新規試験法提案書

ER STTA 法（hERα-HeLa-9903 細胞を用いたエストロゲン受容体恒常発現系転写活性化試験法）

平成28年12月

国立医薬品食品衛生研究所

新規試験法提案書

平成 28 年 12 月 6 日
No.　2016-01

ER STTA 法（hERα-HeLa-9903 細胞を用いたエストロゲン受容体恒常発現系転写活性化試験法）に関する提案

平成 28 年 12 月 6 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： ER STTA 法（hERα-HeLa-9903 細胞を用いたエストロゲン受容体恒常発現系転写活性化試験法）は培養細胞を用いる *in vitro* 試験法であり、化学物質のエストロゲン受容体への作用の有無を評価でき、誤評価が少ないことから、類似試験法である BG1Luc ER TA 法と同じ程度に、行政上利用が可能であると考える。

この提案書は、Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 455 Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and AntagonistsおよびThe Validation Report of the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity, Part A (agonist assay), Part B (antagonist assay)をもとに、内分泌かく乱試験資料編纂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 ER STTA 法の使用を提案するものである。

大野泰雄

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

大 野 泰 雄　（運営委員会推薦）：座長
飯 塚 尚 文　（独立行政法人　医薬品医療機器総合機構）
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吉 田 武 美　（日本毒性学会）
吉 村　　功　（座長推薦）

任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日

JaCVAM 評価会議

大 野 泰 雄　（運営委員会推薦）：座長
飯 塚 尚 文　（独立行政法人　医薬品医療機器総合機構）
五十嵐良明　（国立医薬品食品衛生研究所）
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任期：平成 28 年 4 月 1 日～平成 30 年 3 月 31 日

JaCVAM 運営委員会

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加藤　篤　（国立感染症研究所）
日下部哲也　（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）
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動物管理室）
東野正明　（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）
中村高敏　（独立行政法人　医薬品医療機器総合機構）
日田　充　（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）
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
安全性予測評価部）
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）
三澤　馨　（厚生労働省　医薬・生活衛生局　医薬品審査管理課）
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
安全性予測評価部　第二室）：事務局

JaCVAM statement on the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity

At a meeting held on 6 December 2016 at the National Institute of Health Sciences (NIHS) in Tokyo, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Proposal: The Stably transfected Transcriptional Activation Assay to Detect ER mediated activity (ER STTA) is an *in vitro* test method that uses cultured cells to assess the effect of chemical substances on estrogen receptors. A high reliability with regard to positive/negative classification means that the ER STTA is considered equivalent to the BG1 Luc ER antagonist assay as a suitable test method for use in a regulatory context.

This statement was prepared following a review of the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 455 Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and Antagonists as well as of a validation report on the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity, Part A (agonist assay) and Part B (antagonist assay), together with other materials prepared by the Endocrine Disruption Testing JaCVAM Editorial Committee, to acknowledge that the results of a review and study by the JaCVAM Regulatory Acceptance Board have confirmed the usefulness of this assay.
Based on the above, we propose the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity as a useful means for assessing ocular irritation potency during safety assessments by regulatory agencies.

Yasuo Ohno
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

6 December 2016

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. Yasuo Ohno (nominee by JaCVAM Steering Committee) : Chairperson
Mr. Naofumi Iizuka (Pharmaceuticals and Medical Devices Agency)
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Yuji Ishii (Biological Safety Research Center: BSRC, NIHS)
Ms. Yumiko Iwase (Japan Pharmaceutical Manufacturers Association)
Mr. Kazuhiro Kaneko (Japan Chemical Industry Association)
Mr. Eiji Maki (Japanese Society of Immunotoxicology)
Mr. Takeshi Morita (Japanese Environmental Mutagen Society)
Mr. Akiyoshi Nishikawa (BSRC, NIHS)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Ms. Koko Tanigawa (Japanese Society for Alternatives to Animal Experiments)
Mr. Takashi Yamada (National Institute of Technology and Evaluation)
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)
Mr. Takemi Yoshida (Japanese Society of Toxicology)
Mr. Isao Yoshimura (nominee by Chairperson)

Term: From 1st April 2014 to 31st March 2016

Mr. Yasuo Ohno (nominee by JaCVAM Steering Committee) : Chairperson
Mr. Naofumi Iizuka (Pharmaceuticals and Medical Devices Agency)
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Noriyasu Imai (Japanese Society for Alternatives to Animal Experiments)
Mr. Tomoaki Inoue (Japanese Society of Immunotoxicology)
Mr. Yuji Ishii (BSRC, NIHS)
Ms. Yumiko Iwase (Japan Pharmaceutical Manufacturers Association)
Mr. Takeshi Morita (Japanese Environmental Mutagen Society)
Mr. Shunji Nakai (Japan Chemical Industry Association)
Ms. Ruriko Nakamura (National Institute of Technology and Evaluation)
Mr. Akiyoshi Nishikawa (BSRC, NIHS)
Mr. Satoshi Numazawa (Japanese Society of Toxicology)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)

Term: From 1st April 2016 to 31st March 2018

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. Toru Kawanishi (NIHS)
Mr. Mitsuru Hida (Ministry of Health, Labour and Welfare)
Ms. Yoko Hirabayashi (Division of Toxicology, BSRC, NIHS)
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)
Mr. Atsushi Kato (National Institute of Infectious Diseases)
Mr. Tetsuya Kusakabe (Ministry of Health, Labour and Welfare)
Mr. Kaoru Misawa (Ministry of Health, Labour and Welfare)
Mr. Takatoshi Nakamura (Pharmaceutical & Medical Devices Agency)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Ms. Yuko Sekino (Division of Pharmacology, BSRC, NIHS)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Mr. Atsuya Takagi (Animal Management Section of the Division of Toxicology, BSRC, NIHS)
Mr. Masaaki Tsukano (Ministry of Health, Labour and Welfare)
Mr. Hajime Kojima (Division of Risk Assessment, BSRC, NIHS): Secretary

ER STTA 法

（hERα-HeLa-9903 細胞を用いたエストロゲン受容体恒常発現系転写活性化試験法）

目　　次

評価会議報告書

ER STTA 法
（hERα-HeLa-9903 細胞を用いたエストロゲン受容体恒常発現系転写活性化試験法）

JaCVAM 評価会議

平成 28 年（2016 年） 12 月 6 日

JaCVAM 評価会議

大 野 泰 雄　（運営委員会推薦）：座長
飯 塚 尚 文　（独立行政法人　医薬品医療機器総合機構）
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任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日

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任期：平成 28 年 4 月 1 日～平成 30 年 3 月 31 日

hERα-HeLa-9903 細胞を用いたエストロゲン受容体（Estrogen receptor：ER）恒常発現系転写活性化試験法：The Stably Transfected TA assay using the human ERα-HeLa-9903 cell line（ER STTA 法、以下、本試験法）は、化学物質のエストロゲン（および抗エストロゲン）活性を化学発光により検出する *in vitro* 試験法の一つで、内分泌かく乱物質対策のために開発されたものである。本試験法は HeLa 細胞に導入された ERα の活性化によって起こるレポーター遺伝子の転写活性の変化を化学発光により定量的に測定する試験系である。

本試験法のバリデーション研究については、ERα アゴニストと ERα アンタゴニストについて、それぞれ独立した施設間バリデーション研究が実施された。アゴニスト試験バリデーション報告書[1)]は、OECD（Organisation for Economic Co-operation and Development）に 2006 年に提出され、アンタゴニスト試験バリデーション報告書[2)]は、2014 年に提出された。前者は 2009 年に試験法ガイドライン（Test Guideline: TG）455 として承認され[3)]、後者は 2015 年に TG455 の改訂版として TG となった[4)]。JaCVAM（Japanese Center for Validation of Alternative Methods）評価会議は、内分泌かく乱試験法資料編纂委員会により作成された「ER STTA 法：hERα-HeLa-9903 細胞を用いたエストロゲン受容体恒常発現系転写活性化試験法の評価報告書」（平成 28 年 10 月 21 日）を用いて、本試験法の妥当性について検討した。

１．試験法の定義

名称：　ER STTA 法（hERα-HeLa-9903 細胞を用いたエストロゲン受容体恒常発現系転写活性化試験法）

代替する対象毒性試験：　*in vivo* 試験法の「げっ歯類を用いる子宮肥大試験」（OECD TG440, 2007）[5)]。を代替する試験法である。類似の試験法として、*in vitro* 試験法の「ラット子宮エストロゲン受容体結合性試験」（OECD TG 493, 2015）[6)]と「BG1Luc4E2 細胞を用いるエストロゲン受容体転写活性化試験 : BG1Luc estrogen receptor transactivation assay（BG1Luc ER TA）」（OECD TG 457, 2012）[7)]がある。

試験法の概略：　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するために、本試験法は、HeLa 細胞にヒト ERα を恒常的に発現するプラスミドとエストロゲン応答配列（Estrogen responsive element：ERE）の下流にルシフェラーゼ遺伝子を繋いだレポータープラスミドを導入・安定発現させた細胞（hERα-HeLa-9903 細胞）を用いる。この細胞に被験物質を曝露した後のルシフェラーゼ活性の変化を測定する。

２．評価に用いた資料および評価内容の科学的妥当性

アゴニストを評価する試験法に関しては、（一財）化学物質評価研究機構が中心となって、4 試験施設が参加したバリデーション研究が実施された。その報告書が 2006 年に OECD に

提出され、2009 年に TG455 として承認された。この TG455 は、2012 年に BG1Luc ER TA 法のアゴニスト試験法を包含する性能準拠試験法ガイドライン（Performance-Based Test Guideline：PBTG）[8]に更新され、あわせてアゴニスト試験の性能基準（Performance Standards：PS）が公開された[9]。アンタゴニストを評価する試験法に関しては、JaCVAM が中心となって、最終的に 4 試験施設でバリデーション研究が実施された。アンタゴニストバリデーション報告書は、先に提出されたアゴニストについてのバリデーション報告書の追加文書として提出され、最終的にアゴニスト評価法（part A）とアンタゴニスト評価法（part B）を含む単一のバリデーション報告書として公開された[2]。これらのバリデーション報告書に基づき、2014 年にアンタゴニスト試験を包含する TG455 改訂案が OECD に提出され、承認された。これらの資料を用いて、JaCVAM 内分泌かく乱試験法資料編纂委員会が評価し、報告書としてまとめたものを評価資料とした。

本試験法は HeLa 細胞にヒト ERα を導入し、強制発現させた hER α-HeLa-9903 細胞を用い、アゴニストおよびアンタゴニストの両者を検出できるような *in vitro* 試験法にしたものである。水や Dimethyl sulfoxide（DMSO）等に溶解する広範な被験物質に適用できる点で価値が高い。類似の ER 転写活性化試験法である BG1Luc ER TA で用いられる BG1Luc 細胞は、ERα 型／ERβ 型双方を発現しており、両者を介した作用が検出可能であるとされている。これに対して、本試験法では、純粋にヒト ERα のみを介した作用を検出可能であるが、2 種の ER アイソフォーム（ERα、ERβ）に対して完全な選択性を示す化学物質は知られておらず、定性的評価において両試験法の結果は同等と考えられ、ER を介して作用する化学物質をスクリーニングする方法として科学的な妥当性があると考えられる。

３．本試験法の有用性と適用限界

本試験法は単にアンタゴニストと ER との結合にとどまらずアゴニストの活性を抑制する効果を検出できる点が優れている。ICCVAM（Interagency Coordinating Committee on the Validation of Alternative Methods）参照分類、ER 結合試験、子宮肥大試験等の結果との一致度、本試験法の感度、特異度は良好であり、偽陽性や偽陰性は少ないという結果を示している。

BG1Luc ER TA 試験法に比し感度が高く、アゴニスト、アンタゴニストを 1/100 の濃度で検出できる。

本試験法による化学物質のアゴニスト活性・アンタゴニスト活性のスクリーニングの結果を ICCVAM 参照分類との比較した結果、一致率は 100％であった。エストロゲン活性の検出に用いられる既存の細胞系（内在性のヒト ER を利用する BG1 細胞）などと比較しても、陽性物質や陰性物質の識別性は良好である。本試験法で用いている hERα-HeLa-9903 細胞は増殖が早いため、試験準備のための時間を短くすることができ、多数の化学物質のスクリーニングに向いている。

レポーターとして用いているルシフェラーゼ活性に影響を与える化学物質では、ER 非特

異的な亢進や阻害等により偽陽性（もしくは偽陰性）反応を惹起する可能性があるため注意が必要である。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が、細胞の継代によって ER の反応性が変化しないことを確認しておく必要がある。OECD TG で示された ERαアゴニストおよび ERαアンタゴニスト試験それぞれの熟達度確認物質 14 物質と 10 物質を用いて用量-応答性を調べておくことが必要である。また、溶媒として水や Ethanol (>95%)あるいは DMSO が用いられているが、DMSO に溶けにくい物質については、他の有用な溶媒を検討し、それが hERα-HeLa-9903 細胞に影響しないことを検証する必要がある。

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。今後の検討が期待される。また、代謝されてから ERαアゴニストおよび ERαアンタゴニスト作用を示す物質の評価も行えない。

４．目的とする物質又は製品の毒性を評価する試験法としての、社会的受け入れ性および行政上の利用の可能性

社会的受け入れ性：

本試験法は遺伝子組み換えにより作製された hERα-HeLa-9903 細胞を用いる試験法であり、生きた動物を用いないという点で、3Rs の精神に合致している。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。また、本試験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、高価なものでない。細胞も公的な細胞バンクから入手可能である。以上より、本試験法の社会的受け入れ性は高いと考える。

行政上の利用性：

本試験法は培養細胞を用いる *in vitro* 試験法であり、化学物質のエストロゲン受容体への作用の有無を評価でき、誤評価が少ないことから、類似試験法である BG1Luc ER TA 法と同じ程度に [10]、行政上利用が可能であると考える。

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ER STTA 法：hERα-HeLa-9903 細胞を用いたエストロゲン受容体恒常発現系転写活性化試験法の評価報告書

平成 28 年 10 月 21 日

内分泌かく乱試験法資料編纂委員会

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（横浜市立大学、元 基礎生物学研究所）

小野　敦（岡山大学、元 国立医薬品食品衛生研究所）

用語集

Accuracy：正確性

Concordance：一致度

17β-estradiol (E2)：17β-エストラジオール

ER：Estrogen receptor

ER α/β：エストロゲン受容体 α/β

ER STTA：hERα-HeLa-9903 細胞を用いたエストロゲン受容体恒常発現系転写活性化試験法

Estrogen responsive element (ERE)：核内のエストロゲン応答配列

European Centre for Validation of Alternative Methods (ECVAM)：欧州代替法評価センター

False negative rate：偽陰性率

False positive rate：偽陽性率

GLP: Good Laboratory Practice

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：施設間バリデーション研究

Intra-laboratory validation study：施設内バリデーション研究

Japanese Center for Validation of Alternative Methods (JaCVAM)：日本動物実験代替法評価センター

Performance-Based Test Guideline (PBTG)：性能準拠試験法ガイドライン

Performance Standards (PS)：性能標準

Reliability：信頼性

Test Guideline (TG)：試験法ガイドライン

Transcription activation (TA)：転写活性化

Uterotrophic assay：子宮肥大試験

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

Validation study：バリデーション研究

Validation study management team：バリデーション研究運営委員会

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

ER STTA 法（詳しくは「hERα-HeLa-9903 細胞を用いたエストロゲン受容体(ER)恒常発現系転写活性化試験法」; The Stably Transfected Transcription Activation (STTA) assay using the human ERα-HeLa-9903 cell line）は、化学物質のエストロゲン（および抗エストロゲン）活性を化学発光により検出する *in vitro* 試験法の一つで、内分泌かく乱物質対策のために開発されたものである。本試験法は、我が国（日本）の住友化学株式会社で構築された hERα-HeLa-9903 細胞株を用いて、（一財）化学物質評価研究機構（以下、CERI と記す）においてプロトコルが開発された。hERα-HeLa-9903 細胞は、ヒト子宮頚がん由来の HeLa 細胞にヒト ERα を発現する遺伝子配列と、アフリカツメガエルのビテロジェニン遺伝子由来のエストロゲン応答配列（ERE）の下流に mouse metallothionein（MT）遺伝子の TATA ボックス配列およびレポーター遺伝子となるホタルルシフエラーゼ遺伝子を連結した遺伝子配列を安定的に導入した細胞であり、ER STTA 法は導入された ERα の活性化によって起るレポーター遺伝子の転写活性の変化を化学発光により定量的に測定する試験系である。hERα-HeLa-9903 細胞は、国立研究開発法人医薬基盤・健康・栄養研究所 JCRB 細胞バンク（http://cellbank.nibiohn.go.jp/）より入手可能である（細胞登録番号：JCRB1318）。ER STTA 法については、厚生労働省・経済産業省共同で OECD に提案され、その後実施されたバリデーション試験の結果をもとに、試験法ガイドライン（TG）の提案に至った。

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（内分泌かく乱物質と呼ばれる）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「嚙歯類を用いる子宮肥大試験」が確立され、バリデーション試験を行った上で国際的な TG（OECD TG440）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結合反応の測定法があり、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 17β-estradiol (E2)と比較して定量的に決定するものである。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。

ER STTA 法は､エストロゲン活性物質による ER 結合に続く下流遺伝子の転写活性化をエンドポイントとして検出する方法である。細胞には転写活性化を調べるために、当該受容体特異的応答配列と連結した位置（下流）にレポーター遺伝子を組み込んでおき、転写活性化によるレポータータンパクの発現を発光基質の添加により検出する。このような細胞としては、すでに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 ERE の下流に安定的に導入した BG1Luc4E2 細胞が開発されており、この細胞を用いた試験法「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（BG1Luc ER TA 法）」が OECD TG に収載されている（OECD TG 457, 2012）。

ER STTA 法と BG1Luc ER TA 法の相違は、(1) 本法が hERα を HeLa 細胞に組み込んだものであるのに対し、BG1Luc ER TA 法は BG1 細胞に内在する ER を利用する、(2) 本法では組み込まれた受容体はヒト ERα のみであるが、BG1 細胞は ERα と ERβ の双方を発現している。ただし、2 種の ER アイソフォーム（ERα、ERβ）に対して完全な選択性を示す化学物質は知られておらず、定性的評価において両測定系の結果は同等と考えられる。(3) BG1Luc ER TA 法のアンタゴニスト試験では、バリデーション試験において高濃度領域でのルシフェラーゼ活性の非特異的阻害が認められたことから、評価可能な被験物質の最大濃度は、20μg/mL (約 10μM)に制限されているのに対して、本法では、媒体への溶解性が良好かつ細胞毒性が認められない場合、アゴニスト・アンタゴニスト試験とも最大 1mM まで評価可能である。

ER STTA 法のバリデーションについては、アゴニスト試験とアンタゴニスト試験について別々に独立した施設間バリデーション試験が実施された。

アゴニスト試験に関しては、2004 年に CERI が中心となって、住友化学(株)(以下、住友

化学と記す)、大塚製薬（株）(以下、大塚製薬と記す)、(株)カネカテクノリサーチ(以下、カネカと記す)の国内 4 施設の参加によるバリデーション試験が実施された。アゴニストバリデーション試験の予試験では、事前検討として陽性 2 物質（E2, Bisphenol A）の 2 回繰り返し測定を行い、参加試験施設の測定技術および実験手法の技術移転の評価が行われた。本試験では、陽性対照を含むコード化された 10 物質の 3 回繰り返し測定により施設内、施設間再現性についての検討が行われた。また、リードラボで実施された 86 物質（多施設バリデーションでの測定実施 10 物質を含む）の測定結果から結合試験との比較、子宮肥大試験との比較、ICCVAM 報告との比較が行われた。バリデーション試験の結果、4 試験施設のデータは高い一致率を示し、ICCVAM 報告の分類（陽性・陰性）との一致率も高いものであった。この結果から、ER STTA 法は、化学物質の *in vitro* ER アゴニスト活性を正確に検出する試験法であると評価された。このアゴニスト試験バリデーション報告書は、2006 年にアゴニスト試験法のみの試験法ガイドライン案とともに OECD に提出され、2009 年に成立した最初の TG455 をサポートするデータとなった。その後、TG455 は、2012 年に BG1Luc ER TA 法のアゴニスト試験法を包含する性能準拠試験法ガイドライン（PBTG）に更新され、あわせてアゴニスト試験の性能基準（PS）が公開された(1)。

