses, but the primary evaluation was based on two comparisons: (1) the extent to which the result of the test method corresponds to the ICCVAM reference classification for each substance and (2) the accuracy of the BG1Luc ER TA test method compared to that of the EPA OPPTS 890.1300/OECD TG 455 (EPA 2009; OECD 2009)[1] assay.

Test Method Accuracy – Agonist Assay

Thirty-five substances (28 positive, 7 negative) were used to evaluate the accuracy of the BG1Luc ER TA agonist assay. The consensus classification obtained from all BG1Luc ER TA tests for these 35 substances yielded the following statistics: concordance of 97% (34/35), sensitivity of 96% (27/28), specificity of 100% (7/7), a false positive rate of 0% (0/7), and a false negative rate of 4% (1/28). Similar results were obtained when the results from each laboratory were used instead of the consensus classification.

EPA OPPTS 890.1300/OECD TG 455 is the only test guideline published by a U.S. regulatory agency for generating ER TA data. Therefore, BG1Luc ER TA test method concordance with EPA OPPTS 890.1300/OECD TG 455 was also evaluated using the 26 reference substances for which data are available from both BG1Luc ER TA and EPA OPPTS 890.1300/OECD TG 455 assays. Accuracy statistics for the two test methods were identical: concordance of 96% (25/26), sensitivity of 95% (21/22), specificity of 100% (4/4), a false positive rate of 0% (0/4), and a false negative rate of 5% (1/22).

Test Method Accuracy – Antagonist Assay

To evaluate the accuracy of the BG1Luc ER TA antagonist assay, 25 substances (3 positive, 22 negative) were used. The consensus classification obtained from all BG1Luc ER TA tests for these 25 substances yielded the following statistics: concordance of 100% (25/25), sensitivity of 100% (3/3), specificity of 100% (22/22), a false positive rate of 0% (0/22), and a false negative rate of 0% (0/3). Similar results were obtained when the results from each laboratory were used instead of the consensus classification.

Because there currently is no valid EPA OPPTS 890.1300/OECD TG 455 antagonist protocol, no comparison with the BG1Luc ER TA antagonist results was conducted.

Concordance with Other Endocrine Disruptor Assays

Although the primary goal of the BG1Luc ER TA test method is to provide a qualitative assessment of estrogenic/anti-estrogenic activity, quantitative measures of activity are usually obtained for positive results. The values obtained from BG1Luc ER TA test results (half-maximal

[1] The EPA OPPTS 890.1300/OECD TG 455 (OECD 2009) assay uses the hERα-HeLa-9903 human cervical cancer cell line to detect estrogen agonist activity mediated through human ER alpha (hERα).

effective concentration [EC_{50}] and half-maximal inhibitory concentration [IC_{50}]), were compared to median values from other ER TA test methods reported in the literature. This comparison found a high correlation. There was 97% (33/34) concordance between the BG1Luc ER TA test method and ER binding data. The only discordant substance (medroxyprogesterone acetate) was positive in the BG1Luc ER TA test method and negative based on ER binding data. Similarly, based on a comparison with available data in the *in vivo* uterotrophic assay, there was 92% (12/13) concordance between the BG1Luc ER TA test method and ER binding data. The only discordant substance (butylbenzyl phthalate) was positive in the BG1Luc ER TA test method and negative based on uterotrophic data.

Test Method Reliability

Intralaboratory reproducibility (whether multiple tests of the same substance at a single laboratory produce the same results) of the BG1Luc ER TA agonist and antagonist test methods was assessed by comparing (1) reference standard and control results for all plates tested within each laboratory during the course of the validation study and (2) results from Phase 2 testing, during which 12 substances were tested in at least three independent experiments in each of the three laboratories. Intralaboratory agreement for agonist and antagonist classification was determined for the 12 substances that were tested at least three times at each laboratory.

In the agonist testing, mean induction in each laboratory ranged from 4.6 to 7.8 fold, and 17β-estradiol (E2) reference standard EC_{50} values ranged from 8.0×10^{-12} to 1.2×10^{-11} M. There was 100% agreement within each laboratory for each of the three repeat tests, although the agonist classifications for some of the 12 test substances differed among the different laboratories.

In the antagonist testing, mean reduction ranged from 8.0 to 9.9 fold, and raloxifene reference standard IC_{50} values ranged from 1.1×10^{-9} to 1.3×10^{-9} M. There was 100% agreement within each laboratory for each of the three repeat tests, although the antagonist classifications for some of the 12 test substances differed among the different laboratories.

Interlaboratory reproducibility (whether tests of a single substance run at different laboratories produce the same results) of the BG1Luc ER TA agonist and antagonist test methods was determined for the 12 substances that were tested at least three times for agonist and antagonist activity during Phase 2 at each of the three laboratories. The three laboratories agreed on 67% (8/12) of the substances tested for agonist activity and on 100% (12/12) of the substances tested for antagonist activity.

Interlaboratory reproducibility was also determined for 41 substances that were tested once for agonist and antagonist activity during Phase 3 testing at each of the three laboratories. Five of the 41 substances produced inadequate results for agonist activity and could not be considered in the evaluation. Among the 36 remaining substances that produced a definitive test result in at least two laboratories, there was 100% agreement. All 41 substances produced definitive results for antagonist activity. The three laboratories agreed on 93% (38/41) of these substances.

ICCVAM Recommendations: Performance Standards

Based on the results of this study, NICEATM and the EDWG developed performance standards applicable to methods that are functionally and mechanistically similar to the BG1Luc ER TA test method. These performance standards can also be used by laboratories with no experience with the BG1Luc ER TA test method to demonstrate technical proficiency.

Essential Test Method Components

In order to be considered functionally and mechanistically similar to the BG1Luc ER TA test method, a modified ER TA test method protocol must include the following components to ensure that the same biological effect is being measured:

- The test method should be based on a cell line that endogenously expresses ER.
- Reference standards, controls, and test substances should be dissolved in a solvent that mixes well with cell culture media at concentrations that are noncytotoxic and that do not otherwise interfere with the test system.
- The maximum test substance concentration should be 1 mM for ER TA agonist testing and 10 μM for ER TA antagonist testing unless otherwise limited by solubility, cytotoxicity, or other mechanisms that interfere with assay performance.
- At least seven concentrations spaced at logarithmic (log_{10}) intervals, up to the limit concentration, should be tested.
- An evaluation of cytotoxicity should be included, and only data from concentrations at or above 80% viability should be used for data analyses.
- A reference estrogen and a reference anti-estrogen should be used to demonstrate the adequacy of the test method for detecting ER TA agonist and antagonist activity.
- The ability of the reference estrogen to induce ER TA activity and the ability of the reference anti-estrogen to inhibit ER TA activity should be demonstrated by generating a full concentration–response curve in each experiment that provides a minimum threefold estrogenic induction and a minimum threefold anti-estrogenic reduction.
- A set of concurrent controls should be included. For agonist assays, this would include the vehicle control and a weak agonist. For antagonist assays, this would include the vehicle control, weak antagonist, and reference estrogen.
- Test substances that are positive for ER agonist activity should have a concentration–response curve consisting of a baseline, followed by a positive slope, with a response peak of at least 20% of the average maximal value of the reference estrogen response.
- Test substances are negative for agonist activity if all data points are below 20% of the average maximal value of the reference estrogen response.
- Test substances that are positive for ER antagonist activity should have a concentration–response curve consisting of a baseline, followed by a negative slope, with a response decrease to at least 80% of the average maximal value of the reference estrogen response.
- Test substances are negative for ER antagonist activity if all data points are above 80% of the average maximal value of the reference estrogen response.

Test method protocols should incorporate the essential components listed above. Modifications should be detailed and scientifically justified, and the modified test method should perform as well as or better than the BG1Luc ER TA test method.

Reference Substances

ICCVAM recommends for test method validation a subset of those substances that were definitively classified as positive or negative for ER TA activity in the scientific literature and that were tested in the BG1Luc ER TA validation study. The reference substances include a range of chemical and product classes commonly associated with endocrine disruption.

Test Method Accuracy and Reliability

When evaluated using this minimum list of recommended reference substances, a proposed ER TA test method should have accuracy (i.e., sensitivity, specificity, false positive rates, and false negative rates) and reliability characteristics equal to or better than those of the BG1Luc ER TA test method. Any misclassified reference substances should be addressed in terms of the test

method's ability to accurately classify other substances with similar potencies and from the same chemical/product classes.

Using the Performance Standards

Test method developers are encouraged to consult directly with ICCVAM before using these performance standards to conduct a validation study for a proposed test method. Developers are also encouraged to submit results of validation studies to ICCVAM for an evaluation of the validation status. Upon completing its evaluation in accordance with the ICCVAM Authorization Act (42 U.S.C. 285*l*-3), ICCVAM will forward recommendations to ICCVAM agencies regarding the usefulness and limitations of the test method.

ICCVAM Consideration of the Independent Peer Review Panel Report and Other Comments

The ICCVAM evaluation process incorporates scientific peer review and a high level of transparency. The evaluation process for the BG1Luc ER TA test method included a public review meeting by an independent scientific peer review panel, multiple opportunities for public comments, and comments from SACATM. ICCVAM and the EDWG considered the Panel report, SACATM comments, and all public comments before finalizing the ICCVAM test method evaluation report and final BRD for the BG1Luc ER TA test method.

1.0 Introduction

In vitro estrogen receptor (ER) transcriptional activation (TA) assays are designed to identify agonist or antagonist substances that might interfere with estrogen activity *in vivo*. Unlike receptor binding assays, TA assays can distinguish between agonist and antagonist activity. The BG1Luc ER TA test method utilizes an ER-responsive reporter gene (*luc*) in the human ovarian adenocarcinoma cell line BG-1 to detect substances with *in vitro* ER agonist or antagonist activity. ER-mediated transcription of the *luc* gene results in the production of luciferase, the activity of which is quantified using a luminometer. A concentration–response curve can be established to provide qualitative and quantitative information regarding the *in vitro* estrogenic activity of a test substance (Rogers and Denison 2000).

The Federal Food, Drug, and Cosmetic Act; the Food Quality Protection Act; and the Safe Drinking Water Act all aim to identify potential endocrine disruptors and thereby protect humans and animals (7 U.S.C. 136; 21 U.S.C. 301 et seq.; 110 Stat 1613). The U.S. Environmental Protection Agency (EPA) was specifically required to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or such other endocrine effect as the Administrator may designate" (21 U.S.C. 346a[p][1]). In 1996, the EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), a committee of scientists and stakeholders that was charged by the EPA to provide recommendations on how to implement its Endocrine Disruptor Screening Program (EDSP). The EDSP is described in detail at http://www.epa.gov/scipoly/oscpendo/.

The EPA accepted EDSTAC's recommendations for a two-tier screening program as proposed in the *Federal Register* (63 FR 71542). The purpose of Tier 1, which consists of *in vivo* and *in vitro* test methods, is to identify the potential of chemicals to interact with the estrogen, androgen, or thyroid hormonal systems. Tier 1 currently includes EPA OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line [HeLa-9903]) (EPA 2009). EPA OPPTS 890.1300 is an ER TA test method validated for the detection of *in vitro* ER agonists.

In 2004, Xenobiotic Detection Systems, Inc. (XDS), nominated their LUMI-CELL® ER test method (hereafter BG1Luc ER TA test method) to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for validation. ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) recommended that the BG1Luc ER TA test method should be considered a high priority for interlaboratory validation studies based on the lack of adequately validated test methods and the regulatory and public health need for such test methods.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) led and coordinated an international validation study with its counterparts in Europe (the European Centre for the Validation of Alternative Methods [ECVAM]) and Japan (the Japanese Center for the Evaluation of Alternative Methods [JaCVAM]) using laboratories sponsored by each validation organization. NICEATM organized a Study Management Team (SMT) to oversee the scientific aspects of the validation study and coordinate the day-to-day activities among the participating laboratories (XDS, ECVAM, and Hiyoshi). A representative from the recently established Korean Center for the Validation of Alternative Methods (KoCVAM) joined the SMT in 2010.

The validation study proceeded in four phases. During Phase 1, each of the three participating centers (ICCVAM, ECVAM, and JaCVAM) selected validation laboratories. The protocols were reviewed, and the laboratories demonstrated proficiency with the test method by successfully completing 10 replicate agonist and 10 replicate antagonist tests. In Phases 2 through 4, the

protocols were evaluated and refined, and 78 ICCVAM reference substances that should be used to standardize and validate *in vitro* ER and AR binding and TA test methods were tested.

Based on the results of this study, ICCVAM reviewed the validation status of the BG1Luc ER TA test method for identification of substances with *in vitro* ER agonist or antagonist activity. NICEATM and the ICCVAM Interagency Endocrine Disruptor Working Group (EDWG) prepared a draft background review document (BRD) that provides a comprehensive description and the data from the validation study used to assess the accuracy and reliability of the BG1Luc ER TA test method.

On January 24, 2011, ICCVAM announced the availability of the draft BRD to the public and a public Panel meeting to review the validation status of the BG1Luc ER TA test method as a screening test to identify *in vitro* ER agonists and antagonists (76 FR 4113[2]). All of the information provided to the Panel, including the draft BRD, ICCVAM draft test method recommendations, and all public comments received before the Panel meeting, were made publicly available via the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/).

The public Panel meeting was held on March 29–30, 2011. The Panel evaluated (1) the extent to which the draft BRD addressed established validation and acceptance criteria and (2) the extent to which the draft BRD supported ICCVAM's draft test method recommendations. Interested stakeholders from the public were provided opportunities to comment at the Panel meeting. After considering all public comments, the Panel agreed with the ICCVAM draft recommendation that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* ER agonist and antagonist activity. On May 18, 2011, ICCVAM posted a report of the Panel's recommendations[3] (see **Appendix D**) on the NICEATM–ICCVAM website for public review and comment (announced in 76 FR 28781).

ICCVAM provided SACATM with the draft BRD and test method recommendations, the Panel report, and all public comments for discussion at their meeting on June 16–17, 2011, where public stakeholders were given another opportunity to comment.

ICCVAM and the EDWG considered the SACATM comments, the Panel report, and all public comments before finalizing ICCVAM test method recommendations for use of the BG1Luc ER TA test method as a screening test to identify substances with *in vitro* ER agonist and antagonist activity. ICCVAM's recommendations (see **Section 2.0**) and the final BRD (see **Appendix C**) are incorporated in this test method evaluation report. As required by the ICCVAM Authorization Act of 2000 (42 U.S.C. 285*l*-3), ICCVAM will forward this report and its recommendations to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/), and agency responses will also be made available as they are received.

[2] *Federal Register* notices published by NICEATM–ICCVAM during evaluation of the BG1Luc ER TA test method are available in Appendix E and from the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/).

[3] http://iccvam.niehs.nih.gov/docs/endo_docs/EDPRPRept2011.pdf

2.0 ICCVAM Recommendations: Usefulness and Limitations of the BG1Luc ER TA Test Method

2.1 Background and Introduction

ICCVAM has completed its evaluation of the validation status of the BG1Luc ER TA test method, an *in vitro* method proposed to identify potential agonist or antagonist substances that might interfere with normal estrogen activity. NICEATM and ICCVAM prepared a comprehensive BRD that includes the data and information available to characterize the validity of this proposed use of the BG1Luc ER TA test method. The information included in the BRD (**Appendix C**) is based on an international validation study that utilized 78 reference substances that should be used to standardize and validate *in vitro* ER and androgen receptor (AR) binding and TA test methods. Based on the results of this study, ICCVAM developed these draft test method recommendations on the usefulness and limitations of the BG1Luc ER TA test method for identifying potential ER agonists or antagonists. ICCVAM also developed draft recommendations for standardized test method protocols, future studies, and performance standards.

2.2 ICCVAM Recommendations: Test Method Usefulness and Limitations

2.2.1 Evaluation as a Screening Test to Identify Substances with Estrogen Receptor Agonist Activity

ICCVAM concludes that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* ER agonist activity. This recommendation is based on an evaluation of available validation study data and corresponding accuracy and reliability. ICCVAM concludes that the accuracy of this assay is at least equivalent to that of EPA OPPTS 890.1300, part of the EDSP Tier 1 screening battery. The supporting accuracy analysis used 35 ICCVAM reference substances, which produced the following definitive results in agonist testing when compared with existing reference data from other *in vitro* ER TA assays:

- Concordance of 97% (34/35)
- Sensitivity of 96% (27/28)
- Specificity of 100% (7/7)
- False positive rate of 0% (0/7)
- False negative rate of 4% (1/28)

Only L-thyroxine was false negative in the BG1Luc ER TA test method when compared to the ICCVAM reference classification. This reference substance is classified as positive (2/3) based on two reports of positive agonist activity and one report of no agonist activity. The two positive results were in GH3 cells (rat pituitary adenoma) and HeLa cells (human cervical carcinoma), whereas MCF-7 cells (human breast adenocarcinoma) showed no estrogenic response when exposed to L-thyroxine. These results indicate a possible tissue-specific response to L-thyroxine, which may explain the lack of ER agonist activity observed in this experiment with BG-1 cells (human ovarian carcinoma).

During Phase 1, 12 substances were tested in each of the three laboratories (XDS, ECVAM, and Hiyoshi) to evaluate intralaboratory reproducibility. Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the three repeat tests. When results were compared *across* laboratories for these 12 substances, all three laboratories agreed on 67% (8/12) of the substances. An additional 36 substances tested for agonist activity once in each laboratory produced a definitive result in at

least two laboratories. There was 100% agreement among the laboratories for 83% (30/36) of these substances.

Only one *in vitro* ER TA test method is currently accepted to assess ERα agonist activity of test substances. This test method was validated by the Chemicals Evaluation and Research Institute (CERI) and is described in Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 455: the Stably Transfected Human Estrogen Receptor-α Transcriptional Activation (STTA) Assay for the Detection of Estrogenic Agonist-Activity (OECD 2009). Adopted by the EPA as OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line [HeLa-9903]) (EPA 2009), it is considered adequately validated by national and international regulatory agencies.

Because the BG1Luc ER TA test method is another STTA assay that could be considered for regulatory use, a comparison of test method accuracy between these two test methods was conducted based on a list of ICCVAM-recommended agonist reference substances for which definitive classifications have been produced in both methods. These results show identical levels of accuracy when both methods tested the same agonist reference chemicals: concordance of 95% (24/25), sensitivity of 95% (21/22), and specificity of 100% (4/4). Overall, these data indicate that the BG1Luc ER TA test method is equivalent to the EPA OPPTS 890.1300/OECD TG 455 method for assessing ERα agonist activity.

Based on these results, the BG1Luc ER TA agonist test method can be applied to a wide range of substances, provided they (1) can be dissolved in dimethyl sulfoxide (DMSO), (2) do not react with DMSO or the cell culture medium, and (3) are not toxic to the cells. Although this method may apply to mixtures, none was evaluated in this validation study. Volatile substances may yield acceptable results if CO_2-permeable plastic film is used to seal the test plates, but no volatile substances were evaluated in this validation study. Although relatively few are known, substances with endogenous luminescence or that naturally inhibit luciferase activity cannot be used in this or any other luciferase-based test method. The demonstrated performance of the BG1Luc ER TA agonist test method suggests that data generated with this test method could be routinely considered for prioritization of substances for further testing.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concluded that the available data and test method performance support the ICCVAM draft recommendation that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* ER agonist activity. However, the Panel emphasized that, because there has been no clear regulatory guidance on how ER TA test methods will be used in the EPA EDSP Program, the use of the BG1Luc ER TA test method in the overall strategy of hazard identification or safety assessment of endocrine-disruptive chemicals is unclear.

2.2.2 Evaluation as a Screening Test to Identify Substances with Estrogen Receptor Antagonist Activity

Based on an evaluation of available data and corresponding performance (accuracy and reliability), ICCVAM recommends that the BG1Luc ER TA test method can be used as a screening test to identify substances with ER antagonist activity. The accuracy analysis, conducted with 25 reference substances, produced the following definitive results in antagonist testing:

- Accuracy of 100% (25/25)
- Sensitivity of 100% (3/3)
- Specificity of 100% (22/22)
- False positive rate of 0% (0/22)

- False negative rate of 0% (0/3)

Intralaboratory reproducibility of the BG1Luc ER TA agonist and antagonist test methods was assessed by comparing (1) reference standard and control results for all plates tested within each laboratory during the course of the validation study and (2) results from Phases 2a and 2b testing, during which 12 substances were tested in at least three independent experiments in each of three laboratories. Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the three repeat tests.

When results were compared *across* laboratories for these 12 substances, there was 100% agreement among the three laboratories for all 12 substances. An additional 41 substances tested once in each laboratory for antagonist activity during Phase 3 produced a definitive result in at least two laboratories. There was 100% agreement among the laboratories for 93% (38/41) of the 41 substances.

Based on these results, the limitations of the BG1Luc ER TA antagonist test method appear to be the same as those identified for the agonist test method described above. Although the validation database is somewhat limited in number (n = 25), the demonstrated performance of the BG1Luc ER TA antagonist test method suggests that data generated with this test method could be routinely considered for prioritization of substances for further testing. This is further supported by the fact that so few ER antagonists have been definitively identified, and all three tested in the BG1Luc ER TA antagonist test method were correctly identified.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concluded that the available data and test method performance support the ICCVAM draft recommendation that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* ER antagonist activity. The Panel further concluded that, based upon support of the ICCVAM draft recommendation, the BG1Luc ER TA test method could be considered as a replacement for the currently accepted ER TA assay (EPA OPPTS 890.1300/OECD TG 455) and the rat uterine cytosol binding assays. However, the Panel noted that additional analysis may be necessary to further support this recommendation, particularly regarding the rat uterine cytosol ER binding assay.

2.3 ICCVAM Recommendations: Test Method Protocol for the BG1Luc ER TA Test Method

For use of the BG1Luc ER TA test method as a screening test to identify substances with *in vitro* ER agonist or antagonist activity, ICCVAM recommends using the ICCVAM BG1Luc ER TA agonist and antagonist test method protocols (**Appendix B**). In addition, all future studies intended to further characterize the usefulness and limitations of the BG1Luc ER TA agonist and antagonist test methods should be conducted using these recommended protocols.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concluded that the BG1Luc ER TA test method protocols are complete and adequate in detail for a laboratory to conduct the study (see **Appendix D**). The Panel noted several advantages provided by this assay over the currently accepted test method (EPA OPPTS 890.1300/OECD TG 455). The BG1Luc ER TA test method:

- Has more detailed and complete test method protocols than those provided in EPA OPPTS 890.1300/OECD TG 455
- Is validated for testing up to 1 mM per EPA requirements. EPA OPPTS 890.1300/OECD TG 455 is only validated up to a limit dose of 10 μM.