アンタゴニスト試験に関しては、上記のアゴニスト試験バリデーション報告書の第三者評価における要求を受けて、2008 年に OECD にプロジェクト提案され、 JaCVAM が中心となって、当初、国内 3 施設（CERI、大塚製薬、カネカ）、海外 2 施設（韓国 KFDA、ベルギーVITO）の参加による国際バリデーション試験として開始されたものの、海外 2 施設および国内 1 施設（カネカ）は各施設の事情によりバリデーション試験の途中で撤退したため、バリデーションに必要な試験データの取得のため、2010 年より BG1Luc ER TA 法バリデーション試験に参加経験のある(株)日吉(以下、日吉と記す)が追加で参加した。アンタゴニストバリデーション試験は 3 段階にわけて行われ、第 1 段階では、TG455 で示されたアゴニスト試験の参照物質 3 物質の 3 回繰り返し測定により、参加試験施設の測定技術および実験手法の技術移転の評価が行われ、全ての参加施設が、アゴニストの基準を満たす結果を得た。第 2 段階では、新たに構築されたアンタゴニストのプロトコルに従い、アンタゴニスト測定の参照物質候補 4 物質の測定、第 3 段階では、コード化された 20 物質の測定が行われた。海外 2 施設は第 2 段階の途中で、国内 1 施設（カネカ）は第 3 段階の途中でバリデーション試験より撤退したため、最終的な評価は、CERI、大塚製薬および日吉の測定結果とカネカの測定済み結果より行われた。バリデーション試験の結果、被験物質の定性的評価結果は、施設間で一致するものの、施設によっては参照基準を満たす事が出来なかった。この原因として、細胞培養技術に由来する反応性の変化が示唆されたものの完全には

解消出来なかったことから、陽性物質の定量的評価結果について信頼性を保証出来ない可能性が示された。そのため、本試験系は当初、定量的評価系として開発されたものの定性的評価を主眼としたプロトコルの変更が行われた。既に試験法ガイドラインが成立している BG1Luc ER TA 法も定性的評価系として試験法ガイドライン化されていることから、OECD TG 455 改定案についても定性的評価系として提案された。アンタゴニスト試験バリデーション報告書については、2014 年にアンタゴニスト試験を包含する TG455 改訂案(2)およびアンタゴニスト試験の PS(3)とともに OECD に提出された。アンタゴニスト試験バリデーション報告書は、先に提出されたアゴニスト試験バリデーション報告書の追加文書として提出され、最終的にアゴニスト試験（part A）とアンタゴニスト試験（part B）を含む単一のバリデーション報告書として公開される予定である(4)。なお、TG 455 へのアンタゴニスト試験法の追加により BG1Luc ER TA 法は、ER STTA 法と共に試験法の一つとして TG 455 に収載され、BG1Luc ER TA 法のみの TG である TG 457 は、廃止される予定である。

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かく乱物質のうち ER に作用する化学物質を検索し、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、ER STTA 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するために HeLa 細胞にヒト ERα を恒常的に発現するプラスミドと ERE 下流にルシフェラーゼ遺伝子を繋いだレポータープラスミドを導入・安定発現させ、被験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。OECD TG 455 に添付されたプロトコル（ANNEX 2）で示された培養方法および注意点を忠実に守ること、細胞濃度の調整方法なども含めて、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. 比較対照の設定：被験物質の測定値は全て相対値で比較するので適切な濃度の E2 を常に同一の 96 ウェル・プレート上で測定し、それに対する相対値として表す。

c. 被験物質：被験物質測定時には、プロトコルで規定される陽性・陰性の参照物質を同時に測定することが重要である。

2-2　妥当性の検討

１）総合的検討: ER STTA 法の基となる HeLa 細胞は天然の ER を発現しておらず、ER STTA 法で用いる HeLa-9903 では強制発現されたヒト ERα を介したアゴニストおよびアンタゴニストの両者を検出可能な *in vitro* 系である。溶媒に溶解可能（DMSO 以外を用いる場合もある）な広範な被験物質に適用できる点で非常に価値が高い。同様の ER 転写活性化試験法である BG1Luc 細胞は、ERα 型／β 型双方を発現しており、両者を介した作用が検出可能であるとされるものの、いずれの受容体への作用であるかを区別することは出来ない。これに対して、ER STTA 法（HeLa-9903 細胞）では、純粋にヒト ERα のみを介した作用を検出可能である。

理論的視点から：

アゴニスト活性およびアンタゴニスト作用のある化学物質による ER に対する単なる結合能ではなく転写活性を測定できる。*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：ER STTA 法では HeLa 細胞にヒト ERα を恒常的に強制発現させた HeLa9903 細胞を用いて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。その影響は強制発現させた 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、試験対象物質の測定時には、ER 反応性のチェックを TG で指定された参照（標準）物質の測定によって常に評価する必要がある。

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 活性化によるレポーター遺伝子発現により測定を行う。一部の化学物質は、レポーターとして用いているルシフェラーゼ活性の受容体非特異的な亢進や阻害等により疑陽性（もしくは偽陰性）反応を惹起する可能性がある。また、ER STTA 法では、一部の植物エストロゲンで高濃度において受容体を介しないルシフェラーゼの高活性化

現象が示されることが明らかにされている。これまでに工業用化学物質では、同様の作用が検出された例はないが注意が必要である。

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　ER STTA 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。

使用培養細胞について：細胞の反応性を維持するため、プロトコルでは、測定に使用する細胞の継代数は 40 代までと規定されている。しかし、アンタゴニスト試験のバリデーションにおいては、一部の参加施設で継代数が 40 代以下であっても細胞継代に伴うと思われる反応性の低下や用量反応性の変化が認められており注意が必要である。バリデーション試験では、継代時のトリプシン消化時間の延長が細胞にダメージを与える可能性が示唆されている。また、細胞形態に異常が無くても、反応性が変化することが示唆されており、参照物質の測定結果がプロトコルに示されたクライテリアを連続して逸脱した場合やプレート採用基準を連続して満たさなかった場合、細胞を新たなロット（サブロット）に切替えるべきである。

３．バリデーション試験に用いた物質の分類と妥当性

アゴニスト試験法のバリデーションでは 86 物質（Table 1）が、アンタゴニスト試験法のバリデーションでは 21 物質（Table 2）が用いられた(4)。それらにはステロイド、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含まれている。試験法の感度を評価するため、陰性物質がアゴニスト試験では約 30%（27/86）、アンタゴニスト試験では 60%（12/20）を占めており、選ばれた物質は妥当と判断される。

４．試験法のデータと結果の有用性

アゴニスト試験のバリデーションに用いられた 86 物質のうち、参加 4 施設全てで測定が行われた物質は、陽性物質として測定された E2 及びコード化物質として測定が実施された 9 物質の計 10 物質（陽性 7 物質、陰性 3 物質）であり、これら 10 物質の測定結果をもとに

施設内および施設間再現性の評価が行われ、他の 76 物質については、リードラボである CERI における測定結果をもとに、ICCVAM 参照分類や ER 結合試験、子宮肥大試験結果との比較が行われた（Table 3～6）。

アンタゴニスト試験のバリデーションでは、参照物質を含む 21 物質のうち、バリデーション試験第 3 段階でコード化物質として測定が実施された 20 物質（陽性 8 物質、陰性 10 物質、不明 2 物質）の測定結果をもとに、施設内および施設間再現性の評価、および文献情報などから予想されたアンタゴニスト活性との比較による評価精度のバリデーションが行われた（Table 7）。アンタゴニスト試験のバリデーションにおいては測定物質数が少ないが、アンタゴニスト活性を示すことが明らかにされている化学物質の多くは、医薬品もしくはその類似物質であり、一般工業化学物質で明らかなアンタゴニスト活性が知られている物質は、非常に限られていることによる。バリデーション試験で評価が行われた陽性 8 物質は、今後の新たな試験系評価における重要な参照物質となるであろう。

いずれのバリデーション試験においても用いられた化学物質は、ICCVAM 参照分類リスト等を参考に、広範な種類の物質が選択されているが、バリデーション報告書においては、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。

５．試験方法の再現性

アゴニスト試験では、陽性物質として測定された E2 を含む 10 物質（陽性 7 物質、陰性 3 物質）の参加 4 施設における 3 回の測定結果をもとに施設内および施設間再現性のバリデーションが行われ、いずれも一致率は 100％であった。

アンタゴニスト試験では、バリデーション試験第 3 段階目で撤退したカネカを含む 4 施設における 20 物質（大塚製薬は 19 物質、カネカは 12 物質）の 3 回以上の測定結果をもとに施設内再現性の解析が行われた。施設内再現性についてカネカでは、各物質 5～6 回の測定結果が全て一致したのは 67％(8/12 物質)であったが、他の 4 物質については、測定結果が一致しなかったのは、各 1 測定のみであった。他の施設では 90％以上の再現性を示した（Table 8）。一方、施設間再現性についても同様に 20 物質についての 2～4 施設からの測定結果から解析が行われ、2 物質（Atrazine、Dibenzo[a,h]anthracene）については、それぞれ 1/3 施設で他施設と一致しない結果が示された。その他の被験物質については、施設間で 100％一致する結果を得た。結果として文献情報などから予想されたアンタゴニスト活性（不明 2 物質を除く 18 物質（陽性 8 物質、陰性 10 物質））との比較では、2 施設で一致率 94％、他の 2 施設では 100％であった（Table 9）。

６．試験法の正確性・信頼性

ER STTA 法の正確性を評価するため、アゴニスト試験では、35 物質（陽性 25 物質、陰性 10 物質）の測定結果について ICCVAM 参照分類との比較が行われ一致率は、100%であった（Table 10）。ER 結合試験データと ER STTA 法の比較が 48 物質（結合試験陽性 24 物質、陰性 24 物質）について行われ一致率は 79%（38/48）であった（Table 11）。同様に、*in vivo* 子宮肥大試験との比較が 48 物質（子宮肥大試験陽性 32 物質、陰性 16 物質）について行われ一致率は 85%（41/48）であった（Table 12）。

アンタゴニスト試験については、バリデーションに用いられた 20 物質について、文献情報などから予想されたアンタゴニスト活性（不明 2 物質を除く 18 物質（陽性 8 物質、陰性 10 物質））と 2～4 施設での測定結果を総合的に判断した判定結果との比較が行われ、一致率は、100%であった（Table 9）。

７．データの質

アゴニストバリデーション試験における多施設測定は、リードラボである CERI の信頼性保証システムのもと、GLP（OECD principle of Good Laboratory Practice, November 26, 1997）に準拠して実施された（先行バリデーション試験は、非 GLP で実施された）。アンタゴニストバリデーション試験については、バリデーション報告書に記載は無いが、各施設において GLP に準じて試験が実施された。

８．試験法の有用性、限界および提言

1) アンタゴニストによる結果：ER STTA 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を抑制する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、ER STTA 法の感受性、特異性は良好であり、偽陽性や偽陰性は少ないという結果を示した。
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 濃度で検出できるのでより感度が高く、偽陽性・偽陰性の生じる可能性は少ないと思われる。
3) ER STTA 法による化学物質のアゴニスト活性・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、 BG1 細胞）などと比較しても、陽性や陰性物質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) レポーターとして用いているルシフェラーゼ活性に影響を与える化学物質では、ER 非特異的な亢進や阻害等により疑陽性（もしくは偽陰性）反応を惹起する可能性があるため注意が必要である。

5) 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。細胞の継代によって ER の反応性が変化しないことを確認しておくことが必要である。TG で示された ER アゴニスト試験、アンタゴニスト試験それぞれの参照物質（陽性・陰性）を用いて用量-応答性を調べておくことが必要である。

6) 溶媒として水や Ethanol (>95%)あるいは DMSO が用いられているが、DMSO に溶けにくい物質については、他の有用な溶媒を検討し、それが HeLa-9903 細胞に影響しないことを確認しておく必要がある。

7) 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。

8) 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、ER STTA 法を用いてどのように評価するかの検討が必要である。

9) ER STTA 法は、化学物質の内分泌かく乱性をスクリーニングするための試験法であり、化学物質の安全性評価に用いる際には、他の *in vivo* 評価系等との結果と併せて実際の生体影響について総合的に判断を行うべきである。

10) 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。HeLa-9903 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。使用する試験系の参照(標準)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。

11) 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、たとえば顕微鏡写真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

内分泌かく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。一方、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 への作用は、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、導入の一過性型／安定型を問わず、前述の BG1Luc 細胞を始め、酵母、ゼブラフィッシュ肝細胞株、HepG2、CV-1 など種々の細胞で検討されている。

上述の試験のうち、BG1Luc 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、BG1Luc ER TA 法として OECD TG にも収載されているが、これに対する ER STTA 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、バリデーション試験は行われていない。

１０．結論

ER STTA 法は、化学物質の *in vitro* でのアゴニスト活性および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バリデーション試験はアゴニストについては 4 試験施設の測定結果をもとに、また、アンタゴニストについては参加 6 試験施設のうち 4 施設の測定結果をもとにその再現性･信頼性が確認されている。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 RNA 転写活性化まで検出することにより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は、ICCVAM の選定した参照物質、CERI における背景データや文献情報をもとに選定された化学物質（アゴニスト試験では 86 物質、アンタゴニスト試験では 20 物質）を用いてバリデーション試験が行われた。バリデーションの結果をもとに、OECD の第三者評価ではこの試験法の正確性と信頼性が高いと評価されており、OECD-EDTA で提案された化学物質の内分泌かく乱性評価のコンセプチュアルフレームワークのレベル 2 に該当する試験法として内分泌かく乱物質のスクリーニング評価に有用であると認めている。本試験法は定量的評価法として開発され、アゴニスト試験法については、バリデーション試験において定量的評価値の信頼性が評価されたが、アンタゴニスト試験法ではバリデーション試験に

おいて試験施設間で定量値のばらつきが認められたことから定性的評価法として OECD ガイドライン化された。

本試験法の今後の問題点として、内分泌かく乱性がエストロゲン（抗エストロゲン）活性に起因するという考えにおいて、本試験法における陽性反応が実際の生体影響をどの程度反映するものであるかという課題があげられる。本試験法はあくまでもスクリーニング法であり、想定される有害影響を確定評価する試験法と組み合わせて評価を行うことで今後の化学物質管理に大きく貢献すると考えられる。

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Table 1 ER-STTA アゴニストバリデーション試験で測定が行われた 86 物質の測定結果

No	Chemical	CASRN	ER STTA agonist assay			
			PC_{10} (M)	PC_{50} (M)	PC_{10} based class	PC_{50} based class
1	Atrazine	1912-24-9	-	-	N	N
2	Corticosterone	50-22-6	-	-	N	N
3	Haloperidol	52-86-8	-	-	N	N
4	Ketoconazole	65277-42-1	-	-	N	N
5	Linuron	330-55-2	-	-	N	N
6	Phenobarbital(Na)	57-30-7	-	-	N	N
7	Reserpine	50-55-5	-	-	N	N
8	Spironolactone	52-01-7	-	-	N	N
9	Flutamide	13311-84-7	-	-	N	N
10	Procymidone	32809-16-8	-	-	N	N
11	Ethyl paraben	120-47-8	5.00E-06	PC_{10} (noPC_{50})	P	N
12	p,p'-Methoxychlor	72-43-5	1.23E-06	PC_{10} (noPC_{50})	P	N
13	Tamoxifen	10540-29-1	1.49E-07	PC_{10} (noPC_{50})	P	N
14	Clomiphene citrate	50-41-9	3.68E-08	PC_{10} (noPC_{50})	P	N
15	Zearalenone	17924-92-4	2.44E-11	6.44E-10	P	P
16	17β-Estradiol	50-28-2	<1.00E-11	<1.00E-11	P	P
17	Trenbolone	10161-33-8	1.78E-08	2.73E-07	P	P
18	17α-Estradiol	57-91-0	7.24E-11	6.44E-10	P	P
19	17α-Ethinyl estradiol	57-63-6	<1.00E-11	<1.00E-11	P	P
20	4-Cumylphenol	599-64-4	1.49E-07	1.60E-06	P	P
21	4-tert-Octylphenol	140-66-9	1.85E-09	7.37E-08	P	P
22	Apigenin	520-36-5	1.31E-07	5.71E-07	P	P
23	Bisphenol A	80-05-7	2.02E-08	2.94E-07	P	P
24	Bisphenol B	77-40-7	2.36E-08	2.11E-07	P	P
25	Butylbenzyl phthalate	85-68-7	1.14E-06	4.11E-06	P	P
26	Coumestrol	479-13-0	1.23E-09	2.00E-08	P	P
27	Daidzein	486-66-8	1.76E-08	1.51E-07	P	P
28	Diethylstilbestrol	56-53-1	<1.00E-11	2.04E-11	P	P
29	Estrone	53-16-7	3.02E-11	5.88E-10	P	P
30	Genistein	446-72-0	2.24E-09	2.45E-08	P	P
31	Kaempferol	520-18-3	1.36E-07	1.21E-06	P	P
32	Chlordecone (Kepone)	143-50-0	7.11E-07	7.68E-06	P	P
33	Methyl testosterone	58-18-4	1.73E-07	4.11E-06	P	P
34	Morin	480-16-0	5.43E-07	4.16E-06	P	P