- Has a more restrictive set of classification criteria for determination of a positive response, which will reduce the number of false positive results, resulting in fewer follow-up tests conducted in animal studies
- Can detect substances with *in vitro* anti-estrogenic activity
- Endogenously expresses both hERα and hERβ, whereas the HeLa-9903 cell line used in EPA OPPTS 890.1300/OECD TG 455 was transfected only with hERα

2.4 ICCVAM Recommendations: Future Studies for the BG1Luc ER TA Test Method

ICCVAM promotes the scientific validation and regulatory acceptance of new methods that reduce, refine, or replace animal use where scientifically feasible. The rat uterine cytosol ER binding assay, currently listed as part of the EDSP Tier 1 screening battery, requires the use of animals as a source of ERs. Results from the BG1Luc ER TA test method were examined for concordance with published reports of ER binding for 34 reference substances. There was 97% (33/34) concordance between the BG1Luc ER TA test method and ER binding data from the literature, and 100% sensitivity (no false negatives). In light of the excellent degree of agreement between ER binding and BG1Luc ER TA data, it appears that evaluating results from BG1Luc ER TA agonist and antagonist testing may provide a viable alternative to conducting ER binding studies. This cannot currently be accomplished with EPA OPPTS 890.1300/OECD TG 455 due to the inability of this method to assess ER antagonist activity. ICCVAM recommends that additional validation studies could be performed to determine whether or not the BG1Luc ER TA method could replace the rat uterine cytosol ER binding assay.

Results from the BG1Luc ER TA test method were examined for concordance with published data from the uterotrophic bioassay (n = 13 reference substances), which is currently listed as part of the EDSP Tier 1 screening battery. There was 92% (12/13) concordance between the BG1Luc ER TA test method and the uterotrophic bioassay data, and 100% specificity (no false negatives). These data indicate that the BG1Luc ER TA agonist test method has very good agreement with the *in vivo* results obtained with the uterotrophic bioassay. Accordingly, ICCVAM recommends that further work be carried out to determine if the BG1Luc ER TA test method could be used in combination with other methods (to include *in vitro* metabolic activation) in a weight-of-evidence approach to replace the uterotrophic bioassay.

To further characterize the BG1Luc ER TA test method, ICCVAM identified additional studies that may be considered by interested parties:

- Additional studies/evaluations may be conducted to more completely characterize the ratio of ERα and ERβ in the BG-1 cell line and the extent to which these receptor subtypes contribute to the overall performance of the BG1Luc ER TA test method.
- Additional studies/evaluations may be conducted to determine the feasibility of testing volatile substances using CO_2-permeable plastic film or other methods to seal the test plates.
- Additional studies/evaluations may be conducted to determine if substances that are not soluble in DMSO could be tested in another vehicle that would more adequately solubilize the substance in culture media.
- As ER antagonists are identified, additional studies/evaluations may be conducted to expand the database of positive substances tested and thereby better characterize the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with ER antagonist activity.
- ICCVAM encourages users to provide all data that are generated from future studies to ICCVAM so that they may be used to further characterize the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with *in vitro* ER agonist or antagonist activity.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concluded that the available data support the draft ICCVAM-recommended future studies. The Panel encouraged additional studies and evaluations to assess the utility of the current visual assessment of cytotoxicity evaluation for chemicals, as well as efforts to identify a quantitative cytotoxicity method. The Panel also recommended future studies to account for metabolic activation that could expand the utility of this and other ER TA methods. The Panel further recommended an effort to expand the reference substance list and associated BG1Luc ER TA database with additional negative agonist and positive antagonist test substances as they are identified.

2.5 ICCVAM Recommendations: Performance Standards for the BG1Luc ER TA Test Method

ICCVAM has developed test method performance standards so that modified versions of the BG1Luc ER TA test method that are mechanistically and functionally similar can be effectively and efficiently evaluated for their validity by national and international validation organizations (e.g., ICCVAM, ECVAM, and JaCVAM) or other organizations. The ICCVAM-recommended BG1Luc ER TA agonist and antagonist test method protocols are the key references used to establish these performance standards.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concluded that the draft ICCVAM performance standards are adequate, but they proposed modifications that could expand the performance standards' applicability. The Panel suggested that the specific tissue source, type, and species used for the cell system in ER TA test methods may not be critical but recommended that the appropriate cellular machinery be included. The Panel also recommended that, ideally, more negatives should be included. They recognized, however, that data on such substances are not currently available. The Panel also suggested that reference substance classification be based upon reports that have been ranked with a method that focuses on the reliability of the published data (e.g., Klimisch criteria) (Klimisch et al. 1997).

Classification of reference substances was based on the following published guidance from ICCVAM (ICCVAM 2003a, 2006):

- A substance was classified as "positive" if it was reported as positive in >50% of referenced ER TA studies.
- A substance was classified as "presumed positive" if it was positive in 50% or less of referenced ER TA studies.

Prior to the BG1Luc ER TA test method validation study, L-thyroxine was classified as positive because two of three literature citations described estrogenic activity for this compound. Because the BG-1 validation study will be considered a published study, and L-thyroxine was negative in the study, the updated database will reflect that this compound is reported as positive in two of four studies (50%), changing its classification from positive to presumed positive per the guidelines given above. Because only those compounds with definitive classifications (positive or negative) are used as reference substances, L-thyroxine will not be used as a reference substance in future studies.

3.0 Validation Status for Use of the BG1Luc ER TA Test Method as a Screening Test to Identify *In Vitro* ER Agonists and Antagonists

The ICCVAM BRD (see **Appendix C**) provides a comprehensive review of the current validation status of the BG1Luc ER TA test method, including its accuracy and reliability, the substances tested, the rationale for the standardized test method protocol used for the validation study, and all available data supporting its validity. This section provides a brief description and summary of the validation status of the BG1Luc ER TA test method.

3.1 Test Method Description

The BG1Luc ER TA test method uses an ER-responsive reporter gene (*luc*) in the human ovarian adenocarcinoma cell line BG-1 to detect substances with *in vitro* ER agonist or antagonist activity. ER-mediated transcription of the *luc* gene results in the production of luciferase, the activity of which is quantified using a luminometer. A concentration–response curve can be established to provide qualitative and quantitative information regarding the *in vitro* estrogenic activity of a test substance.

3.2 General Test Method Procedures

ICCVAM previously recommended minimum essential test method components for *in vitro* ER TA assays (ICCVAM 2003a), and these components are incorporated into the ICCVAM-recommended BG1Luc ER TA protocols (see **Appendices B1** and **B2**). These protocols include three sequential phases: solubility, range finder, and comprehensive testing. During solubility testing, the maximum test substance concentration that is soluble in 100% DMSO is established in order to set the starting concentration for range finder testing. The test substance concentration range to be included in comprehensive testing is established during range finder testing. Results from comprehensive testing are used to determine the extent to which a test substance influences ER-mediated luciferase transcription as a correlate to *in vitro* ER TA activity. These data can then be used to classify a test substance based on its *in vitro* ER agonist or antagonist activity.

3.3 Validation Database

The validation database used to evaluate the BG1Luc ER TA test method is based upon the list of 78 substances that ICCVAM recommended for use in validation studies for *in vitro* ER and AR binding and TA test methods (ICCVAM 2003a, 2006). The purpose of this list is to ensure that the usefulness and limitations of *in vitro* ER and AR binding and TA assays can be adequately characterized across a broad range of chemical classes and responses. These substances were selected based on information contained in the ICCVAM BRDs for ER and AR binding and TA test methods (ICCVAM 2002d, 2002a, 2002c, 2002b), as well as information obtained from publications reviewed or published after completion of the ICCVAM BRDs. The complete list of substances and their respective reference classifications for agonist and antagonist activity based on available reference data is provided in Section 3-2 of the BG1Luc ER TA BRD (**Appendix C**).

Only those substances that could be definitively classified as positive (POS) or negative (NEG) were used to assess accuracy, resulting in 48 unique substances used to assess accuracy. (Substances classified as presumed positive [PP] or presumed negative [PN] were not considered when evaluating test method accuracy.) Separate lists were generated for evaluating accuracy based on agonist (42 substances: 33 positive, 9 negative) activity and antagonist (25 substances: 3 positive, 22 negative) activity. Nineteen substances appeared on both reference lists. The 42 reference substances used to assess accuracy based on ER agonist activity are provided in **Table 3-1**, and the 25 reference substances used to assess accuracy based on ER antagonist

activity are provided in **Table 3-2**. These tables also include the BG1Luc ER TA results from each of the participating laboratories.

3.4 Test Method Accuracy

Thirty-five substances (28 positive, 7 negative) had definitive results and were used to evaluate test method accuracy for ER agonist activity. The remaining seven (17%) of the 42 substances used to evaluate test method accuracy had inadequate (I) testing results and were therefore excluded from the analysis. Data are classified as inadequate if, because of major qualitative or quantitative limitations, they cannot be interpreted as valid for showing either the presence or absence of agonist activity. The following seven substances had inadequate BG1Luc ER TA agonist test method data:

- Clomiphene citrate
- *p,p'*-DDE
- 5∝-Dihydrotestosterone
- Flutamide
- Procymidone
- Resveratrol
- Tamoxifen

It should be emphasized that the "inadequate" classification is usually a result of poor data quality and would normally require retesting. However, the classification system was revised after testing to include positive, negative, and inadequate classifications. Retesting of these substances was therefore not possible.

These seven substances (clomiphene citrate, *p,p'*-DDE, 5∝-dihydrotestosterone, flutamide, procymidone, resveratrol, and tamoxifen) represent eight chemical classes (two cyclic hydrocarbons, and one each of an amide, amine, carboxylic acid, halogenated hydrocarbon, heterocyclic compound, polycyclic compound, and steroid) and five product classes (four pharmaceuticals and one each of a fungicide, natural product, pesticide intermediate, and veterinary agent). The diversity of chemical and product classes indicates that no one category or class is overrepresented with inadequate data.