35	Norethynodrel	68-23-5	1.11E-10	1.50E-09	P	P
36	Phenolphthalin	81-90-3	-	-	N	N
37	Progesterone	57-83-0	-	-	N	N
38	Cyproterone acetate	427-51-0	-	-	N	N
39	Mifepristone	84371-65-3	-	-	N	N
40	Diethylhexyl phthalate	117-81-7	-	-	N	N
41	L-Thyroxine	51-48-9	1.32E-06	PC_{10} ($noPC_{50}$)	P	N
42	4-Androstenedione	63-05-8	2.56E-07	PC_{10} ($noPC_{50}$)	P	N
43	Testosterone	58-22-0	2.82E-08	9.78E-06	P	P
44	Vinclozolin	50471-44-8	1.33E-07	7.65E-06	P	P
45	Dibutyl phthalate	84-74-2	-	-	N	N
46	Nonylphenol	25154-52-3	1.37E-08	1.58E-07	P	P
47	Phenobarbital	50-06-6	-	-	N	N
48	Medroxyprogesterone	520-85-4	-	-	N	N
49	Dihydrotestosterone (DHT)	521-18-6	1.04E-07	5.28E-07	P	P
50	Testosterone propionate	57-85-2	2.03E-09	2.91E-07	P	P
51	Fenarimol	60168-88-9	-	-	N	N
52	p,p'-DDE	72-55-9	-	-	N	N
53	Hexestrol	84-16-2	<1.00E-11	2.75E-11	P	P
54	(2,4,5-Trichlorophenoxy)acetic acid	93-76-5	-	-	N	N
55	para-sec-butylphenol	99-71-8	1.38E-06	PC10($noPC_{50}$)	P	N
56	Norgestrel	797-63-7	1.05E-07	PC10($noPC_{50}$)	P	N
57	Norethindrone	68-22-4	1.01E-09	4.95E-08	P	P
58	Equilin	474-86-2	<1.00E-11	7.54E-11	P	P
59	Dicyclohexylphthalate	84-61-7	2.53E-06	PC_{10} ($noPC_{50}$)	P	N
60	Diethyl phthalate	84-66-2	4.46E-06	PC_{10} ($noPC_{50}$)	P	N
61	di(2-ethylhexyl)adipate	103-23-1	-	-	N	N
62	p-dodecyl-phenol	104-43-8	2.36E-08	4.10E-07	P	P
63	4-n-octylphenol	1806-26-4	1.26E-06	PC_{10} ($noPC_{50}$)	P	N
64	4-n-amylphenol	14938-35-3	1.78E-07	4.62E-06	P	P
65	Testosterone enanthate	315-37-7	1.71E-08	2.71E-07	P	P
66	p-(tert-pentyl)phenol	80-46-6	4.02E-07	3.46E-06	P	P
67	4-cyclohexylphenol	1131-60-8	6.43E-08	1.51E-06	P	P
68	4-(1-adamantyl)phenol	29799-07-3	1.25E-09	1.86E-08	P	P
69	4-(phenylmethyl)phenol	101-53-1	1.20E-06	4.07E-06	P	P
70	2,2-bis(4-hydroxyphenyl)-4-methyl-n-pentane	6807-17-6	1.89E-09	1.99E-08	P	P
71	4,4'-(Hexafluoroisopropylidene)diphenol	1478-61-1	6.91E-09	8.02E-08	P	P
72	4,4'-(octahydro-4,7-methano-5H-inden-5-lidene)	1943-97-1	3.72E-08	PC_{10}	P	N

	bisphenol			($noPC_{50}$)		
73	4,4'-dimethoxytriphenylmethane	7500-76-7	-	-	N	N
74	Benzophenone	119-61-9	-	-	N	N
75	4-hydroxybenzophenone	1137-42-4	1.10E-06	2.60E-06	P	P
76	4,4'-dihydroxybenzophenone	611-99-4	1.24E-07	1.65E-06	P	P
77	2,4,4'-trihydroxybenzophenone	1470-79-7	4.38E-08	3.75E-07	P	P
78	4,4'-dimethoxybenzophenone	90-96-0	2.50E-06	PC_{10} ($noPC_{50}$)	P	N
79	2,2',4,4'-tetrahydroxybenzophenone	131-55-5	1.06E-07	3.28E-07	P	P
80	4-hydroxyazobenzene	1689-82-3	1.64E-07	1.08E-06	P	P
81	3,3,3',3'-tetramethyl-1,1'-spirobisindane-5,5',6,6'-tetrol	77-08-7	1.43E-07	3.16E-06	P	P
82	4,4'-thiobis-phenol	2664-63-3	2.01E-08	2.14E-07	P	P
83	Diphenyl-p-phenylenediamine	74-31-7	2.30E-06	PC_{10} ($noPC_{50}$)	P	N
84	Octachlorostyrene	29082-74-4	-	-	N	N
85	Hematoxylin	517-28-2	-	-	N	N
86	Tributyltin chloride	1461-22-9	-	-	N	N

Abbreviations: CASRN = Chemical Abstracts Service Registry Number, PC_{10} (PC_{50}) = the concentration of a test chemical at which the response is 10% (or 50% for PC_{50}) of the response induced by the positive control (E2, 1nM) in each plate, P = Positive, N = Negative

※　参考文献(1)の ANNEX2, Table 2 をもとに作成

Table 2 ER-STTA アンタゴニストバリデーション試験で測定が行われた 21 物質の測定結果

No.	Chemical	CASRN	ER STTA [1] antagonist Classification	ER STTA [1] Mean IC_{50}(M)
1	17β-estradiol	50-28-2	NEG	-
2	4,4'-(Hexafluoroisopropylidene)diphenol	1478-61-1	NEG	-
3	4,4'-[1-[4-[1-(4-Hydroxyphenyl)-1-methylethyl]phenyl]ethylidene]bis[phenol]	110726-28-8	POS	2.51×10^{-6}
4	4,4'-Cyclohexylidenebisphenol	843-55-0	NEG	-
5	4-Hydroxytamoxifen	68047-06-3	POS	3.97×10^{-9}
6	Apigenin	520-36-5	NEG	-
7	Atrazine	1912-24-9	NEG	-
8	Clomiphene citrate (cis and trans mixture)	50-41-9	POS	4.26×10^{-7}
9	Dibenzo[a,h]anthracene	53-70-3	POS	No IC_{50}
10	Dibutyl phthalate	84-74-2	NEG	-
11	Fenarimol	60168-88-9	NEG	-
12	Flavone	525-82-6	NEG	-
13	Flutamide	13311-84-7	NEG	-
14	Genistein	446-72-0	NEG	-
15	ICI 182,780	129453-61-8	POS	2.67×10^{-10}
16	Methylpiperidinylpyrazole dihydrochloride	289726-02-9	POS	3.11×10^{-8}
17	Mifepristone (Mifeprex)＝RU-486	84371-65-3	POS	5.61×10^{-6}
18	p-n-nonylphenol	104-40-5	NEG	-
19	Raloxifene HCl	82640-04-8	POS	7.86×10^{-10}
20	Resveratrol	501-36-0	NEG	-
21	Tamoxifen	10540-29-1	POS	4.91×10^{-7}

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; M = molar; IC_{50} = half maximal inhibitory concentration of test chemical; NEG = negative; POS = positive.

[1] The Validation Report of the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity, Part B (9)

※　参考文献(3)の ANNEX2, Table 2 を引用

Table 3 アゴニストバリデーション試験において施設間比較に用いられた 10 物質

Chemical	CAS No.	Manufacturer	Lot. No.	Purity
17β-Estradiol	50-28-2	Wako	ACK5754	99%
17α-Estradiol	57-91-0	Wako	ELJ1532	97% ,HPLC ,for Biochem.
Genistein	446-72-0	Wako	VIR1711	98%
4-tert-Octylphenol	140-66-9	Wako	YWE9213	97% ,cGC ,for Environment Anal.
Bisphenol A	80-05-7	TCI	GF01	>99%
p-tert-Pentylphenol	80-46-6	Wako	KSQ2664	97% ,GC
17α-Methyltestosterone	58-18-4	Wako	TPE6748	97% ,HPLC ,for Biochem.
Hematoxylin	517-28-2	Wako	LDK7723	N.S.
Diethylhexyl phthalate	117-81-7	Wako	ELE1799	97% ,GC
Benzophenone	119-61-9	Wako	RLH9114	99% ,cGC ,for Environment Anal.

TCI：Tokyo Kasei Kogyo Co., Ltd.

Wako：Wako Pure Chemical Industries, Ltd.

N.S.：not specified

※　参考文献(4), Table 9 を引用

Table 4 アゴニストバリデーション試験において ICCVAM 参照分類との比較に用いられた 46 物質

Chemical	CAS No.	Manufacturer	Lot. No.	Purity
17α-Ethinyl estradiol	57-63-6	Wako	KSN3933	98%
Diethylstilbestrol	56-53-1	Wako	7488C	N.S.
17α-Estradiol	57-91-0	Wako	ELJ1532	97%
17β-Estradiol	50-28-2	Wako	ACL1188	>97%
Zearalenone	17924-92-4	Sigma	50K4014	N.S.
Estrone	53-16-7	Wako	TPN4558	98%
Methyl testosterone	58-18-4	Wako	TPE6748	97%
Coumestrol	479-13-0	Fluka	400248	<95%
Genistein	446-72-0	Wako	NNP1712	98%
p- n-Nonylphenol	104-40-5	Wako		N.S.
Bisphenol B	77-40-7	TCI	FIC01	N.S.
Daidzein	486-66-8	Wako	HC-1408	97%
4-Cumylphenol	599-64-4	Wako	PAK1144	98%
Bisphenol A	80-05-7	TCI	GF01	>99%
p,p' -Methoxychlor	72-43-5	Wako	YWL9207	>97%
Apigenin	520-36-5	Aldrich	00902BU	N.S.
Tamoxifen	10540-29-1	ICN	4636C	N.S.
Kepone (Chlordecone)	143-50-0	Wako		99%
Butylbenzyl phthalate	85-68-7	Wako	KSJ8408	99%
Kaempferol	520-18-3	Wako	ELK6128	95%
4-tert- Octylphenol	140-66-9	Wako	YWE9213	97%
Atrazine	1912-24-9	Wako	MSF9593	>98%
Progesterone	57-83-0	Sigma	98H0893	99%
Testosterone	58-22-0	Wako	ACG5233	>97%
Corticosterone	50-22-6	Sigma	128H0744	95%
Phenobarbital	57-30-7	Wako	ACE1373	98%
Vinclozolin	50471-44-8	Wako	HCQ9724	99%
Cyproterone acetate	427-51-0	Sigma	65H0687	N.S.
Flutamide	13311-84-7	Sigma	87H1511	98%
Linuron	330-55-2	Dr.Ehrensofter	70226	100%
Mifepristone	84371-65-3	Sigma	19H0828	98%
Procymidone	32809-16-8	Wako	HCH9638	99%
Clomiphene citrate	50-41-9	Sigma	28 0308	N.S.
Ethyl paraben	120-47-8	Wako	ELH6061	99%
Norethynodrel	68-23-5	Sigma	88F0192	N.S.
4-Androstenedione	63-05-8	Sigma	116H0463	98%
2-*sec*- Butylphenol	89-72-5	Wako	KSR1873	98%
Diethylhexyl phthalate	117-81-7	Wako	ELE1799	97%
Morin	480-16-0	Fluka	404144	N.S.
Phenolphthalin	81-90-3	Wako	ELP7131	N.S.
Haloperidol	52-86-8	ICN	85689	90%
Ketoconazole	65277-42-1	Wako	78353	N.S.

Reserpine	50-55-5	RBI	SNV-494A	97.5%
Spironolactone	52-01-7	Sigma	41K1534	97%
L-Thyroxine	51-48-9	TCI	GF01	98%
17β-Trenbolone	10161-33-8	Sigma aldrich	024K0877	>98%

Aldrich：Aldrich Chemical Co., Inc. (Sigma-Aldrich corp.)

Fluka：Fluka Chemie AG (Sigma-Aldrich corp.) ICN：ICN Biomedicals, Inc.

Kanto：Kanto Chemical Co,. Inc.

Sigma：Sigma Chemical Co. (Sigma-Aldrich corp.) RBI：SIGMA-RBI

TCI：Tokyo Kasei　Kogyo Co., Ltd.

Wako：Wako Pure Chemical Industries, Ltd.

N.S.：not specified

※　参考文献(4), Table 6 を引用

Table 5 アゴニストバリデーション試験において ER 結合試験結果との比較に用いられた 48 物質

Chemical	CAS No.	Manufacturer	Lot. No.	Purity
Ethynyl estradiol	57-63-6	Wako	KSN3933	98%
17β-Estradiol	50-28-2	Wako	ACL1188	>97%
Hexestrol	84-16-2	Wako	LDQ2218	N.S.
Estrone	53-16-7	Wako	TPN4558	98%
17α-Estradiol	57-91-0	Wako	ELJ1532	97%
Norethynodrel	68-23-5	Sigma	88F0192	N.S.
Coumestrol	479-13-0	Fluka	400248	<95%
Genistein	446-72-0	Wako	NNP1712	98%
4-*tert*- Octylphenol	140-66-9	Wako	YWE9213	97%
Daidzein	486-66-8	Wako	HC-1408	97%
Nonylphenol (mixture)	25154-52-3	Aldrich	00504CU	N.S.
Bisphenol B	77-40-7	TCI	FIC01	N.S.
Testosterone propionate	57-85-2	Sigma	98H0566	N.S.
Bisphenol A	80-05-7	TCI	GF01	>99%
5α-Dihydrotestosterone	521-18-6	Wako	TPJ4827	95%
Kaempferol	520-18-3	Wako	ELK6128	95%
4-alpha-Cumylphenol	599-64-4	Wako	PAK1144	98%
17α-Methyltestosterone	58-18-4	Wako	TPE6748	97%
Morin	480-16-0	Fluka	404144	N.S.
Vinclozolin	50471-44-8	Wako	HCQ9724	99%
Testosterone	58-22-0	Wako	ACG5233	>97%
Tamoxifen	10540-29-1	ICN	4636C	N.S.
Clomiphene citrate	50-41-9	Sigma	28 0308	N.S.
di(2-Ethylhexyl)phthalate	117-81-7	Wako	ELE1799	97%
RU-486	84371-65-3	Sigma	19H0828	98%
Methoxychlor	72-43-5	Wako	YWL9207	>97%
Fenarimol	60168-88-9	Kanto	707S7109	97%
para-sec -butylphenol	99-71-8	TCI	FHF01	>98%
Dibutyl phthalate	84-74-2	Wako		for Anal. of Phthalic Acid Esters
Phenolphthalin	81-90-3	Wako	ELP7131	N.S.
Cyproterone acetate	427-51-0	Sigma	65H0687	N.S.
Ethyl *p* -Hydroxybenzoate	120-47-8	Wako	ELH6061	99%
2,4,5-Trichlorophenoxyacetic acid	93-76-5	Wako	HCL9884	98.7%
p,p' -DDE	72-55-9	Wako	YWG9700	99%
Ketoconazol	65277-42-1	Wako	78353	N.S.
Androstenedione	63-05-8	Sigma	116H0463	98%
Progesterone	57-83-0	Sigma	98H0893	99%
Haloperidol	52-86-8	ICN	85689	90%
Medroxyprogesterone	520-85-4	Sigma	59H0579	N.S.
Spironolactone	52-01-7	Sigma	41K1534	97%
L-thyroxine	51-48-9	TCI	GF01	98%

Reserpine	50-55-5	RBI	SNV-494A	97.5%
Corticosterone	50-22-6	Sigma	128H0744	95%
Phenobarbital	50-06-6	Maruishi	8603	N.S.
Linuron = Lorox	330-55-2	Dr.Ehrensofter	70226	100%
Procymidon	32809-16-8	Wako	HCH9638	99%
Atrazine	1912-24-9	Wako	MSF9593	>98%
Flutamide	13311-84-7	Sigma	87H1511	98%

Aldrich：Aldrich Chemical Co., Inc. (Sigma-Aldrich corp.)

Fluka：Fluka Chemie AG (Sigma-Aldrich corp.) ICN：ICN Biomedicals, Inc.

Kanto：Kanto Chemical Co., Inc.

N.S.：not specified

Maruishi：Maruishi Pharmaceutical. Co., Ltd.

Sigma：Sigma Chemical Co. (Sigma-Aldrich corp.) RBI：SIGMA-RBI

TCI：Tokyo Kasei　Kogyo Co., Ltd.

Wako：Wako Pure Chemical Industries, Ltd.

※　参考文献(4), Table 7 を引用

Table 6 アゴニストバリデーション試験において子宮肥大試験結果との比較に用いられた 48 物質

Chemical	Cas No.	Manufacture	Lot No.	Purity
Ethynyl Estradiol	57-63-6	Wako	KSN3933	>97%
Equilin	474-86-2	Sigma	97H1529	100%
Estrone	53-16-7	Wako	TPN4558	98%
17α-Estradiol	57-91-0	Wako	ACL1188	>97%
Zearalenone	17924-92-4	Sigma	50K4014	N.S.
4-(1-Adamantyl)phenol	29799-07-3	Aldrich	11608MR	97%
2,2-bis(4-Hydroxyphenyl)-4-methyl-n-pentane	6807-17-6	Wako	PTM1337	100%
Genistein	446-72-0	Wako	NNP1712	98%
Norethrindrone	68-22-4	Wako	DWM4647	100%
4-*tert* -Octylphenol	140-66-9	Wako	09802JQ	99%
4,4'-(Hexafluoroisopropylidene)diphenol	1478-61-1	Aldrich	05328PI	97%
Daidzein	486-66-8	Wako	HC-1408	97%
Nonylphenol (mixture)	25154-52-3	Kanto	109281	97%
Bisphenol B	77-40-7	TCI	FIC01	100%
4,4'-Thiobisphenol	2664-63-3	TCI	JC01	100%
Testosterone enanthate	315-37-7	Wako	KSL4869	100%
Bisphenol A	80-05-7	TCI	GF01	>99%
2,2',4,4'-Tetrahydroxybenzophenone	131-55-5	Wako	ELN6605	98%
2,4,4'-Trihydroxybenzophenone	1470-79-7	Aldrich	04417JN	95%
p -Dodecyl-phenol	104-43-8	Kanto	209D2209	N.S.
5α-Dihydrotestosterone	521-18-6	Wako	TPJ4827	95%
4-Hydroxyazobenzene	1689-82-3	Wako	LDM7343	96%
4-Cyclohexylphenol	1131-60-8	TCI	FIJ01	100%
4-α-Cumylphenol	599-64-4	Wako	PAK1144	98%
4,4'-Dihydroxybenzophenone	611-99-4	Wako	LDR1808	99%
4-Hydroxybenzophenone	1137-42-4	Aldrich	04419CO	98%
3,3,3',3'-Tetramethyl-1,1'-spirobisindane-5,5',6,6'-tetrol	77-08-7	TCI	GG01	99%
p -(*tert*- Pentyl)phenol	80-46-6	Wako	ELF1567	100%
4-(Phenylmethyl)phenol	101-53-1	TCI	FHG01	100%
17α-Methyltestosterone	58-18-4	Wako	ELG7538	100%
4-*n* -Amylphenol	14938-35-3	TCI	FIF01	99%
4,4'-(Octahydro-4,7-methano-5H-inden-5-ylidene) bisphenol	1943-97-1	ACROS	A008394601	100%
Levonorogestrel	797-63-7	Sigma	30K0711	99%
Methoxychlor	72-43-5	Wako	YWL9207	>97%
4-*n* -Octylphenol	1806-26-4	Wako	JSL9944	99%
Diphenyl-*p* -Phenylenediamine	74-31-7	Wako	ELH7269	97%
4,4'-Dimethoxybenzophenone	90-96-0	TCI	FIH01	100%
Dicyclohexyl phthalate	84-61-7	Wako	RIG9061	100%
Diethyl phthalate	84-66-2	Wako	ELH6895	99%
di-*n* -Butyl phthalate	84-74-2	Wako	ACE7193	N.S.
di(2-Ethylhexyl)adipate	103-23-1	Wako	LDR4958	100%

p-n -Nonylphenol	104-40-5	TCI	10425	99%
di(2-Ethylhexyl)phthalate	117-81-7	Wako	ELH6895	99%
Benzophenone	119-61-9	Wako	HCM9879	100%
Tributyltin chloride	1461-22-9	Wako	LDN5508	98%
Octachlorostyrene	29082-74-4	Kanto	106121	100%
Hematoxylin	517-28-2	Sigma	99H3645	N.S.
4,4'-Dimethoxytriphenylmethane	7500-76-7	ERC	1040701	100%

Aldrich：Aldrich Chemical Co., Inc. (Sigma-Aldrich corp.)

Fluka：Fluka Chemie AG (Sigma-Aldrich corp.) ICN：ICN Biomedicals, Inc.

Kanto：Kanto Chemical Co,. Inc.

N.S.：not specified

Sigma：Sigma Chemical Co. (Sigma-Aldrich corp.)

RBI：SIGMA-RBI

TCI：Tokyo Kasei Kogyo Co., Ltd.

Wako：Wako Pure Chemical Industries, Ltd.

※　参考文献(4), Table 8 を引用

Table 7 アンタゴニストバリデーション試験 Task3 において施設内再現性、施設間再現性の評価の用いられた 20 物質

Code	Chemical name	CASRN	Manufacturer	Catalog ID.	Lot No.
ATG001	ICI 182,780	129453-61-8	Sigma	I4409	068K4711
ATG002	Mifepristone (Mifeprex, RU-486)	84371-65-3	Sigma	M8046	125K1071
ATG003	4,4'-(Hexafluoroisopropylidene) diphenol	1478-61-1	TCI	B0945	MMSFBTQ
ATG004	Methylpiperdinylpyrazole dihydrochloride	289726-02-9	Sigma-Aldrich	M7068	076K47051
ATG005	4-Hydroxytamoxifen	68047-06-3	Sigma	H7904	018K4132
ATG006	Raloxifene HCl	82640-04-8	Wako (LKT Labs, Inc)	513-22901(R0243)	23923004
ATG007	Clomiphene citrate(cis and trans mixture)	50-41-9	Sigma	C6272	126K1525
ATG008	Dibutyl phthalate	84-74-2	Wako	047-16521	ALQ8564
ATG009	Atrazine	1912-24-9	Wako	010-15631	TSG9681
ATG010	Flutamide	13311-84-7	Wako Wako	069-04851 069-04851	WKR0694 PEF2340
ATG011	4,4'-Cyclohexylidenebisphenol	843-55-0	Wako	028-11071	DPR2259
ATG012	4,4'-[1-[4-[1-(4-Hydroxyphenyl)-1-methylethyl]phenyl]ethylidene]bis [phenol]	110726-28-8	Wako	322-28032	EWH0280
ATG013	Apigenin	520-36-5	Wako	012-18913	PEK4270
ATG014	Genistein	446-72-0	Sigma Sigma	G6649 G6649	018K1203 098K0735
ATG015	Dibenzo[a,h]anthracene	53-70-3	Wako Wako	041-26791 041-26791	HSQ8748 EPQ4053
ATG016	p-n-nonylphenol	104-40-5	Wako	146-06791	DPF8978
ATG017	Flavone	525-82-6	Wako	061-02231	PEM1887
ATG018	Resveratrol	501-36-0	Sigma	R5010	038K5202
ATG019	Fenarimol	60168-88-9	Supelco Supelco	PS1073 PS1073	393-118B 404-67B
ATG020	17β-estradiol	50-28-2	Wako Wako	056-04044 056-04044	ALL2384 ALH5038
	Tamoxifen*	10540-29-1	Sigma Sigma	T5648 T5648	117K1079 089K1381

* Tamoxifen は、参照化合物としてのみ測定された

※　参考文献(4), Part B, Table 2-1,2-2 をもとに作成

Table 8　アンタゴニストバリデーション試験における施設内再現性

Activity per Test	**CERI**	**OTSUKA**	**KANEKA**	**HIYOSHI**
Agreement Within Laboratory	19/20 (95%)	19/19 (100%)	8/12 (67%)	18/20 (90%)
Positive candidate	8/9	9/9	3/5	8/9
Negative candidate	9/9	8/9	6/7	8/9
Unknown	2/2	1/1	0/1	2/2
Discordance Within Laboratory	1/20 (5%)	0/19 (0%)	4/12 (33%)	2/20 (10%)
Positive candidate (Results*)	1/9 (NNP)*	0/9	2/5 (PPPPNP) (PPPNP)	1/9 (NPP)
Negative candidate (Results)	0/9	1/9 (PNN)	1/6 (NNNPN)	1/9 (NPP)
Unknown (Results)	0/2	0/1	1/1 (PNNNNN)	0/2

*: Details of discordant results, (NNP) means two negatives and one positive.

※　参考文献(3), ANNEX2, Table 5 を引用

Table 9 アンタゴニストバリデーション試験における施設間再現性および文献情報等から予想された作用との比較

Code	Chemical name	Candidate effect	CERI	OTSUKA	KANEKA	HIYOSHI	Total
ATG001	ICI 182,780	**strong**	Positive	Positive	Positive	Positive	Positive (4/4)
ATG002	Mifepristone(Mifeprex) ＝ RU-486	**mild**	Positive	Positive	Positive	Positive	Positive (4/4)
ATG003	4,4'-(Hexafluoroisopropylidene)diphenol	**Negative in ER STTA assay**	Negative	Negative	Negative	Negative	Negative (4/4)
ATG004	Methylpiperdinylpyrazole dihydrochloride	**mild**	Positive	Positive		Positive	Positive (3/3)
ATG005	4-Hydroxytamoxifen	**moderate**	Positive	Positive	Positive	Positive	Positive (4/4)
ATG006	Raloxifene HCl	**moderate**	Positive	Positive		Positive	Positive (3/3)
ATG007	Clomiphene citrate(cis and trans mixture)	**moderate**	Positive	Positive	Positive	Positive	Positive (4/4)
ATG008	Dibutyl phthalate	**Negative**	Negative	Negative	Negative	Negative	Negative (4/4)
ATG009	Atrazine	**Negative**	Negative	Negative		Positive	Negative (2/3)
ATG010	Flutamide	**Negative**	Negative	Negative	Negative	Negative	Negative (4/4)
ATG011	4,4'-Cyclohexylidenebisphenol	**Negative in ER STTA assay**	Negative	Negative	Negative	Negative	Negative (4/4)
ATG012	4,4'-[1-[4-[1-(4-Hydroxyphenyl)-1-methylethyl]phenyl]ethylidene]bis[phenol]	**mild**	Positive	Positive		Positive	Positive (3/3)
ATG013	Apigenin	**Negative**	Negative	Negative		Negative	Negative (3/3)
ATG014	Genistein	**Negative***	Negative	Negative	Negative	Negative	Negative (4/4)
ATG015	Dibenzo[a,h]anthracene	**Positive***	Negative	Positive		Positive	Positive (2/3)
ATG016	p-n-nonylphenol	**not tested**	Negative	Negative	Negative	Negative	Negative (4/4)
ATG017	Flavone	**Negative***	Negative	Negative		Negative	Negative (3/3)
ATG018	Resveratrol	**Negative***	Negative	Negative	Negative	Negative	Negative (4/4)
ATG019	Fenarimol	**not tested**	Negative			Negative	Negative (2/2)
ATG020	17β-estradiol	**Negative***	Negative	Negative	Negative	Negative	Negative (4/4)
2x2 table analysis compared with candidate effects		Accuracy:	94%	100%	100%	94%	100% (97%)**
		Sensitivity:	88%	100%	100%	100%	100% (97%)**
		Specificity :	100%	100%	100%	88%	100% (97%)**

* classified positive/negative according to literature review

**Performance compared with candidate effect excluding chemicals noted as "not tested"(n=18), parentheses indicate the value based on the total number of tests.

※　参考文献(4), Part B, Table19 を引用

Table 10　アゴニストバリデーション試験結果と ICCVAM 参照分類との比較結果

A:　個別試験データ

			ER STTA agonist assay				Updated Classifications of Chemical
No	Chemical	CASRN	PC10 (M)	PC50 (M)	PC10 based class	PC50 based class	ICCVAM class
1	Atrazine	1912-24-9	-	-	N	N	N
2	Corticosterone	50-22-6	-	-	N	N	N
3	Haloperidol	52-86-8	-	-	N	N	N
4	Ketoconazole	65277-42-1	-	-	N	N	N
5	Linuron	330-55-2	-	-	N	N	N
6	Phenobarbital(Na)	57-30-7	-	-	N	N	N
7	Reserpine	50-55-5	-	-	N	N	N
8	Spironolactone	52-01-7	-	-	N	N	N
9	Flutamide	13311-84-7	-	-	N	N	N
10	Procymidone	32809-16-8	-	-	N	N	N
11	Ethyl_paraben	120-47-8	5.00E-06	PC10(noPC50)	P	N	P
12	p,p'-Methoxychlor	72-43-5	1.23E-06	PC10(noPC50)	P	N	P
13	Tamoxifen	10540-29-1	1.49E-07	PC10(noPC50)	P	N	P
14	Clomiphene citrate	50-41-9	3.68E-08	PC10(noPC50)	P	N	P
15	Zearalenone b	17924-92-4	2.44E-11	6.44E-10	P	P	P
16	17β-Estradiol	50-28-2	<1.00E-11	<1.00E-11	P	P	P
17	17β-Trenbolone	10161-33-8	1.78E-08	2.73E-07	P	P	P
18	17α-Estradiol	57-91-0	7.24E-11	6.44E-10	P	P	P
19	17α-Ethinyl estradiol	57-63-6	<1.00E-11	<1.00E-11	P	P	P
20	4-Cumylphenol	599-64-4	1.49E-07	1.60E-06	P	P	P
21	4-tert-Octylphenol	140-66-9	1.85E-09	7.37E-08	P	P	P
22	Apigenin	520-36-5	1.31E-07	5.71E-07	P	P	P
23	BisphenolA	80-05-7	2.02E-08	2.94E-07	P	P	P
24	BisphenolB	77-40-7	2.36E-08	2.11E-07	P	P	P
25	Butylbenzyl phthalate	85-68-7	1.14E-06	4.11E-06	P	P	P
26	Coumestrol	479-13-0	1.23E-09	2.00E-08	P	P	P
27	Daidzein	486-66-8	1.76E-08	1.51E-07	P	P	P
28	Diethylstilbestrol	56-53-1	<1.00E-11	2.04E-11	P	P	P
29	Estrone	53-16-7	3.02E-11	5.88E-10	P	P	P
30	Genistein	446-72-0	2.24E-09	2.45E-08	P	P	P
31	Kaempferol	520-18-3	1.36E-07	1.21E-06	P	P	P
32	Kepone(Chlordecone)	143-50-0	7.11E-07	7.68E-06	P	P	P
33	Methyl testosterone	58-18-4	1.73E-07	4.11E-06	P	P	P
34	Morin	480-16-0	5.43E-07	4.16E-06	P	P	P
35	Norethynodrel	68-23-5	1.11E-10	1.50E-09	P	P	P

Abbreviations: CASRN = Chemical Abstracts Service Registry Number, PC10 (PC50) = the concentration of a test chemical at which the response is 10% (or 50% for PC_{50}) of the response induced by the positive control (E2, 1nM) in each plate, P = Positive, N = Negative

※　参考文献(1)の ANNEX2, Table 2 をもとに作成

B:　集計表

		STTA (PC10 based)		
		Positive	Negative	Total
Updated classification of ICCVAM Chemicals	Positive	25	0	25
	Negative	0	10	10
	Total	25	10	35

Overall Accuracy	100%	35/35
Sensitivity	100%	25/25
Specificity	100%	10/10
False positive	0%	0/10
False negative	0%	0/25
Positive predictivity	100%	25/25
Negative predictivity	100%	10/10

※　参考文献(1)の ANNEX2, Table 8 を引用

Table 11　アゴニストバリデーション試験結果と ER 結合試験データとの比較
A:　個別試験データ

			ER STTA agonist assay				ER Binding Assay	
No	Chemical	CASRN	PC10 (M)	PC50 (M)	PC10 based class	PC50 based class	ER-RBA E2=100%	ER-RBA class
1	Atrazine	1912-24-9	-	-	N	N	N.B.	N
2	Corticosterone	50-22-6	-	-	N	N	N.B.	N
3	Haloperidol	52-86-8	-	-	N	N	N.B.	N
4	Ketoconazole	65277-42-1	-	-	N	N	N.B.	N
5	Linuron	330-55-2	-	-	N	N	N.B.	N
7	Reserpine	50-55-5	-	-	N	N	N.B.	N
8	Spironolactone	52-01-7	-	-	N	N	N.B.	N
9	Flutamide	13311-84-7	-	-	N	N	N.B.	N
10	Procymidone	32809-16-8	-	-	N	N	N.B.	N
11	Ethyl paraben	120-47-8	5.00E-06	PC10(noPC50)	P	N	N.B.	N
12	p,p'-Methoxychlor	72-43-5	1.23E-06	PC10(noPC50)	P	N	0.00238	P
13	Tamoxifen	10540-29-1	1.49E-07	PC10(noPC50)	P	N	47	P
14	Clomiphene_citrate	50-41-9	3.68E-08	PC10(noPC50)	P	N	37	P
16	17β-Estradiol	50-28-2	<1.00E-11	<1.00E-11	P	P	126	P
18	17α-Estradiol	57-91-0	7.24E-11	6.44E-10	P	P	80.1	P
19	17α-Ethinyl estradiol	57-63-6	<1.00E-11	<1.00E-11	P	P	142	P
20	4-Cumylphenol	599-64-4	1.49E-07	1.60E-06	P	P	0.107	P
21	4-tert-Octylphenol	140-66-9	1.85E-09	7.37E-08	P	P	0.124	P
23	BisphenolA	80-05-7	2.02E-08	2.94E-07	P	P	0.195	P
24	BisphenolB	77-40-7	2.36E-08	2.11E-07	P	P	0.593	P
26	Coumestrol	479-13-0	1.23E-09	2.00E-08	P	P	0.264	P
27	Daidzein	486-66-8	1.76E-08	1.51E-07	P	P	0.18	P
29	Estrone	53-16-7	3.02E-11	5.88E-10	P	P	44.2	P
30	Genistein	446-72-0	2.24E-09	2.45E-08	P	P	0.12	P
31	Kaempferol	520-18-3	1.36E-07	1.21E-06	P	P	0.029	P
33	Methyl testosterone	58-18-4	1.73E-07	4.11E-06	P	P	N.D.	N
34	Morin	480-16-0	5.43E-07	4.16E-06	P	P	0.0011	P
35	Norethynodrel	68-23-5	1.11E-10	1.50E-09	P	P	0.282	P
36	Phenolphthalin	81-90-3	-	-	N	N	N.D.	N
37	Progesterone	57-83-0	-	-	N	N	N.B.	N
38	Cyproterone acetate	427-51-0	-	-	N	N	N.D.	N
39	Mifepristone	84371-65-3	-	-	N	N	0.0594	P
40	Diethylhexyl phthalate	117-81-7	-	-	N	N	0.071	P
41	L-Thyroxine	51-48-9	1.32E-06	PC10(noPC50)	P	N	N.B.	N
42	4-Androstenedione	63-05-8	2.56E-07	PC10(noPC50)	P	N	N.B.	N
43	Testosterone	58-22-0	2.82E-08	9.78E-06	P	P	N.D.	N
44	Vinclozolin	50471-44-8	1.33E-07	7.65E-06	P	P	N.B.	N
45	Dibutyl phthalate	84-74-2	-	-	N	N	N.D.	N
46	Nonylphenol	25154-52-3	1.37E-08	1.58E-07	P	P	0.143	P
47	Phenobarbital	50-06-6	-	-	N	N	N.B.	N
48	Medroxyprogesterone	520-85-4	-	-	N	N	N.B.	N
49	DHT	521-18-6	1.04E-07	5.28E-07	P	P	0.0218	P
50	Testosterone propionate	57-85-2	2.03E-09	2.91E-07	P	P	N.B.	N
51	Fenarimol	60168-88-9	-	-	N	N	0.00179	P
52	p,p'-DDE	72-55-9	-	-	N	N	N.B.	N
53	Hexestrol	84-16-2	<1.00E-11	2.75E-11	P	P	37.6	P
54	2,4,5-Trichlorophenoxyaceticacid	93-76-5	-	-	N	N	N.B.	N
55	para-sec-butylphenol	99-71-8	1.38E-06	PC10(noPC50)	P	N	0.00177	P

Abbreviations: CASRN = Chemical Abstracts Service Registry Number, PC10 (PC50) = the concentration of a test chemical at which the response is 10% (or 50% for PC_{50}) of the response induced by the positive control (E2, 1nM) in each plate, P = Positive, N = Negative

※　参考文献(1)の ANNEX2, Table 2 をもとに作成

B: 集計表

		STTA (PC10 based)		
		Positive	Negative	Total
ER binding assay	Positive	21	3	24
	Negative	7	17	24
	Total	28	20	48

Overall accuracy	79%	38/48
Sensitivity	88%	21/24
Specificity	71%	17/24
False positive	29%	7/24
False negative	13%	3/24
Positive predictivity	75%	21/28
Negative predictivity	85%	17/20

※ 参考文献(1)の ANNEX2, Table 9 を引用

Table 12　アゴニストバリデーション試験結果と子宮肥大試験との比較

A:　個別試験データ

			ER STTA agonist assay				Uterotrophic Assay
No	Chemical	CASRN	PC10 (M)	PC50 (M)	PC10 based class	PC50 based class	Utero. Class
12	p,p'-Methoxychlor	72-43-5	1.23E-06	PC10(noPC50)	P	N	N
15	Zearalenone_b	17924-92-4	2.44E-11	6.44E-10	P	P	P
18	17α-Estradiol	57-91-0	7.24E-11	6.44E-10	P	P	P
19	17α-Ethinyl_estradiol	57-63-6	<1.00E-11	<1.00E-11	P	P	P
20	4-Cumylphenol	599-64-4	1.49E-07	1.60E-06	P	P	P
21	4-tert-Octylphenol	140-66-9	1.85E-09	7.37E-08	P	P	P
23	BisphenolA	80-05-7	2.02E-08	2.94E-07	P	P	P
24	BisphenolB	77-40-7	2.36E-08	2.11E-07	P	P	P
27	Daidzein	486-66-8	1.76E-08	1.51E-07	P	P	N
29	Estrone	53-16-7	3.02E-11	5.88E-10	P	P	P
30	Genistein	446-72-0	2.24E-09	2.45E-08	P	P	P
33	Methyl_testosterone	58-18-4	1.73E-07	4.11E-06	P	P	P
40	Diethylhexyl_phthalate	117-81-7	-	-	N	N	N
45	Dibutyl phthalate	84-74-2	-	-	N	N	N
46	Nonylphenol	25154-52-3	1.37E-08	1.58E-07	P	P	P
49	DHT	521-18-6	1.04E-07	5.28E-07	P	P	P
56	Norgestrel	797-63-7	1.05E-07	PC10(noPC50)	P	N	P
57	Norethindrone	68-22-4	1.01E-09	4.95E-08	P	P	P
58	Equilin	474-86-2	<1.00E-11	7.54E-11	P	P	P
59	Dicyclohexyl phthalate	84-61-7	2.53E-06	PC10(noPC50)	P	N	N
60	Diethyl phthalate	84-66-2	4.46E-06	PC10(noPC50)	P	N	N
61	di(2-ethylhexyl)adipate	103-23-1	-	-	N	N	N
62	p-dodecyl-phenol	104-43-8	2.36E-08	4.10E-07	P	P	P
63	4-n-octylphenol	1806-26-4	1.26E-06	PC10(noPC50)	P	N	N
64	4-n-amylphenol	14938-35-3	1.78E-07	4.62E-06	P	P	P
65	Testosterone_enanthate	315-37-7	1.71E-08	2.71E-07	P	P	P
66	p-(tert-pentyl)phenol	80-46-6	4.02E-07	3.46E-06	P	P	P
67	4-cyclohexylphenol	1131-60-8	6.43E-08	1.51E-06	P	P	P
68	4-(1-adamantyl)phenol	29799-07-3	1.25E-09	1.86E-08	P	P	P
69	4-(phenylmethyl)-phenol	101-53-1	1.20E-06	4.07E-06	P	P	P
70	2,2-bis(4-hydroxyphenyl)-4-methyl-n-pentane	6807-17-6	1.89E-09	1.99E-08	P	P	P
71	4,4'-exafluoroisopropylidene)diphenol	1478-61-1	6.91E-09	8.02E-08	P	P	P
72	4,4'-(octahydro-4,7-methano-5H-inden-5-lidene)bisphenol	1943-97-1	3.72E-08	PC10(noPC50)	P	N	P
73	4,4'-dimethoxytriphenylmethane	7500-76-7	-	-	N	N	N
74	Benzophenone	119-61-9	-	-	N	N	N
75	4-hydroxybenzophenone	1137-42-4	1.10E-06	2.60E-06	P	P	P
76	4,4'-dihydroxybenzophenone	611-99-4	1.24E-07	1.65E-06	P	P	P
77	2,4,4'-trihydroxybenzophenone	1470-79-7	4.38E-08	3.75E-07	P	P	P
78	4,4'-dimethoxybenzophenone	90-96-0	2.50E-06	PC10(noPC50)	P	N	N
79	2,2',4,4'-tetrahydroxybenzophenone	131-55-5	1.06E-07	3.28E-07	P	P	P
80	4-hydroxyazobenzene	1689-82-3	1.64E-07	1.08E-06	P	P	P
81	3,3,3',3'-tetramethyl-1,1'-spirobisindane-5,5',6,6'-tetrol	77-08-7	1.43E-07	3.16E-06	P	P	N
82	4,4'-thiobis-phenol	2664-63-3	2.01E-08	2.14E-07	P	P	P
83	Diphenyl-pphenylenediamine	74-31-7	2.30E-06	PC10(noPC50)	P	N	P
84	Octachlorostyrene	29082-74-4	-	-	N	N	N
85	Hematoxylin	517-28-2	-	-	N	N	N
86	Tributyltin-chloride	1461-22-9	-	-	N	N	N