Table 3-1 42 ICCVAM-Recommended Substances Used to Evaluate ER Agonist Accuracy

Substance	CASRN	Classification[a]				
		ICCVAM Consensus	BG1Luc ER TA Consensus[b]	XDS	ECVAM	Hiyoshi
17∝-Estradiol	57-91-0	POS	POS	POS (1/1)	POS (3/3)	POS (2/2)
17∝-Ethinyl estradiol	57-63-6	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
17ß-Estradiol	50-28-2	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
19-Nortestosterone	434-22-0	POS	POS	POS (1/1)	NT	NT
4-Cumylphenol	599-64-4	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
4-*tert*-Octylphenol	140-66-9	POS	POS	I (1/1)	POS (1/1)	POS (2/2)
5∝-Dihydrotestosterone	521-18-6	POS	I	I (1/1)	I (1/1)	POS (1/1)
Apigenin	520-36-5	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Atrazine	1912-24-9	NEG	NEG	NEG (3/3)	POS (3/3)	NEG (3/3)
Bicalutamide	90357-06-5	NEG	NEG	NEG (1/1)	NT	NT
Bisphenol A	80-05-7	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Bisphenol B	77-40-7	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Butylbenzyl phthalate	85-68-7	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Chrysin	480-40-0	POS	POS	POS (2/2)	NT	NT
Clomiphene citrate	50-41-9	POS	I	I (1/1)	NEG (1/1)	POS (1/1)
Corticosterone	50-22-6	NEG	NEG	NEG (3/3)	POS (3/3)	NEG (4/4)
Coumestrol	479-13-0	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Daidzein	486-66-8	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Dicofol	115-32-2	POS	POS	POS (1/1)	NEG (1/1)	POS (1/1)
Diethylstilbestrol	56-53-1	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
Estrone	53-16-7	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Ethyl paraben	120-47-8	POS	POS	I (1)	POS (1/1)	POS (1/1)
Fenarimol	60168-88-9	POS	POS	POS (1/1)	NT	NT
Flutamide	13311-84-7	NEG	I	I (1)	NT	NT
Genistein	446-72-0	POS	POS	POS (3/3)	POS (3/3)	POS (4/4)
Hydroxyflutamide	52806-53-8	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Kaempferol	520-18-3	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Kepone	143-50-0	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
L-Thyroxine	51-48-9	POS	NEG	NEG (1/1)	NT	NT
Linuron	330-55-2	NEG	NEG	NEG (1/1)	NT	NT
meso-Hexestrol	84-16-2	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Methyl testosterone	58-18-4	POS	POS	POS (3/3)	POS (1/1)	POS (2/2)

Substance	CASRN	Classification[a]				
		ICCVAM Consensus	BG1Luc ER TA Consensus[b]	XDS	ECVAM	Hiyoshi
Norethynodrel	68-23-5	POS	POS	POS (2/2)	POS (1/1)	POS (2/2)
o,*p*'-DDT	789-02-6	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
p-n-Nonylphenol	104-40-5	POS	POS	POS (3/3)	POS (3/3)	POS (3/3)
p,*p*'-DDE	72-55-9	POS	I	I (1/1)	I (1/1)	NEG (1/1)
p,*p*'- Methoxychlor	72-43-5	POS	POS	POS (1/1)	POS (1/1)	POS (2/2)
Phenobarbital	50-06-6	NEG	NEG	NEG (1/1)	NEG (1/1)	NT
Procymidone	32809-16-8	NEG	I	I (1/1)	NT	NT
Resveratrol	501-36-0	POS	I	POS (1/1)	I (1/1)	NEG (2/3)
Spironolactone	52-01-7	NEG	NEG	NEG (1/1)	NT	NT
Tamoxifen	10540-29-1	POS	I	I (1/1)	I (1/1)	POS (1/1)

Abbreviations: BG1Luc ER TA = LUMI-CELL BG1Luc4E2 ER TA test method; CASRN = CAS Registry Number (American Chemical Society); ECVAM = European Centre for the Validation of Alternative Methods; I = inadequate (positive or negative classification could not be determined because of poor-quality data); NEG = negative; NT = not tested; POS = positive; XDS = Xenobiotic Detection Systems, Inc.

[a] Number in parentheses represents test results (POS, NEG, or I) over the total number of trials that met test plate acceptance criteria.

[b] BG1Luc ER TA consensus classification represents the majority classification among the three validation laboratories.

Definitive classifications (positive or negative) were obtained for all 25 substances used to evaluate test method accuracy for ER antagonist activity, allowing all 25 substances to be used to assess antagonist accuracy.

Table 3-2 25 ICCVAM-Recommended Substances Used to Evaluate ER Antagonist Accuracy

Substance	CASRN	Classification[a]				
		ICCVAM Consensus	BG1Luc ER TA Consensus[b]	XDS	ECVAM	Hiyoshi
17α–Ethinyl estradiol	57-63-6	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
4-Hydroxytamoxifen	68047-06-3	POS	POS	POS (1/1)	I (2/2)	POS (1/1)
5α-Dihydrotestosterone	521-18-6	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Apigenin	520-36-5	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (4/4)
Bisphenol A	80-05-7	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Butylbenzyl phthalate	85-68-7	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (4/4)
Chrysin	480-40-0	NEG	NEG	NEG (1/1)	NT	NT
Coumestrol	479-13-0	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Daidzein	486-66-8	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Di-*n*-butyl phthalate	84-74-2	NEG	NEG	NEG (2/2)	NEG (1/1)	NEG (1/1)
Dicofol	115-32-2	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Diethylhexyl phthalate	117-81-7	NEG	NEG	NEG (1/1)	NEG (2/2)	NEG (1/1)
Diethylstilbestrol	56-53-1	NEG	NEG	NEG (1/1)	NEG (1/1)	POS (1/1)
Genistein	446-72-0	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
Kaempferol	520-18-3	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Kepone	143-50-0	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Mifepristone	84371-65-3	NEG	NEG	NEG (1/1)	NT	NT
Norethynodrel	68-23-5	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
o.p'-DDT	789-02-6	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (4/4)
p-n-Nonylphenol	104-40-5	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
p.p'-DDE	72-55-9	NEG	NEG	NEG (1/1)	NEG (1/1)	NEG (1/1)
Progesterone	57-83-0	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
Raloxifene HCl	82640-04-8	POS	POS	POS (1/1)	POS (1/1)	POS (1/1)
Resveratrol	501-36-0	NEG	NEG	NEG (3/3)	NEG (3/3)	NEG (3/3)
Tamoxifen	10540-29-1	POS	POS	POS (4/4)	POS (3/3)	POS (3/3)

Abbreviations: BG1Luc ER TA = LUMI-CELL BG1Luc4E2 ER TA test method; CASRN = CAS Registry Number (American Chemical Society); ECVAM = European Centre for the Validation of Alternative Methods; I = inadequate (positive or negative classification could not be determined because of poor-quality data); NEG = negative; NT = not tested; POS = positive; XDS = Xenobiotic Detection Systems, Inc.

[a] Number in parentheses represents test results (POS, NEG, or I) over the total number of trials that met test plate acceptance criteria.

[b] BG1Luc ER TA consensus classification represents the majority classification among the three validation laboratories.

The accuracy analysis using the 35 ICCVAM reference substances that produced a definitive BG1Luc ER TA result in agonist testing indicated accuracy of 97% (34/35), sensitivity of 96% (27/28), specificity of 100% (7/7), false positive rate of 0% (0/7), and false negative rate of 4% (1/28) (**Table 3-3**). Analysis of accuracy using individual laboratory results indicated accuracy ranging from 86% (25/29) to 97% (33/34), sensitivity from 92% (23/25) to 96% (27/28),

specificity from 50% (2/4) to 100% (6/6), false positive rates from 0% (0/6) to 50% (2/4), and false negative rates from 4% (1/28) to 8% (2/25).

Table 3-3 Accuracy of the BG1Luc ER TA Agonist Data

Laboratory	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
Combined	35[a]	97% (34/35)	96% (27/28)	100% (7/7)	0% (0/7)	4% (1/28)
XDS	34	97% (33/34)	96% (27/28)	100% (6/6)	0% (0/6)	4% (1/28)
ECVAM	29	86% (25/29)	92% (23/25)	50% (2/4)	50% (2/4)	8% (2/25)
Hiyoshi	32	94% (30/32)	93% (27/29)	100% (3/3)	0% (0/3)	7% (2/29)

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; N = number; XDS = Xenobiotic Detection Systems, Inc.

[a] A total of 42 substances were evaluated in the BG1Luc ER TA agonist test method. Seven substances did not produce a consensus classification and were omitted, leaving 35 substances for analysis.

The antagonist accuracy analysis indicated an overall accuracy of 100% (25/25), sensitivity of 100% (3/3), specificity of 100% (22/22), false positive rate of 0% (0/22), and false negative rate of 0% (0/3) (**Table 3-4**). Similarly, individual laboratory results indicated accuracy ranging from 96% (22/23) to 100% (25/25), sensitivity of 100% (3/3), and specificity of 95% (19/20) to 100% (22/22).

Table 3-4 Accuracy of the BG1Luc ER TA Antagonist Data

Laboratory	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
Combined	25	100% (25/25)	100% (3/3)	100% (22/22)	0% (0/22)	0% (0/3)
XDS	25	100% (25/25)	100% (3/3)	100% (22/22)	0% (0/22)	0% (0/3)
ECVAM	23	100% (23/23)	100% (3/3)	100% (20/20)	0% (0/20)	0% (0/3)
Hiyoshi	23	96% (22/23)	100% (3/3)	95% (19/20)	5% (1/20)	0% (0/3)

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; N = number; XDS = Xenobiotic Detection Systems, Inc.

3.5 Test Method Reliability

Intralaboratory reproducibility of the BG1Luc ER TA agonist and antagonist test methods was assessed quantitatively by comparing the following:

- Relative light unit (RLU) values for the agonist and antagonist DMSO control and the antagonist E2 control for all plates tested within each laboratory during the course of the validation study

- Results from Phases 2a and 2b testing, during which 12 substances were tested in at least three independent experiments in each of the three laboratories

Because DMSO control RLU values are not normalized, they vary considerably between test plates and across time. Therefore, intralaboratory reproducibility was evaluated by comparing the within-plate variability of the four replicate DMSO control RLU values for all test plates that passed acceptance criteria (i.e., coefficient of variation [CV] associated with within-plate DMSO control RLU values). The range of means and CV values for within-plate DMSO control RLU values are provided in **Table 3-5**. Mean plate DMSO RLU values ranged from a low of 511 to a high of 9885, with a mean of 3749. However, within-plate variability of DMSO RLU control values between replicate DMSO wells was low. Coefficients of variation ranged from 1% to 43%, with a mean of 8%. Of the 218 agonist test plates that passed acceptance criteria, only six plates had within-plate CV values greater than 20%.

Table 3-5 Agonist Within-Plate DMSO Control Data

Laboratory	Mean and Range of DMSO Control RLU Values	Mean and Range of CV (%)	N
Combined	3749 (511-9885)	8 (1-43)	218
XDS	2800 (511-9885)	8 (1-43)	93
ECVAM	3379 (828-7306)	8 (1-33)	60
Hiyoshi	5465 (1362-9383)	6 (1-24)	65

Abbreviations: CV = coefficient of variation; DMSO = dimethyl sulfoxide; ECVAM = European Centre for the Validation of Alternative Methods; N = number of plates that passed acceptance criteria; RLU = relative light unit; XDS = Xenobiotic Detection Systems, Inc.

For the antagonist assay, although mean plate DMSO RLU values ranged from a low of 132 to a high of 8451 (mean = 3299), within-plate variability of DMSO RLU control values between replicate DMSO wells was low, with CV values ranging from 1% to 52% (mean = 8%) (**Table 3-6**). Of the 194 antagonist test plates that passed acceptance criteria, only eight plates had within-plate CV values greater than 20%.

Table 3-6 Antagonist Within-Plate DMSO Control Data

Laboratory	Mean and Range of DMSO Control RLU Values	Mean and Range of CV (%)	N
Combined	3299 (132-8451)	8 (1-52)	194
XDS	2230 (132-6860)	9 (1-52)	79
ECVAM	3622 (1352-7333)	9 (1-37)	62
Hiyoshi	4030 (1625-8451)	6 (1-20)	53

Abbreviations: CV = coefficient of variation; DMSO = dimethyl sulfoxide; ECVAM = European Centre for the Validation of Alternative Methods; N = number of plates that passed acceptance criteria; RLU = relative light unit; XDS = Xenobiotic Detection Systems, Inc.

Normalized and adjusted antagonist E2 control RLU values were used as acceptance criteria throughout the validation study. The mean, standard deviation (SD), and CV values calculated for the E2 control RLU value from all antagonist test plates that passed acceptance criteria are provided in **Table 3-7**. Mean E2 control RLU values ranged from 5793 at Hiyoshi to 9246 at ECVAM. Variability was low, with associated CV values ranging from 9% at ECVAM to 19% at XDS.

Table 3-7 Antagonist E2 Control Values

Laboratory	Mean RLU	SD	CV (%)	N
XDS	7524	1443	19	79
ECVAM	9246	805	9	62
Hiyoshi	5793	791	14	53

Abbreviations: CV = coefficient of variation; ECVAM = European Centre for the Validation of Alternative Methods; N = number of plates that passed acceptance criteria; RLU = relative light unit; SD = standard deviation; XDS = Xenobiotic Detection Systems, Inc.

Test substances are classified as positive or negative for agonist activity based on a specific set of criteria. The resulting classifications for each of the 12 substances that were tested at least three times at each laboratory were used to evaluate the extent of intralaboratory agreement (see **Table 3-8**). Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the three repeat tests. There were no “inadequate” data generated at any laboratory during this phase of the validation study.

Table 3-8 Intralaboratory Agreement for Multiple Testing of the 12 Phase 2 Agonist Substances Tested Independently at Least Three Times at Each Laboratory

Activity per Test	XDS	ECVAM	Hiyoshi
Agreement within laboratory	12/12 (100%)	12/12 (100%)	12/12 (100%)
+++	8/12	12/12	9/12
–––	4/12	0/12	3/12
Discordance within laboratory	0/12 (0%)	0/12 (0%)	0/12 (0%)
++–	0/12	0/12	0/12
+––	0/12	0/12	0/12

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; XDS = Xenobiotic Detection Systems, Inc.

+ denotes a positive test result.

- denotes a negative test result.

+++ indicates that each of three replicate tests within each laboratory had a classification as positive.

--- indicates that each of three replicate tests within each laboratory had a classification as negative.

++- indicates that in two of three replicate tests, a test substance was classified as positive. The substance was classified as negative in a third replicate test.

+-- indicates that in one of three replicate tests, the test substance was classified as positive. The substance was classified as negative in the remaining two tests.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The BG1Luc ER TA test method utilizes cultured human ovary adenocarcinoma cells that endogenously express human ER and contain an estrogen-inducible gene expression system. Except for the fetal bovine sera used as part of the cell culture media, the test method does not require the use of animals.

The BG1Luc ER TA test method is being proposed as an independent part of a weight-of-evidence approach to prioritize potentially endocrine-active substances for further testing. Therefore, like the EPA OPPTS 890.1300/OECD TG 455 method, the test does not directly reduce, refine, or replace animal use. However, regulators currently use the following three *in vivo* methods to assess the estrogenic potential of substances: (1) rat uterotrophic assay, (2) rat pubertal female assay, and (3) fish short-term reproduction assay. In addition, the "*in vitro*" rat uterine cytosol ER binding assay also requires the use of animals as a source of ER.

Results from the BG1Luc ER TA test method were examined for concordance with published reports of ER binding. There was 97% (33/34) concordance between the BG1Luc ER TA test method and ER binding data. In light of the excellent degree of agreement between ER binding and BG1Luc ER TA test method results (with no false negative results), it appears that evaluating results from BG1Luc ER TA agonist and antagonist testing may provide a viable alternative to conducting ER binding studies, which use animals as a source of ER. This cannot currently be accomplished with the only accepted ER TA method because of the inability of the EPA OPPTS 890.1300/OECD TG 455 method to assess ER antagonist activity.

Results from the BG1Luc ER TA test method were examined for concordance with published data from the uterotrophic assay. Based on a comparison with the *in vivo* uterotrophic assay classification, the 13 substances with data from the uterotrophic assay and conclusive test results in the BG1Luc ER TA agonist test method produced overall concordance of 92% (12/13). All

substances found positive in the uterotrophic assay were also positive in the BG1Luc ER TA test method. The only discordant substance, butylbenzyl phthalate, was positive for ER agonist activity in the BG1Luc ER TA agonist test method and negative in the uterotrophic assay. These data indicate that the BG1Luc ER TA agonist test method had very good agreement with the *in vivo* results obtained with the uterotrophic assay, with no false negative results.

The development of a battery of *in vitro* and *in silico* methods that can replace animal testing for detecting potential EDs is a biologically complex challenge. The experience derived from validating and using the *in vitro* BG1Luc ER TA test method is expected to contribute to our knowledge and promote progress toward this goal.

4.0 BG1Luc ER TA Test Method Performance Standards

Prior to the acceptance of a new test method for regulatory testing applications, validation studies are conducted to assess its reliability (i.e., the extent of intra- and interlaboratory reproducibility) and its relevance (i.e., the ability of the test method to correctly predict or measure the biological effect of interest) (ICCVAM 1997, 2003b; OECD 1996, 2005). The purpose of performance standards is to communicate the basis by which new proprietary and nonproprietary test methods have been determined to have sufficient accuracy and reliability for a specific testing purpose. These performance standards can then be used to evaluate the accuracy and reliability of other proposed test methods that are considered functionally and mechanistically similar to the accepted test method.

4.1 Elements of ICCVAM Performance Standards

Performance standards are based on an adequately validated test method and provide a basis for evaluating the comparability of a proposed test method that is functionally and mechanistically similar (ICCVAM 2003b). The three elements of performance standards are the following:

- Essential test method components: These consist of essential structural, functional, and procedural elements of a validated test method. They should be included in the protocol of a proposed test method that is functionally and mechanistically similar to the validated method. Essential test method components include unique characteristics of the test method, critical procedural details, and quality control measures.
- A minimum list of reference substances: Reference substances are used to assess the accuracy and reliability of a proposed functionally and mechanistically similar test method. These substances are a representative subset of those used to demonstrate the accuracy and reliability of the validated test method.
- Accuracy and reliability values: These are the standards for accuracy and reliability that the proposed test method should meet or exceed when evaluated using the minimum list of reference substances.

4.2 LUMI-CELL (BG1Luc ER TA) Test Method Performance Standards

4.2.1 Background

The BG1Luc ER TA test method uses an ER-responsive reporter gene (*luc*) in the human ovarian adenocarcinoma cell line BG-1 to detect substances with *in vitro* ER agonist or antagonist activity. The primary objective of this test method is to provide a qualitative assessment of *in vitro* estrogenic activity (i.e., whether a substance is positive or negative for estrogenic activity). Quantitative analysis is also performed to provide additional information on the estrogenic potency of test substances. For example, quantitative analysis can determine the half-maximal effective concentration (EC_{50}) or the half-maximal inhibitory concentration (IC_{50}). Separate protocols are used to identify substances that possess ER agonist or antagonist activity, although the two protocols share most major components (see **Appendices B1** and **B2**).

NICEATM coordinated and led an international validation study of the BG1Luc ER TA test method with ECVAM and JaCVAM. The study proceeded in four phases, during which 78 reference substances were tested (see **Appendix C**). Results from this validation study served as the basis for the BG1Luc ER TA test method performance standards, which are applicable for assessing the validity of methods that are functionally and mechanistically similar to the BG1Luc ER TA test method. These performance standards can also be used by naïve laboratories to demonstrate technical proficiency in performing the BG1Luc ER TA test method. The

performance standards consist of (1) essential test method components, (2) reference substances, and (3) an assessment of accuracy and reliability.

4.2.2 BG1Luc ER TA Essential Test Method Components and Other Validation Considerations

Certain principles are important in delineating the essential test method components that determine whether a modified test is functionally and mechanistically similar to the BG1Luc ER TA test method. *In vitro* ER TA assays are designed to identify substances that might interfere with estrogenic homeostasis *in vivo*. The interaction of estrogens with cellular ERs initiates a cascade of events. A number of *in vitro* endpoints can be used to assess ER–ligand interactions, including receptor binding, cellular proliferation, and transcriptional activation (reporter gene). Unlike receptor binding assays, TA assays can identify whether ligand–receptor association potentiates (agonist) or inhibits (antagonist) estrogenic signaling (Davenport and Russell 1996).

In the BG1Luc ER TA test method, ER-mediated transcription of the *luc* gene results in the production of luciferase, the activity of which is quantified using a luminometer. A concentration–response curve can be established to provide qualitative and quantitative information regarding the *in vitro* estrogenic activity of a test substance.

4.2.2.1 Essential Test Method Components

ICCVAM previously recommended minimum essential test method components for *in vitro* ER TA test method protocols (ICCVAM 2003a). These components were incorporated into the BG1Luc ER TA test method protocols during a protocol standardization study. During the protocol standardization study, protocols were developed for use in the international validation study (see **Appendices B1** and **B2**). During the multiphase validation study, the protocols were refined, ultimately resulting in optimized protocols for agonist and antagonist testing. In order to be considered functionally and mechanistically similar to the BG1Luc ER TA test method, a modified ER TA test method protocol must include the following components, which are based on the optimized test method protocols, to ensure that the same biological effect is being measured. If any of these criteria are not met, then these performance standards cannot be used for validation of the modified test method.

Cell Line

The BG1Luc ER TA test method is based on a human ovarian adenocarcinoma cell line that endogenously expresses ERα (90%) and ERβ (10%) (Pujol et al. 1998) and uses a stably transfected luciferase-based reporter gene system. Other cell lines that endogenously express human ERs and are stably transfected with a reporter gene system may be appropriate for validation using these performance standards.

Solvent

Reference standards, controls, and test substances should be dissolved in a solvent (e.g., 1% DMSO) that is miscible with cell culture media at concentrations that are not cytotoxic and that do not otherwise interfere with the test system.

Limit Concentration and Cytotoxicity

The maximum test substance concentration should be 1 mM for ER TA agonist testing and 10 μM for ER TA antagonist testing unless otherwise limited by solubility, cytotoxicity, or other mechanisms that interfere with assay performance. A minimum of seven concentrations spaced at logarithmic (log10) intervals, up to the limit concentration, should be tested. An evaluation of cytotoxicity and how it is applied to the test method should be included in each study. Any

concentration of test substance that reduces viability by greater than 20% should not be considered in the analysis of the data.

Reference Standards

A reference estrogen (e.g., 17β-estradiol [E2]) and a reference anti-estrogen (e.g., raloxifene HCl) should be used as reference standards to demonstrate the adequacy of the test method for detecting ER TA agonist and antagonist activity, respectively. The ability of the reference estrogen to induce ER TA activity and the reference anti-estrogen to inhibit ER TA activity should be demonstrated by generating a full concentration–response curve in each experiment. At a minimum, the E2 reference standard should provide a threefold induction relative to the solvent control. For antagonist testing, a minimum threefold reduction in the reference anti-estrogenic standard response (e.g., raloxifene HCl) should be demonstrated.