Abbreviations: CASRN = Chemical Abstracts Service Registry Number, PC10 (PC50) = the concentration of a test chemical at which the response is 10% (or 50% for PC_{50}) of the response induced by the positive control (E2, 1nM) in each plate, P = Positive, N = Negative

※　参考文献(1)の ANNEX2, Table 2 をもとに作成

B: 集計表

		STTA (PC10 based)		
		Positive	Negative	Total
Uterotrophic assay	Positive	32	0	32
	Negative	7	9	16
	Total	39	9	48

Overall accuracy	85%	41/48
Sensitivity	100%	32/32
Specificity	56%	9/16
False positive	44%	7/16
False negative	0%	0/32
Positive predictivity	82%	32/39
Negative predictivity	100%	9/9

※ 参考文献(1)の ANNEX2, Table 10 を引用

Adopted:
28 July 2015

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Performance-Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists

GENERAL INTRODUCTION

Performance-Based Test Guideline

1. This Performance-Based Test Guideline (PBTG) describes the methodology of Stably Transfected Transactivation *In Vitro* Assays to detect Estrogen Receptor Agonists and Antagonists (ER TA assays). It comprises several mechanistically and functionally similar test methods for the identification of estrogen receptor (i.e. ERα, and/or ERβ) agonists and antagonists and should facilitate the development of new similar or modified test methods in accordance with the principles for validation set forth in the OECD Guidance Document (GD) on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (1). The fully validated reference test methods (Annex 2 and Annex 3) that provide the basis for this PBTG are:

- The Stably Transfected TA (STTA) assay (2) using the (h) ERα-HeLa-9903 cell line; and
- The BG1Luc ER TA assay (3) using the BG1Luc-4E2 cell line which predominately expresses hERα with some contribution from hERβ (4) (5).

Performance standards (PS) (6) (7) are available to facilitate the development and validation of similar test methods for the same hazard endpoint and allow for timely amendment of this PBTG so that new similar test methods can be added to an updated PBTG; however, similar test methods will only be added after review and agreement that performance standards are met. The test methods included in this Test Guideline can be used indiscriminately to address countries' requirements for test results on estrogen receptor transactivation while benefiting from the Mutual Acceptance of Data.

Background and principles of the test methods included in the PBTG

2. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new, Test Guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD conceptual framework (CF) for testing and assessment of potential endocrine disrupting chemicals 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 (8). The CF comprises five levels, each level corresponding to a different level of biological complexity. The ER Transactivation (TA) assays described in this PBTG are level 2, which includes "*in vitro assays providing data about selected endocrine mechanism(s)/pathway(s)*. This PBTG is for *in vitro* Transactivation (TA) test methods designed to identify estrogen receptor (ER) agonists and antagonists.

3. The interaction of estrogens with ERs can affect transcription of estrogen-controlled genes, which can lead to the induction or inhibition of cellular processes, including those necessary for cell proliferation,

normal fetal development, and reproductive function (9) (10) (11). Perturbation of normal estrogenic systems may have the potential to trigger adverse effects on normal development (ontogenesis), reproductive health and the integrity of the reproductive system.

4. *In vitro* TA assays are based on a direct or indirect interaction of the substances with a specific receptor that regulates the transcription of a reporter gene product. Such assays have been used extensively to evaluate gene expression regulated by specific nuclear receptors, such as ERs (12) (13) (14) (15) (16). They have been proposed for the detection of estrogenic transactivation regulated by the ER (17) (18) (19). 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 (20) (21) (22) (23) (24) (25) (26). Nuclear ERα mediates the classic estrogenic response (27) (28) (29) (30), and therefore most models currently being developed to measure ER activation or inhibition are specific to ERα. The assays are used to identify chemicals that activate (or inhibit) the ER following ligand binding, after which the receptor-ligand complex binds to specific DNA response elements and transactivates a reporter gene, resulting in increased cellular expression of a marker protein. Different reporter responses can be used in these test methods. In luciferase based systems, the luciferase enzyme transforms the luciferin substrate to a bioluminescent product that can be quantitatively measured with a luminometer. Other examples of common reporters are fluorescent protein and the *LacZ* gene, which encodes β-galactosidase, an enzyme that can transform the colourless substrate X-gal (5- bromo-4-chloro-indolyl-galactopyranoside) into a blue product that can be quantified with a spectrophotometer. These reporters can be evaluated quickly and inexpensively with commercially available test kits.

5. Validation studies of the STTA and the BG1Luc TA assays have demonstrated their relevance and reliability for their intended purpose (3) (4) (5) (30). Performance standards for luminescence-based ER TA assays using ovarian cells lines are included in ICCVAM Test Method Evaluation Report on the LUMI-CELL® ER (BG1Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals (3). These performance standards have been modified to be applicable to both the STTA and BG1Luc TA test methods (2).

6. Definitions and abbreviations used in this Test Guideline are described in Annex 1.

Scope and limitations related to the TA assays

7. These test methods are being proposed for screening and prioritisation purposes, but can also provide mechanistic information that can be used in a weight of evidence approach. They address TA induced by chemical binding to the ERs in an *in vitro* system. Thus, results should not be directly extrapolated to the complex signaling and regulation of the intact endocrine system *in vivo*.

8. 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 with other receptors and enzymatic systems within the endocrine system, (ii) hormone synthesis, (iii) metabolic activation and/or inactivation of hormones, (iv) distribution of hormones to target tissues, and (v) clearance of hormones from the body. None of the test methods under this PBTG addresses these modes of action.

9. This PBTG addresses the ability of chemicals to activate (i.e. act as agonists) and also to suppress (i.e. act as antagonists) ER- dependent transcription. Chemicals that are negative in these test methods should be evaluated in an ER binding assay before concluding that the chemical does not bind to the receptor. In addition, the assay is only likely to inform on the activity of the parent molecule bearing in mind the limited metabolising capacities of the *in vitro* cell systems. Considering that only single substances were used during the validation, the applicability to test mixtures has not been addressed. The test method is nevertheless theoretically applicable to the testing of multi-constituent substances and

mixtures. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

10. For informational purposes, Table 1 provides the agonist test results for the 34 substances that were tested in both of the fully validated test methods described in this PBTG. Of these substances, 26 are classified as definitive ER agonists and 8 negatives based upon published reports, including *in vitro* assays for ER binding and TA, and/or the uterotrophic assay (3) (18) (31) (33) (34) (35) (36). Table 2 provides the antagonist test results for the 15 substances that were tested in both of the fully validated test methods described in this PBTG. Of these substances, 4 are classified as definitive/presumed ER antagonists and 10 negatives based upon published reports, including in vitro assays for ER binding and TA (3) (18) (31) (33). In reference to the data summarised in table 1 and table 2, there was 100% agreement between the two test methods on the classifications of all the substances except for one substance (Mifepristone) for antagonist assay, and each substance was correctly classified as an ER agonist/antagonist or negative. Supplementary information on this group of chemicals as well as additional chemicals tested in the STTA and BG1Luc ER TA test methods during the validation studies is provided in the Performance Standards for the ERTA (6) (7), Annex 2 (Tables 1, 2 and 3).

Table 1: Comparison of Results from STTA and BG1Luc ER TA Assays for Substances Tested in Both Agonist Assays and Classified as ER Agonists (POS) or Negatives (NEG)

	Substance	CASRN	STTA Assay[1]			BG1Luc ER TA Assay[2]		Data Source For Classification[4]		
			ER TA Activity	PC_{10} Value (M)	PC_{50} Value[b] (M)	ER TA Activity	EC_{50} Value [b,3] (M)	Other ER TAs[c]	ER Binding	Uterotrophic
1	17-ß Estradiol[a]	50-28-2	POS	$<1.00 \times 10^{-11}$	$<1.00 \times 10^{-11}$	POS	5.63×10^{-12}	POS (227/227)	POS	POS
2	17-α Estradiol[a]	57-91-0	POS	7.24×10^{-11}	6.44×10^{-10}	POS	1.40×10^{-9}	POS(11/11)	POS	POS
3	17-α Ethinylestradiol[a]	57-63-6	POS	$<1.00 \times 10^{-11}$	$<1.00 \times 10^{-11}$	POS	7.31×10^{-12}	POS(22/22)	POS	POS
4	17-β-Trenbolone	10161-33-8	POS	1.78×10^{-8}	2.73×10^{-7}	POS	4.20×10^{-8}	POS (2/2)	NT	NT
5	19-Nortestosterone[a]	434-22-0	POS	9.64×10^{-9}	2.71×10^{-7}	POS	1.80×10^{-6}	POS(4/4)	POS	POS
6	4-Cumylphenol[a]	599-64-4	POS	1.49×10^{-7}	1.60×10^{-6}	POS	3.20×10^{-7}	POS(5/5)	POS	NT
7	4-*tert*-Octylphenol[a]	140-66-9	POS	1.85×10^{-9}	7.37×10^{-8}	POS	3.19×10^{-8}	POS(21/24)	POS	POS
8	Apigenin[a]	520-36-5	POS	1.31×10^{-7}	5.71×10^{-7}	POS	1.60×10^{-6}	POS(26/26)	POS	NT
9	Atrazine[a]	1912-24-9	NEG	-	-	NEG	-	NEG (30/30)	NEG	NT
10	Bisphenol A[a]	80-05-7	POS	2.02×10^{-8}	2.94×10^{-7}	POS	5.33×10^{-7}	POS(65/65)	POS	POS
11	Bisphenol B[a]	77-40-7	POS	2.36×10^{-8}	2.11×10^{-7}	POS	1.95×10^{-7}	POS(6/6)	POS	POS
12	Butylbenzyl phthalate[a]	85-68-7	POS	1.14×10^{-6}	4.11×10^{-6}	POS	1.98×10^{-6}	POS(12/14)	POS	NEG
13	Corticosterone[a]	50-22-6	NEG	-	-	NEG	-	NEG(6/6)	NEG	NT
14	Coumestrol[a]	479-13-0	POS	1.23×10^{-9}	2.00×10^{-8}	POS	1.32×10^{-7}	POS(30/30)	POS	NT
15	Daidzein[a]	486-66-8	POS	1.76×10^{-8}	1.51×10^{-7}	POS	7.95×10^{-7}	POS(39/39)	POS	POS
16	Diethylstilbestrol[a]	56-53-1	POS	$<1.00 \times 10^{-11}$	2.04×10^{-11}	POS	3.34×10^{-11}	POS(42/42)	POS	NT
17	Di-n-butyl phthalate	84-74-2	POS	4.09×10^{-6}		POS	4.09×10^{-6}	POS(6/11)	POS	NEG
18	Ethyl paraben	120-47-8	POS	5.00×10^{-6}	(no PC_{50})	POS	2.48×10^{-5}	POS		NT
19	Estrone[a]	53-16-7	POS	3.02×10^{-11}	5.88×10^{-10}	POS	2.34×10^{-10}	POS(26/28)	POS	POS
20	Genistein[a]	446-72-0	POS	2.24×10^{-9}	2.45×10^{-8}	POS	2.71×10^{-7}	POS(100/102)	POS	POS
21	Haloperidol	52-86-8	NEG	-	-	NEG	-	NEG (2/2)	NEG	NT
22	Kaempferol[a]	520-18-3	POS	1.36×10^{-7}	1.21×10^{-6}	POS	3.99×10^{-6}	POS(23/23)	POS	NT
23	Kepone[a]	143-50-0	POS	7.11×10^{-7}	7.68×10^{-6}	POS	4.91×10^{-7}	POS(14/18)	POS	NT
24	Ketoconazole	65277-42-1	NEG	-	-	NEG	-	NEG (2/2)	NEG	NT
25	Linuron[a]	330-55-2	NEG	-	-	NEG	-	NEG (8/8)	NEG	NT
26	*meso*-Hexestrol[a]	84-16-2	POS	$<1.00 \times 10^{-11}$	2.75×10^{-11}	POS	1.65×10^{-11}	POS(4/4)	POS	NT
27	Methyl testosterone[a]	58-18-4	POS	1.73×10^{-7}	4.11×10^{-6}	POS	2.68×10^{-6}	POS(5/6)	POS	NT
28	Morin	480-16-0	POS	5.43×10^{-7}	4.16×10^{-6}	POS	2.37×10^{-6}	POS(2/2)	POS	NT
29	Norethynodrel[a]	68-23-5	POS	1.11×10^{-11}	1.50×10^{-9}	POS	9.39×10^{-10}	POS(5/5)	POS	NT
30	*p,p'*-Methoxychlor[a]	72-43-5	POS	1.23×10^{-6}	(no PC_{50})[b]	POS	1.92×10^{-6}	POS(24/27)	POS	POS
31	Phenobarbital[a]	57-30-7	NEG	-	-	NEG	-	NEG(2/2)	NEG	NT
32	Reserpine	50-55-5	NEG	-	-	NEG	-	NEG(4/4)	NEG	NT

33	Spironolactone[a]	52-01-7	NEG	-	-	NEG	-	NEG(4/4)	NEG	NT
34	Testosterone	58-22-0	POS	2.82×10^{-8}	9.78×10^{-6}	POS	1.75×10^{-5}	POS(5/10)	POS	NT

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; M = molar; EC_{50} = half maximal effective concentration of test substance; NEG = negative; POS = positive; NT = Not tested; PC_{10} (and PC_{50}) = the concentration of a test substance at which the response is 10% (or 50 % for PC_{50}) of the response induced by the positive control (E2, 1nM) in each plate.

[a]Common substances tested in the STTA and BG1Luc ER TA assays that were designated as ER agonists or negatives and used to evaluate accuracy in the BG1 Luc ER TA validation study (ICCVAM BG1Luc ER TA Evaluation Report, Table 4-1 (3).

[b]Maximum concentration tested in the absence of limitations due to cytotoxicity or insolubility was 1×10^{-5} M (STTA Assay) and 1×10^{-3} M (BG1Luc ER TA Assay).

[c]Number in parenthesis represents the test results classified as positive (POS) or negative (NEG) over the total number of referenced studies.

[1]Values reported in Draft Report of Pre-validation and Inter-laboratory Validation For Stably Transfected Transcriptional Activation (TA) Assay to Detect Estrogenic Activity - The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-HeLa-9903 Cell Line (31)

[2]ICCVAM Test Method Evaluation Report on the LUMI-CELL® ER (BG1Luc ER TA) Test Method: An *In Vitro* Method for Identifying ER Agonists and Antagonists (3)

[3]Mean EC_{50} values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

[4]Classification as an ER agonist or negative was based upon information in the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (32) as well as information obtained from publications published and reviewed after the completion of the ICCVAM BRDs (3) (18) (31) (33) (34) (35) (36).

Table 2: Comparison of Results from STTA and BG1Luc ER TA assays for Substances Tested in Both Antagonist Assays and Classified as ER Antagonists (POS) or Negatives (NEG)

			ER STTA assay[1]		BG1Luc ER TA assay[2]					
	Substance[a]	CASRN	ER TA Activity	IC_{50} Value[b] (M)	ER TA Activity	IC_{50} Value[b,3] (M)	ER STTA candidate effects[4]	ICCVAM [5] Consensus Classification	MeSH[6] Chemical Class	Product Class[7]
1	4-hydroxytamoxifen	68047-06-3	POS	3.97×10^{-9}	POS	2.08×10^{-7}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
2	Dibenzo[a.h] anthracene	53-70-3	POS	No IC_{50}	POS	No IC_{50}	POS	PP	Polycyclic Compound	Laboratory Chemical, Natural Product
3	Mifepristone	84371-65-3	POS	5.61×10^{-6}	NEG	-	mild POS	NEG	Steroid	Pharmaceutical
4	Raloxifene HCl	82640-04-8	POS	7.86×10^{-10}	POS	1.19×10^{-9}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
5	Tamoxifen	10540-29-1	POS	4.91×10^{-7}	POS	8.17×10^{-7}	POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
6	17-b estradiol	50-28-2	NEG	-	NEG	-	PN	PN	Steroid	Pharmaceutical, Veterinary Agent
7	Apigenin	520-36-5	NEG	-	NEG	-	NEG	NEG	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
8	Atrazine	1912-24-9	NEG	-	NEG	-	NEG	PN	Heterocyclic Compound	Herbicide
9	Di-n-butyl phthalate	84-74-2	NEG	-	NEG	-	NEG	NEG	Ester, Phthalic Acid	Cosmetic Ingredient, Industrial Chemical, Plasticizer
10	Fenarimol	60168-88-9	NEG	-	NEG	-	not tested	PN	Heterocyclic Compound, Pyrimidine	Fungicide
11	Flavone	525-82-6	NEG	-	NEG	-	PN	PN	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
12	Flutamide	13311-84-7	NEG	-	NEG	-	NEG	PN	Amide	Pharmaceutical, Veterinary Agent
13	Genistein	446-72-0	NEG	-	NEG	-	PN	NEG	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
14	p-n-nonylphenol	104-40-5	NEG	-	NEG	-	not tested	NEG	Phenol	Chemical Intermediate
15	Resveratrol	501-36-0	NEG	-	NEG	-	PN	NEG	Hydrocarbon (Cyclic)	Natural Product

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; M = molar; IC_{50} = half maximal inhibitory concentration of test substance; NEG = negative; PN = presumed negative; POS = positive; PP = presumed positive.

[a] Common substances tested in the STTA and BG1Luc ER TA assays that were designated as ER antagonists or negatives and used to evaluate accuracy in the BG1 Luc ER TA validation study

(3) (31).
[b] Maximum concentration tested in the absence of limitations due to cytotoxicity or insolubility was 1 x 10-3 M (STTA Assay) and 1 x 10-5 M (BG1Luc ER TA Assay).
[1] The Validation Report of the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity, Part B (31)
[2] ICCVAM Test Method Evaluation Report on the LUMI-CELL ER (BG1Luc ER TA) Test Method: An In Vitro Method for Identifying ER Agonists and Antagonists (3).
[3] Mean IC_{50} values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).
[4] ER STTA activity assumed from their reported effects known from the CERI historical data of ER receptor binding assay, the uterotrophic assay and information collated from the open literature (31)[5] Classification as an ER antagonist or negative was based upon information in the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (32) as well as information obtained from publications published and reviewed after the completion of the ICCVAM BRDs (3) (18) (31) (33).
[6] Substances were assigned to one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognised standardised classification scheme (available at http://www.nlm.nih.gov/mesh).
[7] 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).