Controls

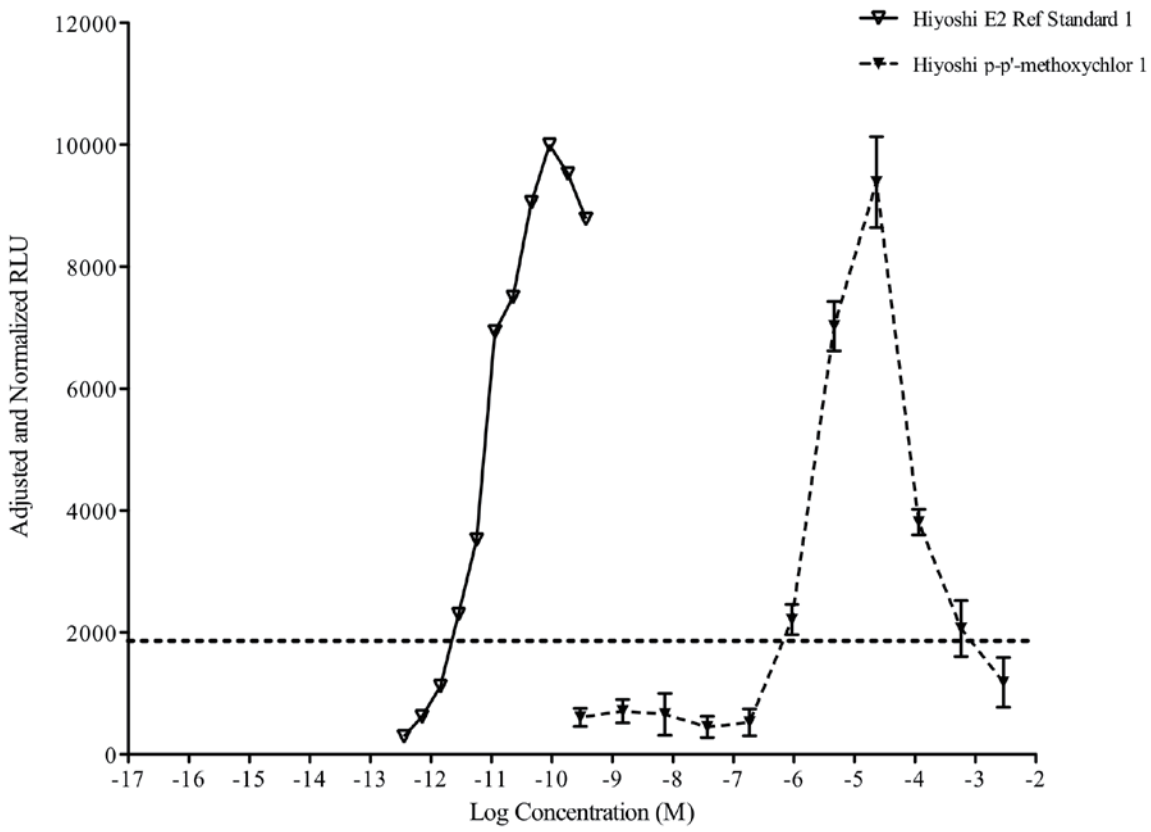
A set of concurrent controls (i.e., solvent, cell culture media) should be included in each experiment to provide a measure of ER TA activity in the absence of reference standards or test substances. A weak positive agonist control (e.g., *p.p'*-methoxychlor) with an EC_{50} five to six orders of magnitude higher than the reference estrogen should be included in each ER TA agonist study to demonstrate that the test method is functioning properly and is sufficiently sensitive to detect weak ER agonists. A weak positive antagonist control (e.g., tamoxifen) that demonstrates ER TA antagonist activity slightly below the 10 μM limit concentration should be included in each ER TA antagonist study to demonstrate that the test method is functioning properly and is sufficiently sensitive to detect weak ER antagonists. In addition, ER TA antagonist studies should include a concurrent control using the reference estrogen (e.g., E2) to establish a baseline level of induction (~80% of E2 maximum) against which antagonistic activity of test substances can be assessed.

Interpretation of Results

For ER TA agonist testing:

- All test substances classified as positive for ER TA agonist activity should have a concentration–response curve consisting of a baseline followed by a positive slope, concluding in a plateau or peak. In some cases, only two of these characteristics (baseline–slope or slope–peak) may be defined.
- The line defining the positive slope must contain at least three points with nonoverlapping error bars (mean ± SD). Points forming the baseline are excluded, but the linear portion of the curve may include the peak or first point of the plateau.
- A positive classification requires a response amplitude, the difference between baseline and peak, of at least 20% of the average maximal value of the reference estrogen, e.g., 2000 RLUs when the maximal response value of the reference estrogen is adjusted to 10,000 RLUs. (See **Figure 4-1** for an example of a concentration–response curve for a substance that is positive for ER TA agonist activity.)
- If possible, an EC_{50} value should be calculated for each positive substance.
- For all concentration–response curves that fail to meet the criteria for a positive response, test substances are classified as negative for agonist activity if all data points are below 20% of the maximal value for the reference estrogen, e.g., 2000 RLUs when the maximal response value of the reference estrogen is adjusted to 10,000 RLUs.

Figure 4-1 Example Concentration–Response Curve for an ER TA Agonist

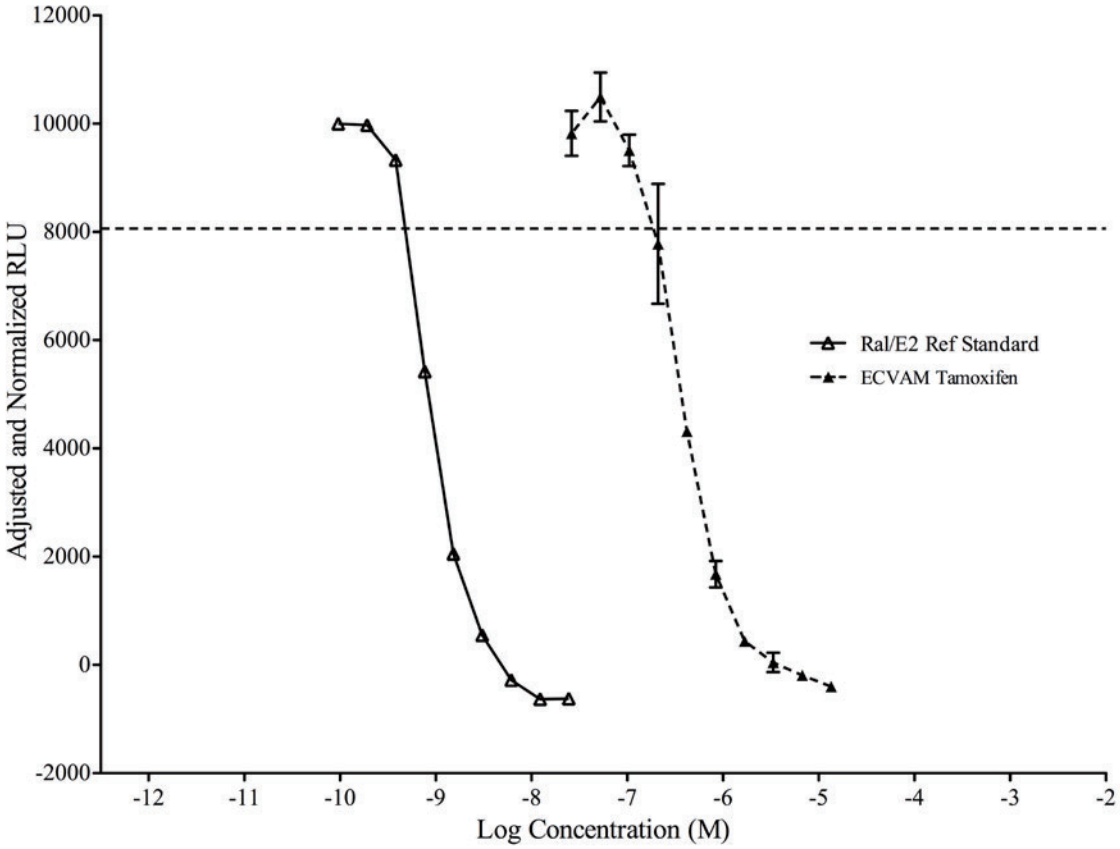


Abbreviations: E2 = 17β-estradiol; M = molar; RLU = relative light unit.
Horizontal dotted line represents 20% of the maximum response of the E2 reference standard.
Test substance shown is *p,p'*-methoxychlor.
E2 reference standard data is presented as the mean value of duplicate wells.
p,p'-Methoxychlor data are presented as the mean and SD values of three replicate wells.

For ER TA antagonist testing:

- All substances classified as positive for ER antagonist activity should have a concentration–response curve consisting of a baseline followed by a negative slope.
- The line defining the negative slope must contain at least three points with nonoverlapping error bars (representative of means ± SDs). Points forming the baseline are excluded, but the linear portion of the curve may include the first point of the plateau.
- A positive classification requires a response amplitude of less than 80% of the value for the reference estrogen. The response amplitude is defined as the difference between the baseline, established by the reference estrogen, and the bottom of the dose–response curve.
- The highest noncytotoxic concentrations of the test substance should be less than or equal to 10 μM. (See **Figure 4-2** for an example of a concentration–response curve for a substance that is positive for ER TA antagonist activity.)
- Test substances are classified as negative for ER antagonist activity if all data points are above 80% of the reference estrogen response, or 8000 RLUs.

Figure 4-2 Example Concentration–Response Curve for an ER TA Antagonist



Abbreviations: M = molar; RLU = relative light unit.

Horizontal dotted line represents 80% of the response of the 17β-estradiol reference estrogen.

Test substance shown is tamoxifen.

Ral/E2 reference standard data are presented as the mean value of duplicate wells.

Tamoxifen data are presented as the mean and SD values of three replicate wells.

Data and Reporting

The validation report should include the following information:

—Reporter Plasmid (if different than that used in BG1Luc ER TA test method)

- Type and structure of ER response elements
- Description of promoter region
- Name, identification, and source of original plasmid used to make construct
- Description and methodology used to make the transfected plasmid
- Nomenclature and genetic components comprising the reporter construct

—Cell Line

- Source and nomenclature of the cell line and protocol for its maintenance before and after transfection
- Source of cell culture media, materials, and supplies
- Passage number of subcultures used in the study
- Methods for maintaining stably transfected cell line

- Methods used to monitor the stability of the cell line used for testing
- Rationale, based on data, for deciding on the number of passages a cell line can undergo without a decrease in activity
- Details regarding selection requirements needed to maintain stable cell lines
- If known, details regarding the relative amounts of ERα and ERβ

—Test Method Conditions

- Composition of media and reagents used
- Incubation volume, duration, and temperature
- Method used to measure ER TA activity
- Methods used to evaluate data, determine response, and calculate EC_{50} or IC_{50} values

—Reference Standards, Controls, and Test Substances

- Name, chemical structure, CAS Registry Number (CASRN), purity, and supplier
- Physicochemical properties relevant to the study (e.g., solubility, pH, stability, volatility)
- Concentrations and volumes used

—Solvent

- Name, CASRN, purity, and supplier
- Justification for choice of solvent
- Information on the solubility of test substances in solvent used
- Information to demonstrate that the solvent, at the maximum volume used, is not cytotoxic and does not otherwise interfere with the study

—Criteria for an Acceptable Test

- Concurrent reference standard and control data
- Laboratory-specific historical ranges of reference standard and control data
- Definition of exclusion criteria and description of the impact of any excluded data

—Results

- Reference standard and control results
- Test substance solubility results
- Test substance cell viability results
- Calculated reference standard and test substance EC_{50} and IC_{50} values
- Graphically presented reference standard, control, and test substance results

—Discussion of Results

- Impact of solubility and cytotoxicity on test results
- Reproducibility of reference standard and control data

—Conclusion

- Classification of test substances with regard to *in vitro* ER TA agonist or antagonist activity

Other Validation Considerations

The following additional points should be considered during the validation of test methods that are functionally and mechanistically similar to the BG1Luc ER TA test method:

- Appropriate quality assurance systems (i.e., in accordance with Good Laboratory Practice guidelines (EPA 2006b, 2006a; FDA 2009; OECD 1998) are required.

- The study should be conducted according to U.S. (ICCVAM 1997) and international validation principles (OECD Guidance Document 34) (OECD 2005).

4.2.3 Reference Substances for *In Vitro* ER TA Test Methods

To ensure that a proposed *in vitro* ER TA test method possesses reliability and accuracy characteristics similar to those of the validated test method (in this case the BG1Luc ER TA test method), the proposed test method should use at least the agonist reference substances listed in **Table 4-1** and the antagonist reference substances listed in **Table 4-2**. All substances should be tested in a coded/blinded manner. When evaluated using these reference substances, the accuracy (i.e., sensitivity, specificity, false positive rates, and false negative rates) and reliability of the proposed ER TA test method should approximate those of the validated ER TA test method, as detailed in **Section 4.2.4**. Although it is not realistic to expect test methods to perform identically, discordant results should be addressed in terms of the test method's ability to accurately classify other substances with similar potencies and from the same chemical/product classes.

4.2.3.1 Criteria for Selection of Reference Substances

ICCVAM previously compiled and recommended a list of 78 substances for use in validation studies for *in vitro* ER and AR binding and TA test methods (ICCVAM 2003a, 2006). These substances were selected based on information contained in the ICCVAM BRDs for AR and ER binding and TA test methods (ICCVAM 2002d, 2002a, 2002c, 2002b), as well as information obtained from publications reviewed or published after completion of the ICCVAM BRDs. Factors and criteria considered necessary for selecting reference substances included:

- A well-defined chemical structure
- Comparatively low systemic toxicity
- Good availability from commercial sources
- A concentration–response range that could be measured or predicted by the test method
- Minimal disposal cost

Because the BG1Luc ER TA test method is used only to detect substances with *in vitro* ER TA agonist or antagonist activity, the following criteria were used to classify each reference substance with respect to ER TA agonist and antagonist activity:

- A substance was classified as POS if it was reported as positive in >50% of referenced ER TA studies.
- A substance was classified as NEG if it was reported as negative in all referenced ER TA studies (at least two studies were required for negative classification).
- A substance was classified as PP (presumed positive) if it was positive in 50% or fewer referenced ER TA studies, or if it was reported positive in the single study conducted.
- A substance was classified as PN (presumed negative) if it was reported negative in a single ER TA study.
- Substances without data were classified as PP or PN based on other available information, including their known mechanism of action or their responses in other ER assays.

Only those substances that could be definitively classified as POS or NEG were used to assess accuracy (substances classified as PP or PN were not considered when evaluating test method accuracy). Accordingly, this subset of substances was used to select the final list of reference substances listed in **Tables 4-1** and **4-2**. Recognizing that the number of available reference substances that are definitively negative for agonist activity (**Table 4-1**) or definitively positive for antagonist activity (**Table 4-2**) is limited, these lists may be updated as additional substances with these characteristics are identified. Accordingly, users should be aware that the reference substance list could be revised based on any additional studies that are conducted in the future.

ICCVAM recommends that users consult the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/) to ensure use of the most current reference substance list.

Table 4-1 34 Reference Substances for Evaluation of ER Agonist Accuracy

Substance[a]	CASRN	ICCVAM Consensus	BG1Luc ER TA Consensus[b]	BG1Luc ER TA Mean EC_{50} (M)[c]	MeSH Chemical Class[d]	Product Class[e]
Ethyl paraben	120-47-8	POS	POS	2.48×10^{-5}	Carboxylic Acid, Phenol	Pharmaceutical, Preservative
Fenarimol	60168-88-9	POS	POS	4.59×10^{-6}	Heterocyclic Compound, Pyrimidine	Fungicide
Kaempferol	520-18-3	POS	POS	3.99×10^{-6}	Flavonoid, Heterocyclic Compound	Natural Product
Methyl testosterone	58-18-4	POS	POS	3.29×10^{-6}	Steroid	Pharmaceutical, Veterinary Agent
Chrysin	480-40-0	POS	POS	3.20×10^{-6}	Flavonoid, Heterocyclic Compound	Natural Product
p-n-Nonylphenol	104-40-5	POS	POS	3.06×10^{-6}	Phenol	Chemical Intermediate
Dicofol	115-32-2	POS	POS	2.22×10^{-6}	Hydrocarbon (Cyclic), Hydrocarbon (Halogenated)	Pesticide
Butylbenzyl phthalate	85-68-7	POS	POS	1.98×10^{-6}	Carboxylic Acid, Ester, Phthalic Acid	Plasticizer, Industrial Chemical
p,*p*'-Methoxychlor	72-43-5	POS	POS	1.92×10^{-6}	Hydrocarbon (Halogenated)	Pesticide, Veterinary Agent
Apigenin	520-36-5	POS	POS	1.85×10^{-6}	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
19-Nortestosterone	434-22-0	POS	POS	1.80×10^{-6}	Steroid	Pharmaceutical, Veterinary Agent
Daidzein	486-66-8	POS	POS	8.71×10^{-7}	Flavonoid, Heterocyclic Compound	Natural Product
Bisphenol A	80-05-7	POS	POS	5.33×10^{-7}	Phenol	Chemical Intermediate, Flame Retardant, Fungicide
Kepone	143-50-0	POS	POS	4.91×10^{-7}	Hydrocarbon (Halogenated)	Pesticide
o,*p*'-DDT	789-02-6	POS	POS	3.94×10^{-7}	Hydrocarbon (Halogenated)	Pesticide
4-Cumylphenol	599-64-4	POS	POS	3.20×10^{-7}	Phenol	Chemical Intermediate
Genistein	446-72-0	POS	POS	2.71×10^{-7}	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical

Substance[a]	CASRN	ICCVAM Consensus	BG1Luc ER TA Consensus[b]	BG1Luc ER TA Mean EC_{50} (M)[c]	MeSH Chemical Class[d]	Product Class[e]
Bisphenol B	77-40-7	POS	POS	1.67×10^{-7}	Phenol	Chemical Intermediate, Flame Retardant, Fungicide
Coumestrol	479-13-0	POS	POS	8.77×10^{-8}	Heterocyclic Compound	Natural Product
4-*tert*-Octylphenol	140-66-9	POS	POS	3.19×10^{-8}	Phenol	Chemical Intermediate, Pharmaceutical Intermediate
17∝-Estradiol	57-91-0	POS	POS	1.54×10^{-9}	Steroid	Pharmaceutical, Veterinary Agent
Norethynodrel	68-23-5	POS	POS	9.39×10^{-10}	Steroid	Pharmaceutical
Estrone	53-16-7	POS	POS	2.57×10^{-10}	Steroid	Pharmaceutical, Veterinary Agent
Diethylstilbestrol	56-53-1	POS	POS	3.34×10^{-11}	Hydrocarbon (Cyclic)	Pharmaceutical, Veterinary Agent
meso-Hexestrol	84-16-2	POS	POS	1.65×10^{-11}	Steroid	Pharmaceutical, Veterinary Agent
17ß-Estradiol	50-28-2	POS	POS	8.37×10^{-12}	Steroid	Pharmaceutical, Veterinary Agent
17∝-Ethinyl estradiol	57-63-6	POS	POS	7.31×10^{-12}	Steroid	Pharmaceutical, Veterinary Agent
Atrazine	1912-24-9	NEG	NEG	-	Heterocyclic Compound	Herbicide
Bicalutamide	90357-06-5	NEG	NEG	-	Amide	Pharmaceutical
Corticosterone	50-22-6	NEG	NEG	-	Steroid	Pharmaceutical
Hydroxyflutamide	52806-53-8	NEG	NEG	-	Amide	Pharmaceutical
Linuron	330-55-2	NEG	NEG	-	Urea	Herbicide
Phenobarbital	50-06-6	NEG	NEG	-	Heterocyclic Compound, Pyrimidine	Pharmaceutical, Veterinary Agent
Spironolactone	52-01-7	NEG	NEG	-	Lactone, Steroid	Pharmaceutical

Abbreviations: BG1Luc ER TA = LUMI-CELL BG1Luc4E2 ER TA test method; CASRN = CAS Registry Number (American Chemical Society); EC_{50} = half-maximal effective concentration of a test substance; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; M = molar; MeSH = Medical Subject Headings (U.S. National Library of Medicine); NEG = negative; POS = positive.

[a] Substances are listed in order based upon EC_{50} values.

[b] BG1Luc ER TA consensus classification represents the majority classification among the three validation laboratories.

[c] Mean EC_{50} values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi).

[d] Substances were assigned to one or more chemical or product classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at http://www.nlm.nih.gov/mesh).

[e] Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

Table 4-2 10 Reference Substances for Evaluation of ER Antagonist Accuracy

Substance[a]	CASRN	ICCVAM Consensus[b]	BG1Luc ER TA Consensus	BG1Luc ER TA Mean IC_{50} (M)[c]	MeSH Chemical Class[d]	Product Class[d]
Tamoxifen	10540-29-1	POS	POS	8.17×10^{-7}	Hydrocarbon (Cyclic)	Pharmaceutical
4-Hydroxytamoxifen	68047-06-3	POS	POS	2.08×10^{-7}	Hydrocarbon (Cyclic)	Pharmaceutical
Raloxifene HCl	82640-04-8	POS	POS	1.19×10^{-9}	Hydrocarbon (Cyclic)	Pharmaceutical
17∝-Ethinyl estradiol	57-63-6	NEG	NEG	-	Steroid	Pharmaceutical, Veterinary Agent
Apigenin	520-36-5	NEG	NEG	-	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
Chrysin	480-40-0	NEG	NEG	-	Flavonoid, Heterocyclic Compound	Natural Product
Coumestrol	479-13-0	NEG	NEG	-	Heterocyclic Compound	Natural Product
Genistein	446-72-0	NEG	NEG	-	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
Kaempferol	520-18-3	NEG	NEG	-	Flavonoid, Heterocyclic Compound	Natural Product
Resveratrol	501-36-0	NEG	NEG	-	Hydrocarbon (Cyclic)	Natural Product

Abbreviations: BG1Luc ER TA = LUMI-CELL BG1Luc4E2 ER TA test method; CASRN = CAS Registry Number (American Chemical Society); IC_{50} = half-maximal inhibitory concentration; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; M = molar; MeSH = Medical Subject Headings (U.S. National Library of Medicine); NEG = negative; POS = positive.

[a] Substances are listed in order based upon IC_{50} values.

[b] BG1Luc ER TA consensus classification represents the majority classification among the three validation laboratories.

[c] Mean IC_{50} values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi).

[d] Substances were assigned to one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at http://www.nlm.nih.gov/mesh).

[e] Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

4.2.3.2 Characteristics of Selected Reference Substances

The reference substances include a range of chemical and product classes representative of the classes commonly associated with endocrine disruption.

Agonist and antagonist test method intralaboratory reproducibility was evaluated using nine substances and four substances, respectively, that were each tested three times on three separate days at each laboratory. Agonist and antagonist test method interlaboratory reproducibility was

evaluated using 27 and 8 substances, respectively, that were tested at least once in each laboratory during the validation study.