ER TA TEST METHOD COMPONENTS

Essential Test Method Components

11. This PBTG applies to methods using a stably transfected or endogenous ERα receptor and stably transfected reporter gene construct under the control of one or more estrogen response elements; however, other receptors such as ERβ may be present. These are essential test method components.

Control substances

12. The basis for the proposed concurrent reference substances for each of agonist and antagonist assay should be described. Concurrent controls (negative, solvent, and positive), as appropriate, serve as an indication that the test method is operative under the test conditions and provide a basis for experiment-to-experiment comparisons; they are usually part of the acceptability criteria for a given experiment (1).

Standard Quality Control Procedures

13. Standard quality control procedures should be performed as described for each assay to ensure the cell line remains stable through multiple passages, remains mycoplasma-free, and retains the ability to provide the expected ER-mediated responses over time. Cell lines should be further checked for their correct identity as well as for other contaminants (e.g. fungi, yeast and viruses).

Demonstration of Laboratory Proficiency

14. Prior to testing unknown chemicals with any of the test methods under this PBTG, each laboratory should demonstrate proficiency in using the test method by testing of the 14 proficiency substances listed in Table 3 for agonist assay and 10 proficiency substances in Table 4 for antagonist assay. This proficiency testing will also confirm the responsiveness of the test system. The list of proficiency substances is a subset of the reference substances provided in the Performance Standards for the ER TA assays (6). These substances are commercially available, represent the classes of chemicals commonly associated with ER agonist or antagonist activity, exhibit a suitable range of potency expected for ER agonists/antagonists (i.e. strong to weak) and negatives. Testing of these substances should be replicated at least twice, on different days. Proficiency is demonstrated by correct classification (positive/negative) of each proficiency substance. Proficiency testing should be repeated by each technician when learning the test methods.

Table 3: List of (14) Proficiency Substances for agonist assay[8]

N°[7]	Substance Name	CASRN	Expected Response[1]	STTA Assay			BG1Luc ER TA Assay		MeSH Chemical Class[5]	Product Class[6]
				PC_{10} Value (M)[2]	PC_{50} Value (M)[2]	Test concentration range (M)	Bg1Luc EC_{50} Value (M)[3]	Highest Concentration for Range Finder (M)[4]		
14	Diethylstilbestrol	56-53-1	POS	$<1.00 \times 10^{-11}$	2.04×10^{-11}	$10^{-14} – 10^{-8}$	3.34×10^{-11}	3.73×10^{-4}	Hydrocarbon (Cyclic)	Pharmaceutical, Veterinary Agent
12	17∝-Estradiol	57-91-0	POS	4.27×10^{-11}	6.44×10^{-10}	$10^{-11} – 10^{-5}$	1.40×10^{-9}	3.67×10^{-3}	Steroid	Pharmaceutical, Veterinary Agent
15	*meso*-Hexestrol	84-16-2	POS	$<1.00 \times 10^{-11}$	2.75×10^{-11}	$10^{-11} – 10^{-5}$	1.65×10^{-11}	3.70×10^{-3}	Hydrocarbon (Cyclic), Phenol	Pharmaceutical, Veterinary Agent
11	4-*tert*-Octylphenol	140-66-9	POS	1.85×10^{-9}	7.37×10^{-8}	$10^{-11} – 10^{-5}$	3.19×10^{-8}	4.85×10^{-3}	Phenol	Chemical Intermediate
9	Genistein	446-72-0	POS	2.24×10^{-9}	2.45×10^{-8}	$10^{-11} – 10^{-5}$	2.71×10^{-7}	3.70×10^{-4}	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
6	Bisphenol A	80-05-7	POS	2.02×10^{-8}	2.94×10^{-7}	$10^{-11} – 10^{-5}$	5.33×10^{-7}	4.38×10^{-3}	Phenol	Chemical Intermediate
2	Kaempferol	520-18-3	POS	1.36×10^{-7}	1.21×10^{-6}	$10^{-11} – 10^{-5}$	3.99×10^{-6}	3.49×10^{-3}	Flavonoid, Heterocyclic Compound	Natural Product
3	Butylbenzyl phthalate	85-68-7	POS	1.14×10^{-6}	4.11×10^{-6}	$10^{-11} – 10^{-5}$	1.98×10^{-6}	3.20×10^{-4}	Carboxylic Acid, Ester, Phthalic Acid	Plasticizer, Industrial Chemical
4	*p,p'*-Methoxychlor	72-43-5	POS	1.23×10^{-6}	-	$10^{-11} – 10^{-5}$	1.92×10^{-6}	2.89×10^{-3}	Hydrocarbon (Halogenated)	Pesticide, Veterinary Agent
1	Ethyl paraben	120-47-8	POS	5.00×10^{-6}	-	$10^{-11} – 10^{-5}$	2.48×10^{-5}	6.02×10^{-3}	Carboxylic Acid, Phenol	Pharmaceutical, Preservative
17	Atrazine	1912-24-9	NEG	-	-	$10^{-10} – 10^{-4}$	-	4.64×10^{-4}	Heterocyclic Compound	Herbicide
20	Spironolactone	52-01-7	NEG	-	-	$10^{-11} – 10^{-5}$	-	2.40×10^{-3}	Lactone, Steroid	Pharmaceutical

21	Ketoconazole	65277-42-1	NEG	-	-	$10^{-11} – 10^{-5}$	-	$9.41 \prec 10^{-5}$	Heterocyclic Compound	Pharmaceutical
22	Reserpine	50-55-5	NEG	-	-	$10^{-11} – 10^{-5}$	-	$1.64 \prec 10^{-3}$	Heterocyclic Compound, Indole	Pharmaceutical, Veterinary Agent

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; EC_{50} = half maximal effective concentration of test substance; NEG = negative; POS = positive; PC_{10} (and PC_{50}) = the concentration of a test substance at which the response is 10% (or 50 % for PC_{50}) of the response induced by the positive control (E2, 1nM) in each plate.

[1]Classification as positive or negative for ER agonist activity was based upon the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (32) (33) as well as empirical data and other information obtained from referenced studies published and reviewed after the completion of the ICCVAM BRDs (3) (18) (31) (32) (33) (34) (35) (36).

[2]Values reported in Draft Report of Pre-validation and Inter-laboratory Validation For Stably Transfected Transcriptional Activation (TA) Assay to Detect Estrogenic Activity - The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-HeLa-9903 Cell Line (30).

[3]Mean EC_{50} values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

[4]Concentrations reported were the highest concentrations tested (range finder) during the validation of the BG1Luc ER TA Assay. If concentrations differed between the laboratories, the highest concentration is reported. See table 4-10 of 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 (3).

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

[6]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)

[7]From Table 1 (List of Reference Chemicals (22) for Evaluation of ER Agonist Accuracy) of the Performance Standards (6)

[8]If a reference substance is no longer commercially available, a substance with the same classification and, comparable potency, mode of action and chemical class can be used.

Table 4: List of (10) Proficiency Substances for antagonist assay

	Substance[a]	CASRN	ER STTA assay[1]			BG1Luc ER TA assay[2]			ER STTA[1] candidateeffects	ICCVAM[5] Consensus Classification	MeSH[6] Chemical Class	Product Class[7]
			ER TA Activity	IC_{50} Value (M)	Test concentration range(M)	ER TA Activity	IC_{50} Value[3] (M)	Highest Concentration for Range Finder (M)[4]				
1	4-hydroxytamoxifen	68047-06-3	POS	3.97×10^{-9}	$10^{-12} – 10^{-7}$	POS	2.08×10^{-7}	2.58×10^{-4}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
2	Raloxifene HCl	82640-04-8	POS	7.86×10^{-10}	$10^{-12} – 10^{-7}$	POS	1.19×10^{-9}	1.96×10^{-4}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
3	Tamoxifen	10540-29-1	POS	4.91×10^{-7}	$10^{-10} – 10^{-5}$	POS	8.17×10^{-7}	2.69×10^{-4}	POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
4	17β estradiol	50-28-2	NEG	-	$10^{-9} – 10^{-4}$	NEG	-	3.67×10^{-3}	to be negative*	PN	Steroid	Pharmaceutical, Veterinary Agent
5	Apigenin	520-36-5	NEG	-	$10^{-9} – 10^{-4}$	NEG	-	3.70×10^{-4}	NEG	NEG	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
6	Di-n-butyl phthalate	84-74-2	NEG	-	$10^{-8} – 10^{-3}$	NEG	-	3.59×10^{-3}	NEG	NEG	Ester, Phthalic Acid	Cosmetic Ingredient, Industrial Chemical, Plasticizer
7	Flavone	525-82-6	NEG	-	$10^{-8} – 10^{-3}$	NEG	-	4.50×10^{-4}	to be negative*	PN	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical

8	Genistein	446-72-0	NEG	-	10^{-9} – 10^{-4}	NEG	-	3.70×10^{-4}	to be negative*	NEG	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
9	p-n-nonylphenol	104-40-5	NEG	-	10^{-9} – 10^{-4}	NEG	-	4.54×10^{-4}	not tested	NEG	Phenol	Chemical Intermediate
10	Resveratrol	501-36-0	NEG	-	10^{-8} – 10^{-3}	NEG	-	4.38×10^{-4}	to be negative*	NEG	Hydrocarbon (Cyclic)	Natural Product

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; M = molar; IC_{50} = half maximal inhibitory concentration of test substance; NEG = negative; PN = presumed negative; POS = positive.

* classified negative according to literature review (31).

[a] Common substances tested in the STTA and BG1Luc ER TA assays that were designated as ER antagonists or negatives and used to evaluate accuracy in the BG1 Luc ER TA validation study (3) (31).

[1] The Validation Report of the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity, Part B (31)

[2] ICCVAM Test Method Evaluation Report on the LUMI-CELL ER (BG1Luc ER TA) Test Method: An In Vitro Method for Identifying ER Agonists and Antagonists (3).

[3] Mean IC_{50} values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

[4]Concentrations reported were the highest concentrations tested (range finder) during the validation of the BG1Luc ER TA Assay. If concentrations differed between the laboratories, the highest concentration is reported. See table 4-11 of 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 (3).

[5] Classification as an ER antagonist or negative was based upon information in the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (32) as well as information obtained from publications published and reviewed after the completion of the ICCVAM BRDs (3) (18) ((31) (33).

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

[7] 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).

Test Run Acceptability Criteria

15. Acceptance or rejection of a test run is based on the evaluation of results obtained for the reference substances and controls used for each experiment. Values for the $PC_{50}(EC_{50})$ or IC_{50} for the reference substances should meet the acceptability criteria as provided for the selected test method (for STTA see Annex 2, for BG1Luc ER TA see Annex 3), and all positive/negative controls should be correctly classified for each accepted experiment. The ability to consistently conduct the test method should be demonstrated by the development and maintenance of a historical database for the reference substances and controls. Standard deviations (SD) or coefficients of variation (CV) for the means of reference substances curve fitting parameters from multiple experiments may be used as a measure of within-laboratory reproducibility. In addition, the following principles regarding acceptability criteria should be met:

- Data should be sufficient for a quantitative assessment of ER activation (for agonist assay) or suppression (for antagonist assay) (i.e., efficacy and potency).

- The mean reporter activity for the reference concentration of reference estrogen should be at least the minimum specified in the test methods relative to that of the vehicle (solvent) control to ensure adequate sensitivity. For the STTA and BG1Luc ER TA test methods, this is four times that of the mean vehicle control on each plate.

- The concentrations tested should remain within the solubility range of the test chemicals and not demonstrate cytotoxicity.

Analysis of data

16. The defined data interpretation procedure for each test method should be used for classifying a positive and negative response.

17. Meeting the acceptability criteria (paragraph 15) indicates the assay system is operating properly, but it does not ensure that any particular test will produce accurate data. Replicating the correct results of the first test is the best indication that accurate data were produced. If two tests give reproducible results (e.g. both test results indicate a test chemical is positive), it is not necessary to conduct a third test.

18. If two runs do not give reproducible results (e.g. a test chemical is positive in one run and negative in the other run), or if a higher degree of certainty is required regarding the outcome of this assay, at least three independent runs should be conducted.

General Data Interpretation Criteria

19. There is currently no universally agreed method for interpreting ER TA data. However, both qualitative (e.g. positive/negative) and/or quantitative (e.g. EC_{50}, PC_{50}, IC_{50}) assessments of ER-mediated activity should be based on empirical data and sound scientific judgment. Where possible, positive results should be characterised by both the magnitude of the effect as compared to the vehicle (solvent) control or reference estrogen and the concentration at which the effect occurs (e.g. an EC_{50}, PC_{50}, RPC_{Max}, IC_{50} , etc.).

Test Report

20. The test report should include the following information:

Test method:

- Test method used;

Control/Reference/Test chemical

- source, lot number, limit date for use, if available
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known.
- measurement of pH, osmolality and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent/Vehicle:

- characterisation (nature, supplier and lot);
- justification for choice of solvent/vehicle;
- solubility and stability of the test chemical in solvent/vehicle, if known;

Cells:

- type and source of cells:
  - Is ER endogenously expressed? If not, which receptor(s) were Transfected?
  - Reporter construct(s) used (including source species);
  - Transfection method;
  - Selection method for maintenance of stable transfection (where applicable);
  - Is the transfection method relevant for stable lines?
- number of cell passages (from thawing);
- passage number of cells at thawing;
- methods for maintenance of cell cultures;

Test conditions:

- solubility limitations;

- description of the methods of assessing viability applied;
- composition of media, CO_2 concentration;
- concentrations of test chemical;
- volume of vehicle and test chemical added;
- incubation temperature and humidity;
- duration of treatment;
- cell density at the start of - and during treatment;
- positive and negative reference substances;
- reporter reagents (product name, supplier and lot);
- criteria for considering tests as positive, negative or equivocal;

Acceptability check:

- fold inductions for each assay plate and whether they meet the minimum required by the test method based on historical controls;
- actual values for acceptability criteria, e.g. $log_{10}EC_{50}$, $log_{10}PC_{50}$, $logIC_{50}$ and Hillslope values, for concurrent positive controls/reference substances;

Results:

- raw and normalised data;
- the maximum fold induction level;
- cytotoxicity data;
- if it exists, the lowest effective concentration (LEC);
- RPC_{Max}, PC_{Max}, PC_{50}, IC_{50} and/or EC_{50} values, as appropriate;
- concentration-response relationship, where possible;
- statistical analyses, if any, together with a measure of error and confidence (e.g. SEM, SD, CV or 95% CI) and a description of how these values were obtained;

Discussion of the results

Conclusion

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ANNEX 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 (concordance): The closeness of agreement between test method results and an accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with "concordance" to mean the proportion of correct outcomes of a test method (1).

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

Antagonist: A type of receptor ligand or chemical that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses.

Anti-estrogenic activity, the capability of a chemical to suppress the action of 17β-estradiol mediated through estrogen receptors.

BG-1: An immortalised adenocarcinoma cell that endogenously express estrogen receptor.

BG-1Luc4E2: The BG-1Luc4E2 cell line was derived from BG-1 immortalised human-derived adenocarcinoma cells that endogenously express both forms of the estrogen receptor (ERα and ERβ) and have been stably transfected with the plasmid pGudLuc7.ERE. 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 Evaluation 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: Harmful effects to cell structure or function that can ultimately cause cell death and can be reflected by a reduction in the number of cells present in the well at the end of the exposure period or a reduction of the capacity for a measure of cellular function when compared to the concurrent vehicle control.

CV: Coefficient of variation

DCC-FBS: Dextran-coated charcoal treated fetal bovine serum.

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
hERα: Human estrogen receptor alpha

hERß: Human estrogen receptor beta

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

Estrogenic activity: The capability of a chemical to mimic 17β-estradiol in its ability to bind to and activate estrogen receptors. hERα-mediated estrogenic activity can be detected with this PBTG.

FBS: Fetal bovine serum

HeLa: An immortal human cervical cell line

HeLa9903: A HeLa cell subclone into which hERα and a luciferase reporter gene have been stably transfected

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

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

Inter-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same substances, can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the prevalidation and validation processes, and indicates the extent to which a test method can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility (1).

Intra-laboratory reproducibility: A determination of the extent that qualified people within the same laboratory can successfully replicate results using a specific protocol at different times. Also referred to as "within-laboratory reproducibility" (1).

LEC: Lowest effective concentration is the lowest concentration of test chemical that produces a response (i.e. the lowest test chemical concentration at which the fold induction is statistically different from the concurrent vehicle control).

Me-too test: A colloquial expression for a test method that is structurally and functionally similar to a validated and accepted reference test method. Interchangeably used with similar test method

MT: Metallothionein

MMTV: Mouse Mammary Tumor Virus

OHT: 4-Hydroxytamoxifen

PBTG: Performance-Based Test Guideline

PC (Positive control)**:** a strongly active substance, preferably 17ß-estradiol that is included in all tests to help ensure proper functioning of the assay.

PC_{10}: the concentration of a test chemical at which the measured activity in an agonist assay is 10% of the maximum activity induced by the PC (E2 at 1nM for the STTA assay) in each plate.

PC_{50}: the concentration of a test chemical at which the measured activity in an agonist assay is 50% of the maximum activity induced by the PC (E2 at the reference concentration specified in the test method) in each plate.

PC_{Max}: the concentration of a test chemical inducing the RPC_{Max}

Performance standards: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are (1) essential test method components; (2) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (3) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of reference chemicals (1).

Proficiency substances: A subset of the reference substances included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardised 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.

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

Reference estrogen (Positive control, PC): 17β -estradiol (E2, CAS 50-28-2).

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

Reference test methods: The test methods upon which this PBTG is based.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test

method (1).

Reliability: Measure of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility.

RLU: Relative Light Units

RNA: Ribonucleic Acid

RPC_{Max}: maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate

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

SD: Standard deviation.

Sensitivity: The proportion of all positive/active substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (1).

Specificity: The proportion of all negative/inactive substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (1).

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).

STTA Assay: Stably Transfected Transactivation Assay, the ERα transcriptional activation assay using the HeLa 9903 Cell Line.

Substance: Used in the context of the UN GHS (1) as chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

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

Transcription: mRNA synthesis

Validated test method: A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose (1).

Validation: The process by which the reliability and relevance of a particular approach, method, process or

assessment is established for a defined purpose (1).

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

Weak positive control: A weakly active substance selected from the reference chemicals list that is included in all tests to help ensure proper functioning of the assay.

ANNEX 2

Stably Transfected Human Estrogen Receptor-α Transactivation Assay for Detection of Estrogenic Agonist and antagonist Activity of Chemicals using the hERα-HeLa-9903 cell line

INITIAL CONSIDERATIONS AND LIMITATIONS (See also **GENERAL INTRODUCTION**, page 1**)**

1. This transactivation (TA) assay uses the hERα-HeLa-9903 cell line to detect estrogenic agonist activity mediated through human estrogen receptor alpha (hERα). The validation study of the Stably Transfected Transactivation (STTA) Assay by the Japanese Chemicals Evaluation and Research Institute (CERI) using the hERα-HeLa-9903 cell line to detect estrogenic agonist and antagonist activity mediated through human estrogen receptor alpha (hERα) demonstrated the relevance and reliability of the assay for its intended purpose (1).

2. This test method is specifically designed to detect hERα-mediated TA by measuring chemiluminescence as the endpoint. However, 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) (3). 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 (Appendix 1).

3. The "**GENERAL INTRODUCTION**" and "**ER TA TEST METHOD COMPONENTS"** (pages 1-14) should be read before using this test method for regulatory purposes. Definitions and abbreviations used in this TG are described in Annex 1.

PRINCIPLE OF THE TEST METHOD (See also **GENERAL INTRODUCTION**, page 1)

4. The assay is used to signal binding of the estrogen receptor with a ligand. Following ligand binding, the receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer. Luciferase activity can be evaluated quickly and inexpensively with a number of commercially available test kits.

5. The test system utilises the hERα-HeLa-9903 cell line, which is derived from a human cervical tumor, with two stably inserted constructs: (i) the hERα expression construct (encoding the full-length human receptor), and (ii) a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin Estrogen-Responsive Element (ERE) driven by a mouse metallothionein (MT) promoter TATA element. The mouse MT TATA gene construct has been shown to have the best performance, and so is commonly used. Consequently this hERα-HeLa-9903 cell line can measure the ability of a test chemical to induce hERα-mediated transactivation of luciferase gene expression.

6. In case of ER agonist assay, data interpretation is based upon whether or not the maximum response

level induced by a test chemical equals or exceeds an agonist response equal to 10% of that induced by a maximally inducing (1 nM) concentration of the positive control (PC) 17β estradiol (E2) (i.e. the PC_{10}). In case of ER antagonist assay, data interpretation is based upon whether or not the response shows at least a 30% reduction in activity from the response induced by the spike in control (25 pM of E2) without cytotoxicity. Data analysis and interpretation are discussed in detail in paragraphs 34 - 48.

PROCEDURE

Cell Lines

7. The stably transfected hERα-HeLa-9903 cell line should be used for the assay. The cell line can be obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank[1], upon signing a Material Transfer Agreement (MTA).

8. Only cells characterised as mycoplasma-free should be used in testing. RT-PCR (Real Time Polymerase Chain Reaction) is the method of choice for a sensitive detection of mycoplasma infection (4) (5) (6).

Stability of the cell line

9. To monitor the stability of the cell line, E2, 17α-estradiol, 17α-methyltestosterone and corticosterone should be used as the reference substances for agonist assay and a complete concentration-response curve in the test concentration range provided in Table 1 should be measured at least once each time the assay is performed, and the results should be in agreement with the results provided in Table 1.

10. In case of antagonist assay, complete concentration curves for two reference substances, tamoxifen and flutamide, should be measured simultaneously with each run. Correct qualitative classification as positive or negative for the two chemicals should be monitored.

Cell Culture and Plating Conditions

11. Cells should be maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with 60 mg/L of antibiotic kanamycine and 10% dextran-coated-charcoal-treated fetal bovine serum (DCC-FBS), in a CO_2 incubator (5% CO_2) at 37±1˚C. Upon reaching 75 -90% confluency, cells can be subcultured at 10 mL of 0.4 x 10^5 – 1 x 10^5 cells/mL for 100 mm cell culture dish. Cells should be suspended with 10% FBS-EMEM (which is the same as EMEM with DCC-FBS) and then plated into wells of a microplate at a density of 1 x 10^4 cells/(100 μL x well). Next, the cells should be pre-incubated in a 5% CO_2 incubator at 37˚±1˚C for 3 hours before the chemical exposure. The plastic-ware should be free of estrogenic activity.

12. To maintain the integrity of the response, the cells should be grown for more than one passage from the frozen stock in the conditioned media and should not be cultured for more than 40 passages. For the hERα-HeLa-9903 cell line, this will be less than three months. However the performance of cells may be reduced if they are grown in inappropriate culture conditions.

[1] JCRB Cell Bank : National Institute of Biomedical Innovation, 7-6-8 Asagi Saito, Ibaraki-shi, Osaka 567-0085, Japan Fax: +81-72-641-9812

13. The DCC-FBS can be prepared as described in Appendix 2, or obtained from commercial sources.

Acceptability criteria

Positive and negative reference substances for ER agonist assay

14. Prior to and during the study, the responsiveness of the test system should be verified using the appropriate concentrations of a strong estrogen: E2, a weak estrogen (17α-estradiol), a very weak agonist (17α-methyltestosterone), and a negative substance (corticosterone). Acceptable range values derived from the validation study (1) are given in Table 1. These 4 concurrent reference substances should be included with each experiment and the results should fall within the given acceptable limits. If this is not the case, the cause for the failure to meet the acceptability criteria should be determined (e.g. cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. Once the acceptability criteria have been achieved, to ensure minimum variability of EC_{50}, PC_{50} and PC_{10} values, consistent use of materials for cell culturing is essential. The four concurrent reference substances, which should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), can ensure the sensitivity of the assay because the PC_{10}s of the three positive reference substances should fall within the acceptable range, as should the PC_{50}s and EC_{50}s where they can be calculated (see Table 1).

Table 1. Acceptable range values of the four reference substances for the ER agonist assay

Name	logPC_{50}	logPC_{10}	logEC_{50}	Hill slope	Test range
17β-Estradiol (E2) CAS No: 50-28-2	-11.4 ~ -10.1	<-11	-11.3 ~ -10.1	0.7 ~ 1.5	10^{-14} ~ 10^{-8} M
17α-Estradiol CAS No: 57-91-0	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	10^{-12} ~ 10^{-6} M
Corticosterone CAS No: 50-22-6	–	–	–	–	10^{-10} ~ 10^{-4}M
17α-Methyltestosterone CAS No: 58-18-4	-6.0 ~ -5.1	-8.0 ~ -6.2	–	–	10^{-11} ~ 10^{-5} M

Positive and negative reference substances for ER antagonist assay

15. Prior to and during the study, the responsiveness of the test system should be verified using the appropriate concentrations of a positive substance (Tamoxifen), and a negative substance (Flutamide). Acceptable range values derived from the validation study (1) are given in Table 2. These two concurrent reference substances should be included with each experiment and the results should be judged correctly as shown in the criteria. If this is not the case, the cause for the failure to meet the criteria should be determined (e.g. cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. In addition, IC_{50} values for a positive substance (Tamoxifen) should be calculated and the results should fall within the given acceptable limits. Once the acceptability criteria have been achieved, to ensure minimum variability of IC_{50} values, consistent use of materials for cell culturing is essential. The two concurrent reference substances, which should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), can ensure the sensitivity of the assay (see Table 2).

Table 2. Criteria and acceptable range values of the two reference substances for the ER antagonist assay

Name	Criteria	$LogIC_{50}$	Test range
Tamoxifen CAS No: 10540-29-1	Positive: IC_{50} should be calculated	-5.942～ -7.596	10^{-10}～10^{-5} M
Flutamide CAS No: 13311-84-7	Negative: IC_{30} should not be calculated	-	10^{-10} ～10^{-5} M

Positive and Vehicle Controls

16. The positive control (PC) for ER agonist assay (1 nM of E2) and for ER antagonist assay (10μM TAM) should be tested at least in triplicate in each plate. The vehicle that is used to dissolve a test chemical should be tested as a vehicle control (VC) at least in triplicate in each plate. In addition to this VC, if the PC uses a different vehicle than the test chemical, another VC should be tested at least in triplicate on the same plate with the PC.

Quality criteria for ER agonist assay

17. The mean luciferase activity of the positive control (1 nM E2) should be at least 4-fold that of the mean VC on each plate. This criterion is established based on the reliability of the endpoint values from the validation study (historically between four- and 30-fold).

18. With respect to the quality control of the assay, the fold-induction corresponding to the PC_{10} value of the concurrent PC (1 nM E2) should be greater than 1+2SD of the fold-induction value (=1) of the concurrent VC. For prioritisation purposes, the PC_{10} value can be useful to simplify the data analysis required compared to a statistical analysis. Although a statistical analysis provides information on significance, such an analysis is not a quantitative parameter with respect to concentration-based potential, and so is less useful for prioritisation purposes.

Quality criteria for ER antagonist assay

19. The mean luciferase activity of the spike in control (25 pM E2) should be at least 4-fold that of the mean VC on each plate. This criterion is established based on the reliability of the endpoint values from the validation study.

20. With respect to the quality control of the assay, relative transcriptional activation (RTA) of 1 nM E2 should be greater than 100%, RTA of 1μM 4-Hydroxytamoxifen (OHT) should be less than 40.6% and RTA of 100 μM Digitonin (Dig) should be less than 0%.

Substances to Demonstrate Laboratory Proficiency (see paragraph 14 and Table 2 in **« ER TA TEST METHOD COMPONENTS»** of this Test Guideline (pages 6-14)).

Vehicle

21. Dimethyl sulfoxide (DMSO), or appropriate solvent, at the same concentration used for the different

positive and negative controls and the test chemicals should be used as the concurrent VC. Test chemicals should be dissolved in a solvent that solubilises that test chemical 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 0.1% (v/v). For any vehicle, it should be demonstrated that the maximum volume used is not cytotoxic and does not interfere with assay performance.

Preparation of Test Chemicals

22. Generally, the test chemicals should be dissolved in DMSO or other suitable solvent, and serially diluted with the same solvent at a common ratio of 1:10 in order to prepare solutions for dilution with media.

Solubility and Cytotoxicity: Considerations for Range Finding.

23. A preliminary test should be carried out to determine the appropriate concentration range of chemical to be tested, and to ascertain whether the test chemical may have any solubility and cytotoxicity problems. Initially, chemicals are tested up to the maximum concentration of 1 µL/mL, 1 mg/mL, or 1 mM, whichever is the lowest. Based on the extent of cytotoxicity or lack of solubility observed in the preliminary test, the first definite run should test the chemical at log-serial dilutions starting at the maximum acceptable concentration (e.g. 1 mM, 100µM, 10µM, etc.) and the presence of cloudiness or precipitate or cytotoxicity noted. Concentrations in the second, and if necessary third run should be adjusted as appropriate to better characterise the concentration-response curve and to avoid concentrations which are found to be insoluble or to induce excessive cytotoxicity.

24. For ER agonists and antagonists, the presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and should be considered when interpreting the data. Cytotoxicity testing methods that can provide information regarding 80% cell viability should be used, utilising an appropriate assay based upon laboratory experience.

25. Should the results of the cytotoxicity test show that the concentration of the test chemical has reduced the cell number by 20% or more, this concentration should be regarded as cytotoxic, and the concentrations at or above the cytotoxic concentration should be excluded from the evaluation.

Chemical Exposure and Assay Plate Organisation

26. The procedure for chemical dilutions (Steps-1 and 2) and exposure to cells (Step-3) can be conducted as follows:

Step-1: Each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtitre plate to achieve final serial concentrations as determined by the preliminary range finding test (typically in a series of, for example 1 mM, 100 µM, 10 µM, 1µM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-3}-10^{-11} M)) for triplicate testing.

Step-2: Chemical dilution: First dilute 1.5 µL of the test chemical in the solvent to a concentration of 500 µL of media.

Step-3: Chemical exposure of the cells: Add 50 µL of dilution with media (prepared in Step-2) to an assay well containing 10^4 cells/100 µL/well.

The recommended final volume of media required for each well is 150 µL. Test samples and reference substances can be assigned as shown in Table 3 and Table 4.

Table 3: Example of plate concentration assignment of the reference substances in the assay plate in ER agonist assay

Row	17α-Methyltestosterone			Corticosterone			17α-Estradiol			E2		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 µM)	→	→	100 µM	→	→	1 µM	→	→	10 nM	→	→
B	conc 2 (1 µM)	→	→	10 µM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	1 µM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	100 nM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	10 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	1 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	conc 7 (10 pM)	→	→	100 pM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (0.1% DMSO); PC: Positive control (1 nM E2)

27. The reference substances (E2, 17α-Estradiol, 17α-methyl testosterone and corticosterone) should be tested in every run (Table 3). PC wells treated with 1 nM of E2 that can produce maximum induction of E2 and VC wells treated with DMSO (or appropriate solvent) alone should be included in each test assay plate (Table 4). If cells from different sources (e.g. different passage number, different lot, etc.) are used in the same experiment, the reference substances should be tested for each cell source.

Table 4: Example of plate concentration assignment of test and plate control chemicals in the assay plate in ER agonist assay

Row	Test Chemical 1			Test Chemical 2			Test Chemical 3			Test Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 µM)	→	→	1 mM	→	→	1 µM	→	→	10 nM	→	→
B	conc 2 (1 µM)	→	→	100 µM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	10 µM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	1 µM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	100 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	10 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	conc 7 (10 pM)	→	→	1 nM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (0.1% DMSO); PC: Positive control (1 nM E2)

Table 5: Example of plate concentration assignment of the reference substances in the assay plate in ER antagonist assay

Row	Tamoxifen			Flutamide			Test Chemical 1			Test Chemical 2		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 µM)	→	→	10 µM	→	→	10 µM	→	→	10 µM	→	→
B	conc 2 (1 µM)	→	→	1 µM	→	→	1 µM	→	→	1 µM	→	→
C	conc 3 (100 nM)	→	→	100 nM	→	→	100 nM	→	→	100 nM	→	→
D	conc 4 (10 nM)	→	→	10 nM	→	→	10 nM	→	→	10 nM	→	→
E	conc 5 (1 nM)	→	→	1 nM	→	→	1 nM	→	→	1 nM	→	→
F	conc 6 (100 pM)	→	→	100 pM	→	→	100 pM	→	→	100 pM	→	→
G	0.1% DMSO	→	→	→	→	→	1 µM OHT	→	→	100 µM Dig	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (0.1% DMSO), PC: Positive control (1 nM E2), OHT :4-Hydroxytamoxifen, Dig: Digitonin.

: Spiked with 25pM E2

28. To evaluate the antagonist activity of chemicals, assay wells located in rows from A to G should be spiked with 25pM E2. The reference substances (Tamoxifen and Flutamide) should be tested in every run. PC wells treated with 1 nM of E2 that can be control quality of hERα-HeLa-9903 cell line, VC wells treated with DMSO (or appropriate solvent), 0.1% DMSO wells treated with DMSO addition to the spiked E2 corresponding to "Spike-in-control", wells treated with final concentration 1 µM OHT and wells treated with 100 µM Dig should be included in each test assay plate (Table 5). Subsequent assay plate should follow the same plate layout without reference substances wells (Table 6). If cells from different sources (e.g. different passage number, different lot, etc.) are used in the same experiment, the reference substances should be tested for each cell source.

Table 6: Example of plate concentration assignment of test and plate control chemicals in the assay plate in ER antagonist assay

Row	Test Chemical 1			Test Chemical 2			Test Chemical 3			Test Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 µM)	→	→	10 µM	→	→	10 µM	→	→	10 µM	→	→
B	conc 2 (1 µM)	→	→	1 µM	→	→	1 µM	→	→	1 µM	→	→
C	conc 3 (100 nM)	→	→	100 nM	→	→	100 nM	→	→	100 nM	→	→
D	conc 4 (10 nM)	→	→	10 nM	→	→	10 nM	→	→	10 nM	→	→
E	conc 5 (1 nM)	→	→	1 nM	→	→	1 nM	→	→	1 nM	→	→
F	conc 6 (100 pM)	→	→	100 pM	→	→	100 pM	→	→	100 pM	→	→
G	0.1% DMSO	→	→	→	→	→	1 µM OHT	→	→	100 µM Dig	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (0.1% DMSO), PC: Positive control (1 nM E2), OHT: 4-Hydroxytamoxifen, Dig: Digitonin.

: Spiked with 25pM E2

29. The lack of edge effects should be confirmed, as appropriate, and if edge effects are suspected, the plate layout should be altered to avoid such effects. For example, a plate layout excluding the edge wells can be employed.

30. After adding the chemicals, the assay plates should be incubated in a 5% CO_2 incubator at 37±1ºC for 20-24 hours to induce the reporter gene products.

31. Special considerations will need to be applied to those compounds that are highly volatile. In such cases, nearby control wells may generate false positives and this should be considered in light of expected and historical control values. In the few cases where volatility may be of concern, the use of "plate sealers" may help to effectively isolate individual wells during testing, and is therefore recommended in such cases.

32. Repeat definitive tests for the same chemical should be conducted on different days, to ensure independence.

Luciferase assay

33. A commercial luciferase assay reagent [e.g. Steady-Glo® Luciferase Assay System (Promega, E2510, or equivalents)] or a standard luciferase assay system (Promega, E1500, or equivalents) can be used for the assay, as long as the acceptability criteria are met. The assay reagents should be selected based on the sensitivity of the luminometer to be used. When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531, or equivalents) should be used before adding the substrate. The luciferase reagent should be applied following the manufacturers' instructions.

ANALYSIS OF DATA

ER agonist assay

34. In case of ER agonist assay, to obtain the relative transcriptional activity to PC (1 nM of E2), the luminescence signals from the same plate can be analysed according to the following steps (other equivalent mathematical processes are also acceptable):

Step 1. Calculate the mean value for the VC.

Step 2. Subtract the mean value of the VC from each well value to normalise the data.

Step 3. Calculate the mean for the normalised PC.

Step 4. Divide the normalised value of each well in the plate by the mean value of the normalised PC (PC=100%).

The final value of each well is the relative transcriptional activity for that well compared to the PC response.

Step 5. Calculate the mean value of the relative transcriptional activity for each concentration group of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).

EC_{50}, PC_{50} and PC_{10} induction considerations

35. The full concentration-response curve is required for the calculation of the EC_{50}, but this may not

always be achievable or practical due to limitations of the test concentration range (for example due to cytotoxicity or solubility problems). However, as the EC_{50} and maximum induction level (corresponding to the top value of the Hill-equation) are informative parameters, these parameters should be reported where possible. For the calculation of EC_{50} and maximum induction level, appropriate statistical software should be used (e.g. Graphpad Prism statistical software).

36. If the Hill's logistic equation is applicable to the concentration response data, the EC_{50} should be calculated by the following equation (7):

$Y=Bottom + (Top-Bottom) / (1+10 \exp ((\log EC_{50} -X) \times Hill\ slope))$
Where:
X is the logarithm of concentration; and,
Y is the response and Y starts at the Bottom and goes to the Top in a sigmoid curve.
Bottom is fixed at zero in the Hill's logistic equation.

37. For each test chemical, the following should be provided:

(i) The RPC_{Max} which is the maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate, as well as the PCMax (concentration associated with the RPC_{Max}); and

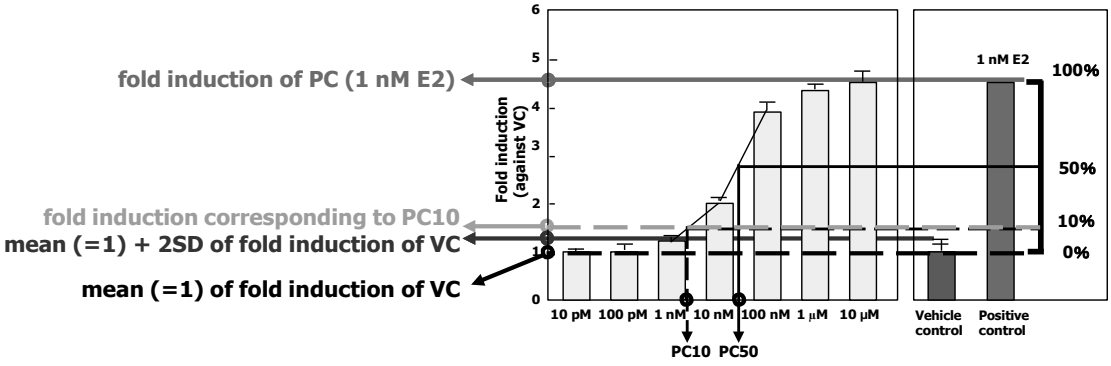
(ii) For positive chemicals, the concentrations that induce the PC_{10} and, if appropriate, the PC_{50}.