4.2.4 Accuracy and Reliability Performance Values

The final elements of performance standards are the accuracy and reliability values (i.e., test method performance) that should be met or exceeded by the proposed test method when evaluated with the reference substances. *Accuracy* is defined as the closeness of agreement between a test method result and an accepted reference value. *Reliability* is the degree to which a test method can be performed reproducibly within and among laboratories over time (ICCVAM 2003b). For these performance standards, the proposed test method should have accuracy and reliability characteristics that approximate those of the validated ER TA test method, which are detailed below. Although it is not realistic to expect test methods to perform identically, discordant results should be addressed in terms of the test method's ability to accurately classify other substances with similar potencies and from the same chemical/product classes.

4.2.4.1 Test Method Accuracy

The analysis of agonist activity for the 34 substances in **Table 4-1** indicated an overall accuracy of 100% (34/34), sensitivity of 100% (27/27), specificity of 100% (7/7), false positive rate of 0% (0/7), and false negative rate of 0% (0/27).

The analysis of antagonist activity for the 10 substances in **Table 4-2** indicated an overall accuracy of 100% (10/10), sensitivity of 100% (3/3), specificity of 100% (7/7), false positive rate of 0% (0/7), and false negative rate of 0% (0/3).

4.2.4.2 Test Method Reliability

For the BG1Luc ER TA agonist test method, there was 100% agreement within each laboratory for each of the three repeat tests for nine reference substances tested in Phase 2 of the agonist validation study. When results were compared across laboratories for these nine substances, there was 78% (7/9) agreement among the three laboratories for the substances. An additional 17 substances tested once in each laboratory for agonist activity produced a definitive result in at least two laboratories. There was agreement among the laboratories for 82% (14/17) of these substances.

For the BG1Luc ER TA antagonist test method, there was 100% agreement within each laboratory for each of the three repeat tests for four reference substances tested in Phase 2 of the antagonist validation study. When results were compared across laboratories for these four substances, there was 100% agreement among the three laboratories for all four substances. An additional five substances tested once in each laboratory for antagonist activity produced a definitive result in at least two laboratories. There was agreement among the laboratories for 80% (4/5) of these substances.

5.0 ICCVAM Consideration of Public Comments

The ICCVAM evaluation process incorporates a high level of scientific peer review and transparency. The evaluation process on the use of the BG1Luc ER TA test method as a screening method to identify *in vitro* ER agonists and antagonists included one public review meeting by an independent scientific peer review panel, multiple opportunities for public comments, and comments from SACATM. ICCVAM and the EDWG considered the Panel report, SACATM comments, and all public comments before finalizing the ICCVAM test method evaluation report and BRD for the use of the BG1Luc ER TA test method. This section summarizes the ICCVAM consideration of public comments (see **Appendix E**).

5.1 ICCVAM Consideration of Public and SACATM Comments

Six opportunities for public comment were provided during the ICCVAM evaluation of the BG1Luc ER TA test method (**Table 5-1**). A total of nine comments were submitted. *Federal Register* notices published by NICEATM–ICCVAM during evaluation of the BG1Luc ER TA test method are available in **Appendix E** and from the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/). Comments received in response to or related to the *Federal Register* notices are available on the NICEATM–ICCVAM website.[4] The following sections, delineated by *Federal Register* notice and public meeting, briefly discuss the public comments received.

Table 5-1 Opportunities for Public Comments

Opportunity for Public Comment	Date	Number of Public Comments Received
69 FR 21564 - *In Vitro* Endocrine Disruptor Test Methods: Request for Comments and Nominations	April 21, 2004	0
71 FR 13597 - Notice of Availability of a Revised List of Recommended Reference Substances for Validation of *In Vitro* Estrogen and Androgen Receptor Binding and Transcriptional Activation Assays: Request for Comments and Submission of *In Vivo* and *In Vitro* Data	March 16, 2006	0
74 FR 62317 - Evaluation of *In Vitro* Estrogen Receptor Transcriptional Activation and *In Vitro* Cell Proliferation Assays for Endocrine Disruptor Chemical Screening: Request for Nominations for an Independent Expert Peer Review Panel and Submission of Relevant *In Vitro* and *In Vivo* Data	November 27, 2009	6
76 FR 4113 - Announcement of an Independent Scientific Peer Review Panel Meeting on an *In Vitro* Estrogen Receptor Transcriptional Activation Test Method for Endocrine Disruptor Chemical Screening; Availability of Draft Background Review Document (BRD); Request for Comments	January 24, 2011	1
76 FR 23323 - Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	April 26, 2011	2
76 FR 28781 - Independent Scientific Peer Review Panel Report: Evaluation of the Validation Status of an *In Vitro* Estrogen Receptor Transcriptional Activation Test Method for Endocrine Disruptor Chemical Screening: Notice of Availability and Request for Public Comments	May 18, 2011	0

[4] http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm

5.1.1 Public Comments in Response to 69 FR 21564 (April 21, 2004)

***In Vitro* Endocrine Disruptor Test Methods: Request for Comments and Nominations**
NICEATM requested nomination of ER and AR binding and TA test methods for validation studies.

No public comments were received in response to this *Federal Register* notice.

5.1.2 Public Comments in Response to 71 FR 13597 (March 16, 2006)

Notice of Availability of a Revised List of Recommended Reference Substances for Validation of *In Vitro* Estrogen and Androgen Receptor Binding and Transcriptional Activation Assays: Request for Comments and Submission of *In Vivo* and *In Vitro* Data
NICEATM announced the availability of an addendum (ICCVAM 2006) to the *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays* (ICCVAM 2003a). The addendum describes the rationale for proposed revisions to the original list of recommended reference substances for validation of *in vitro* ER and AR binding and TA assays. NICEATM requested public comments on the substances proposed as substitutes for six of the 78 substances in the original list. Data were also requested from *in vitro* and *in vivo* studies evaluating the estrogenic and androgenic activity of the 78 substances in the revised list of reference substances.

No public comments were received in response to this *Federal Register* notice.

5.1.3 Public Comments in Response to 74 FR 62317 (November 27, 2009)

Evaluation of *In Vitro* Estrogen Receptor Transcriptional Activation and *In Vitro* Cell Proliferation Assays for Endocrine Disruptor Chemical Screening: Request for Nominations for an Independent Expert Peer Review Panel and Submission of Relevant *In Vitro* and *In Vivo* Data
NICEATM requested:

- Nominations of expert scientists for consideration as potential Panel members
- Submission of existing data from the LUMI–CELL ER and the CertiChem MCF–7 cell proliferation assays
- Submission of data from *in vivo* or other *in vitro* assessments for the 78 reference substances recommended by ICCVAM for the validation of *in vitro* ER and AR binding and TA test methods

NICEATM received six public comments in which nine potential panelists were nominated for consideration. The nominees were included in the database of experts from which the Panel was selected.

5.1.4 Public Comments in Response to 76 FR 4113 (January 24, 2011)

Announcement of an Independent Scientific Peer Review Panel Meeting on an *In Vitro* Estrogen Receptor Transcriptional Activation Test Method for Endocrine Disruptor Chemical Screening; Availability of Draft Background Review Document (BRD); Request for Comments
NICEATM invited public comments on the draft BRD and draft ICCVAM test method recommendations. One public comment was received that included a number of suggestions.

The commenter proposed assigning a level of confidence ranking to the reference data. Substances for which there is a low degree of confidence in the reference data should be deleted

from the reference list and omitted from validation studies. With regard to specific test substances, the commenter stated that the discordant results among laboratories for atrazine, corticosterone, and dicofol were not fully explained.

ICCVAM Response
The independent scientific peer review panel concluded that it is reasonable to use the majority classification criteria among published study results (i.e., >50%) to establish the consensus reference classification for each reference substance. The Panel suggested that this approach could be improved by a ranking method, such as Klimisch criteria (Klimisch et al. 1997), that focuses primarily on the reliability of the data. Such a method would clarify the relative quality of the reference data and strengthen the classification. ICCVAM concurred that additional review and ranking of the published reports would strengthen the utility of literature citations for classifying the reference substances and agreed to take this into consideration in future evaluations.

The commenter questioned the use of flavone as the weak positive control in the antagonist protocol. The commenter further stated that differences among the laboratories in range finder starting concentrations were not fully explained.

ICCVAM Response
During protocol standardization, a number of substances were evaluated for use as the weak antagonist control. Flavone produced a dose response and an $IC_{50} = 4.3 \times 10^{-7}$ M, which was consistent with the single literature reference for this compound (reported $IC_{50} = \sim 15$ μM) and was two times below that of raloxifene. Based on these results, flavone was chosen as the weak antagonist control for the validation study. However, after review of the data from the completed study, it was apparent that the vast majority of test substances classified as "negative" or "presumed negative" produced a "positive" response at concentrations above ~10 μM. Use of flavone as a weak antagonist control was therefore reconsidered.

The commenter suggested including quantitative comparison of test substances (such as EC_{50} values) and indicated that it would be helpful to include data presented as a relative potency index (the EC_{50} of the positive control divided by the EC_{50} of the test substances, multiplied by 100).

ICCVAM Response
Quantitative measures of activity (i.e., EC_{50} and IC_{50} values) were generated and presented in the BRD. The independent scientific peer review panel considered the descriptive approach for evaluating test method reliability acceptable but also suggested additional statistical analyses that could be performed to better characterize and clarify variability. The Panel suggested that a quantitative measure of activity should be included in each future study report, and the uncertainty associated with these estimates should also be reported.

5.1.5 Public Comments in Response to 76 FR 23323 (April 26, 2011)

Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)
NICEATM announced the SACATM meeting and requested written and public oral comments on the agenda topics. Two public comments were received.

One commenter supported the validation of the BG1Luc ER TA test method and recommended modifications of the protocol that would allow for the implementation of a liquid handling system. The commenter felt that the use of a liquid handling system would greatly increase sample throughput.

ICCVAM Response
The use of a liquid handling system represents a potential improvement to the protocol that could

increase throughput. Use of a liquid handling system at the lead laboratory was considered during the initial phases of the validation study. However, because of difficulties experienced with the system that was acquired at the outset of the study, a decision was made to focus on the "benchtop" version of the assay and perhaps reconsider incorporating automated procedures into the assay at a later time.

A second commenter also supported the validation of the BG1Luc ER TA test method and recommended improvements. The commenter recommended a quantitative comparison of the BG1Luc ER TA data to EPA OPPTS 890.1300/OECD TG 455 data and development of a relative potency index for the reference substances.

ICCVAM Response
As stated above, ICCVAM concurred that additional review and ranking of the published reports would strengthen the utility of literature citations for classifying the reference substances. A comparison of median EC_{50} and IC_{50} values from the BG1Luc ER TA test method and literature references is provided in the BRD (**Appendix C**). A relative potency index for the reference substances has not been calculated; however, data provided in the current review permit calculation of such an index.

5.1.6 Public Comments in Response to 76 FR 28781 (May 18, 2011)

Independent Scientific Peer Review Panel Report: Evaluation of the Validation Status of an *In Vitro* Estrogen Receptor Transcriptional Activation Test Method for Endocrine Disruptor Chemical Screening: Notice of Availability and Request for Public Comments
NICEATM requested submission of written public comments on the *Peer Review Panel Report: Evaluation of the LUMI-CELL ER® (BG1Luc ER TA) Test Method* (**Appendix D2**). No comments were received in response to this request.

6.0 References

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