38. The PCx value can be calculated by interpolating between 2 points on the X-Y coordinate, one immediately above and one immediately below a PCx value. Where the data points lying immediately above and below the PCx value have the coordinates (a,b) and (c,d) respectively, then the PCx value may be calculated using the following equation:

$$\log[PCx] = \log[c]+(x-d)/(d-b)$$

39. Descriptions of PC values are provided in Figure 1 below.

Figure 1: Example of how to derive PC-values. The PC (1 nM of E2) is included on each assay plate



ER antagonist assay

40. In case of ER antagonist assay, to obtain the relative transcriptional activity (RTA) to spike in control (25 pM of E2), the luminescence signals from the same plate can be analysed according to the following steps (other equivalent mathematical processes are also acceptable):

Step 1. Calculate the mean value for the VC.

Step 2. Subtract the mean value of the VC from each well value to normalise the data.

Step 3. Calculate the mean for the normalised spike in control.

Step 4. Divide the normalised value of each well in the plate by the mean value of the normalised spike in control (spike in control=100%).

The final value of each well is the relative transcriptional activity for that well compared to the spike in control response.

Step 5. Calculate the mean value of the relative transcriptional activity for each treatment.

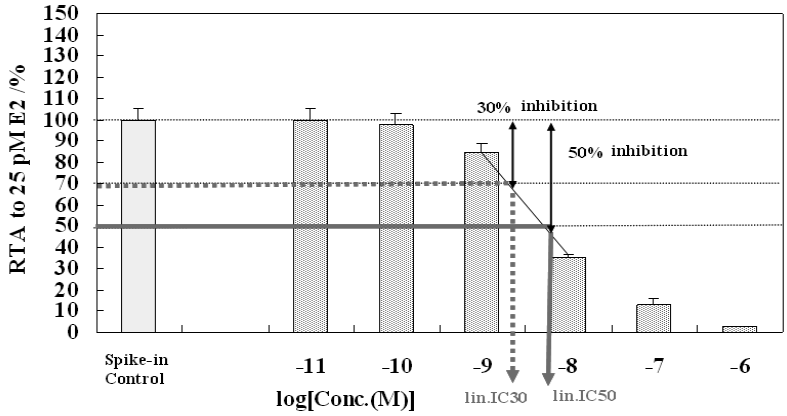
IC_{30} and IC_{50} induction considerations

41. For positive chemicals, the concentrations that induce the IC30 and, if appropriate, the IC50 should be provided.

42. The ICx value can be calculated by interpolating between 2 points on the X-Y coordinate, one immediately above and one immediately below a ICx value. Where the data points lying immediately above and below the ICx value have the coordinates (c,d) and (a,b) respectively, then the ICx value may be calculated using the following equation:

$$\text{lin ICx} = a-(b-(100-x))\,(a-c)\,/(b-d)$$

Figure 2: Example of how to derive IC-values. The spike in control (25 pM of E2) is included on each assay plate



RTA: relative transcriptional activity

43. The results should be based on two (or three) independent runs. If two runs give comparable and therefore reproducible results, it is not necessary to conduct a third run. To be acceptable, the results should:

- Meet the acceptability criteria (see Acceptability criteria para 14-20),
- Be reproducible.

Data Interpretation Criteria

Table 7: Positive and negative decision criteria in ER agonist assay

Positive	If the RPC_{Max} is obtained that is equal to or exceeds 10% of the response of the positive control in at least two of two or two of three runs.
Negative	If the RPC_{Max} fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

Table 8: Positive and negative decision criteria in ER antagonist assay

Positive	If the IC_{30} is calculated in at least two of two or two of three runs.
Negative	If the IC_{30} fails to calculate in two of two or two of three runs.

44. Data interpretation criteria are shown in Tables 7 and 8. Positive results will be characterised by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (PC50) or 10% (PC10) of PC values are reached for the agonist assay, and 50% (IC50) or 30% (IC30) of the spike-in control value is inhibited for the antagonist assay, accomplishes both of these goals. However, a test chemical is determined to be positive, if the maximum response induced by the test chemical (RPCMax) is equal to or exceeds 10% of the response of the PC in at least two of two or two of three runs, while a test chemical is considered negative if the RPCMax fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

45. The calculations of PC_{10}, PC_{50} and PC_{Max} in ER agonist assay and IC_{30} and IC_{50} in ER antagonist assay can be made by using a spreadsheet available with the Test Guideline on the OECD public website[2].

46. It should be sufficient to obtain PC_{10} or PC_{50} and IC_{30} or IC_{50} values at least twice. However, should the resulting base-line for data in the same concentration range show variability with an unacceptably high coefficient of variation (CV; %) the data may not be considered reliable and the source of the high variability should be identified. The CV of the raw data triplicates (i.e. luminescence intensity data) of the data points that are used for the calculation of PC_{10} should be less than 20%.

47. Meeting the acceptability criteria indicates the assay system is operating properly, but it does not

[2] http://www.oecd.org/env/testguidelines

ensure that any particular run will produce accurate data. Duplicating the results of the first run is the best insurance that accurate data were produced.

48. In case of ER agonist assay, where more information is required in addition to the screening and prioritisation purposes of this TG for positive test chemicals, particularly for PC_{10}-PC_{49} chemicals, as well as chemicals suspected to over-stimulate luciferase, it can be confirmed that the observed luciferase-activity is solely an ERα-specific response, using an ERα antagonist (see Appendix 1).

TEST REPORT

49. See paragraph 20 of "**ER TA TEST METHOD COMPONENTS**" (Pages 6-14 of this Test Guideline).

LITERATURE (2)

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Appendix 1

False positives: Assessment of non-receptor mediated luminescence signals

1. False positives in the ER agonist assay might be generated by non-ER-mediated activation of the luciferase gene, or direct activation of the gene product or unrelated fluorescence. Such effects are indicated by an incomplete or unusual dose-response curve. If such effects are suspected, the effect of an ER antagonist (e.g. 4- hydroxytamoxifen (OHT) at non-toxic concentration) on the response should be examined. The pure antagonist ICI 182780 may not be suitable for this purpose as a sufficient concentration of ICI 182780 may decrease the VC value, and this will affect the data analysis.

2. To ensure validity of this approach, the following needs to be tested in the same plate:

- Agonistic activity of the unknown chemical with / without 10 μM of OHT
- VC (in triplicate)
- OHT (in triplicate)
- 1 nM of E2 (in triplicate) as agonist PC
- 1 nM of E2 + OHT (in triplicate)

3. ***Data interpretation criteria***

Note: All wells should be treated with the same concentration of the vehicle.

- If the agonistic activity of the unknown chemical is NOT affected by the treatment with ER antagonist, it is classified as "Negative".
- If the agonistic activity of the unknown chemical is completely inhibited, apply the decision criteria.
- If the agonistic activity at the lowest concentration is equal to, or is exceeding, PC_{10} response the unknown chemical is inhibited equal to or exceeding PC_{10} response. The difference in the responses between the non-treated and treated wells with the ER antagonist is calculated and this difference should be considered as the true response and should be used for the calculation of the appropriate parameters to enable a classification decision to be made.

4. ***Data analysis***

Check the performance standard.

Check the CV between wells treated under the same conditions.

1. Calculate the mean of the VC
2. Subtract the mean of VC from each well value **not** treated with OHT
3. Calculate the mean of OHT
4. Subtract the mean of the VC from each well value treated with OHT
5. Calculate the mean of the PC
6. Calculate the relative transcriptional activity of all other wells relative to the PC.

Appendix 2

Preparation of Serum treated with Dextran Coated Charcoal (DCC)

1. The treatment of serum with dextran-coated charcoal (DCC) is a general method for removal of estrogenic compounds from serum that is added to cell medium, in order to exclude the biased response associated with residual estrogens in serum. 500 mL of fetal bovine serum (FBS) can be treated by this procedure.

Components

2. The following materials and equipment will be required:

Materials
- Activated charcoal
- Dextran
- Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)
- Sucrose
- 1 M HEPES buffer solution (pH 7.4)
- Ultrapure water produced from a filter system

Equipment
- Autoclaved glass container (size should be adjusted as appropriate)
- General Laboratory Centrifuge (that can set temperature at 4°C)

Procedure

3. The following procedure is adjusted for the use of 50 mL centrifuge tubes:

[Day-1] Prepare dextran-coated charcoal suspension with 1 L of ultrapure water containing 1.5 mM of $MgCl_2$, 0.25 M sucrose, 2.5 g of charcoal, 0.25 g dextran and 5 mM of HEPES and stir it at 4°C, overnight.

[Day-2] Dispense the suspension in 50 mL centrifuge tubes and centrifuge at 10000 rpm at 4°C for 10 minutes. Remove the supernatant and store half of the charcoal sediment at 4°C for the use on Day-3. Suspend the other half of the charcoal with FBS that has been gently thawed to avoid precipitation, and heat-inactivated at 56°C for 30 minutes, then transfer into an autoclaved glass container such as an Erlenmeyer flask. Stir this suspension gently at 4°C, overnight.

[Day-3] Dispense the suspension with FBS into centrifuge tubes for centrifugation at 10000 rpm at 4°C for 10 minutes. Collect FBS and transfer into the new charcoal sediment prepared and stored on Day-2. Suspend the charcoal sediment and stir this suspension gently in an autoclaved glass container at 4°C, overnight.

[Day-4] Dispense the suspension for centrifugation at 10000 rpm at 4°C for 10 minutes and sterilise the supernatant by filtration through 0.2 μm sterile filter. This DCC treated FBS should be stored at -20°C and can be used for up a year.

ANNEX 3

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

INITIAL CONSIDERATIONS AND LIMITATIONS (See also **GENERAL INTRODUCTION**, page 1)

1. This assay uses the BG1Luc4E2 cell line. It 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) (1). The BG1Luc cell lines predominantly express endogenous ERα and a minor amount of endogenous ERβ (2) (3) (4).

2. This assay 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 12). 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 prioritisation of substances for further testing.

3. 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 (10). However, the activity of firefly luciferase in cell-based assays can be confounded by substances that inhibit the luciferase enzyme, causing both apparent inhibition or increased luminescence due to protein stabilisation (10). 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 (9) (11). 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 (see Annex 2).

4. The "**GENERAL INTRODUCTION**" and "**ER TA TEST METHOD COMPONENTS"** (pages 1-15) should be read before using this test method for regulatory purposes. Definitions and abbreviations used in this TG are described in Annex 1.

PRINCIPLE OF THE TEST METHOD (See also **GENERAL INTRODUCTION**, page 1)

5. 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. The BG1Luc ER TA utilises 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 tumour 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 (8). Criteria for data interpretation are described in detail in paragraph 41. Briefly, a positive response is identified by a concentration-response curve containing at least three points with non-overlapping error bars (mean ± SD), as well as a change in amplitude (normalised 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

6. The stably transfected BG1Luc4E2 cell line should be used for the assay. The cell line is currently only available with a technical licensing agreement from the University of California, Davis, California, USA[3], and from Xenobiotic Detection Systems Inc., Durham, North Carolina, USA[4].

Stability of the Cell Line

7. 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 (see paragraph 9). 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

8. Procedures specified in the Guidance on Good Cell Culture Practice (5) (6) 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.

9. 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.

10. 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 chemicals 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 with 4.5%

[3] Michael S. Denison, Ph.D. Professor, Dept. of Environmental Toxicology, 4241 Meyer Hall, One Shields Ave, University of California, Davis, CA 95616, E: msdenison@ucdavis.edu, (530) 754-8649

[4] Xenobiotic Detection Systems Inc. 1601 East Geer Street, Suite S, Durham NC, 27704 USA, email: info@dioxins.com, Telephone: 919-688-4804, Fax: 919-688-4404

charcoal/dextran-treated FBS, 1.9% L-glutamine, and 0.9% Pen-Strep. All plasticware should be free of estrogenic activity [see detailed protocol (7)].

Acceptability Criteria

11. 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 should be 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 purpose 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 should be discarded and repeated.

Comprehensive Test

It includes acceptability 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 should 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

Vehicle Control (Agonist and Antagonist Assays)

12. The vehicle that is used to dissolve the test chemicals should be tested as a vehicle control. The vehicle used during the validation of the BG1Luc ER TA assay was 1% (v/v) dimethylsulfoxide (DMSO, CASRN 67-68-5) (see paragraph 24). If a vehicle other than DMSO is used, all reference standards, controls, and test chemicals should be tested in the same vehicle, if appropriate.

Reference Standard (Agonist Range Finder)

13. 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)

14. 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)

15. 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)

16. 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)

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

Weak Positive Control (Antagonist)

18. 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)

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

Fold-Induction (Agonist)

20. 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)

21. 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

22. 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 11).

23. Once the historical databases are compiled, the agonist and antagonist proficiency substances, respectively listed in Tables 3 and 4 of "**ER TA TEST METHOD COMPONENTS**" (Page 9-12 of this Test Guideline), should be tested.

Vehicle

24. Test chemicals should be dissolved in a solvent that solubilises the test chemical 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% (v/v). For any vehicle, it should be demonstrated that the maximum volume used is not cytotoxic and does not interfere with the assay performance. Reference standards and controls are dissolved in 100% solvent and then diluted down to appropriate concentrations in EFM.

Preparation of Test chemicals

25. The test chemicals are dissolved in 100% DMSO (or appropriate solvent), and then diluted down to appropriate concentrations in EFM. All test chemicals should be allowed to equilibrate to room temperature before being dissolved and diluted. Test chemical 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 chemicals should be freshly prepared for each experiment and used within 24 hours of preparation.

Solubility and Cytotoxicity: Considerations for Range Finding

26. Range finder testing consists of seven point - 1:10 serial dilutions run in duplicate. Initially, test chemicals 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. Range finder experiments are used to determine the following:

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

27. An assessment of cell viability/cytotoxicity is included in the agonist and antagonist test method protocols (7) 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 (1) was a scaled qualitative visual observation method; however, a quantitative method for the determination of cytotoxicity can be used (see protocol (7)). Data from test chemical concentrations that cause more than 20% reduction in viability cannot be used.

Test chemical Exposure and Assay Plate Organisation

28. Cells are counted and plated into 96-well tissue culture plates (2 x 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. Special considerations will need to be applied to those substances 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 are therefore recommended.

Range Finder Tests

29. Range finder testing uses all wells of the 96-well plate to test up to six test chemicals 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	TC1-1	TC1-1	TC2-1	TC2-1	TC3-1	TC3-1	TC4-1	TC4-1	TC5-1	TC5-1	TC6-1	TC6-1
B	TC1-2	TC1-2	TC2-2	TC2-2	TC3-2	TC3-2	TC4-2	TC4-2	TC5-2	TC5-2	TC6-2	TC6-2
C	TC1-3	TC1-3	TC2-3	TC2-3	TC3-3	TC3-3	TC4-3	TC4-3	TC5-3	TC5-3	TC6-3	TC6-3
D	TC1-4	TC1-4	TC2-4	TC2-4	TC3-4	TC3-4	TC4-4	TC4-4	TC5-4	TC5-4	TC6-4	TC6-4
E	TC1-5	TC1-5	TC2-5	TC2-5	TC3-5	TC3-5	TC4-5	TC4-5	TC5-5	TC5-5	TC6-5	TC6-5
F	TC1-6	TC1-6	TC2-6	TC2-6	TC3-6	TC3-6	TC4-6	TC4-6	TC5-6	TC5-6	TC6-6	TC6-6
G	TC1-7	TC1-7	TC2-7	TC2-7	TC3-7	TC3-7	TC4-7	TC4-7	TC5-7	TC5-7	TC6-7	TC6-7
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); TC1-1 to TC1-7 = concentrations (from high to low) of test chemical 1 (TC1); TC2-1 to TC2-7 = concentrations (from high to low) of test chemical 2 (TC2); TC3-1 to TC3-7 = concentrations (from high to low) of test chemical 3 (TC3); TC4-1 to TC4-7 = concentrations (from high to low) of test chemical 4 (TC4); TC5-1 to TC5-7 = concentrations (from high to low) of test chemical 5 (TC5); TC6-1 to TC6-7 = concentrations (from high to low) of test chemical 6 (TC6); 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	TC1-1	TC1-1	TC2-1	TC2-1	TC3-1	TC3-1	TC4-1	TC4-1	TC5-1	TC5-1	TC6-1	TC6-1
B	TC1-2	TC1-2	TC2-2	TC2-2	TC3-2	TC3-2	TC4-2	TC4-2	TC5-2	TC5-2	TC6-2	TC6-2
C	TC1-3	TC1-3	TC2-3	TC2-3	TC3-3	TC3-3	TC4-3	TC4-3	TC5-3	TC5-3	TC6-3	TC6-3
D	TC1-4	TC1-4	TC2-4	TC2-4	TC3-4	TC3-4	TC4-4	TC4-4	TC5-4	TC5-4	TC6-4	TC6-4
E	TC1-5	TC1-5	TC2-5	TC2-5	TC3-5	TC3-5	TC4-5	TC4-5	TC5-5	TC5-5	TC6-5	TC6-5
F	TC1-6	TC1-6	TC2-6	TC2-6	TC3-6	TC3-6	TC4-6	TC4-6	TC5-6	TC5-6	TC6-6	TC6-6
G	TC1-7	TC1-7	TC2-7	TC2-7	TC3-7	TC3-7	TC4-7	TC4-7	TC5-7	TC5-7	TC6-7	TC6-7
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); TC1-1 to TC1-7 = concentrations (from high to low) of test chemical 1 (TC1); TC2-1 to TC2-7 = concentrations (from high to low) of test chemical 2 (TC2); TC3-1 to TC3-7 = concentrations (from high to low) of test chemical 3 (TC3); TC4-1 to TC4-7 = concentrations (from high to low) of test chemical 4 (TC4); TC5-1 to TC5-7 = concentrations (from high to low) of test chemical 5 (TC5); TC6-1 to TC6-7 = concentrations (from high to low) of test chemical 6 (TC6); VC = vehicle control (DMSO [1% v/v EFM.]).
Note: All test chemicals are tested in the presence of 9.18×10^{-11} M E2.

30. 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.

31. Determination of starting concentrations for comprehensive ***agonist*** testing is described in depth in the agonist protocol (7). Briefly, the following criteria are used:

- If there are no points on the test chemical 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 chemical 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, use a 1:5 dilution.

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

32. Determination of starting concentrations for comprehensive ***antagonist*** testing is described in depth in the antagonist protocol (7). Briefly, the following criteria are used:

- If there are no points on the test chemical 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 chemical 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 (7), Figure 14-2)
  - The lowest cytotoxic concentration (See antagonist protocol (7), 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

33. 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.

Figure 3: Agonist Comprehensive Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
B	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
C	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
D	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	VC
E	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	Meth
F	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	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: TC11-1 to TC1-11 = concentrations (from high to low) of test chemical 1; TC2-1 to TC2-11 = concentrations (from high to low) of test chemical 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	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
B	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
C	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
D	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	VC
E	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	Tam
F	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	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; TC1-1 to TC1-11 = concentrations (from high to low) of test chemical 1 (TC1); TC2-1 to TC2-11 = concentrations (from high to low) of test chemical 2 (TC2); 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)

34. 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.

Measure of Luminescence

35. 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 (7). Light emission from each well is expressed as RLU per well.

ANALYSIS OF DATA

EC_{50} /IC_{50} determination

36. The EC_{50} value (half maximal effective concentration of a test chemical [agonists]) and the IC_{50} value (half maximal inhibitory concentration of a test chemical [antagonists]) are determined from the concentration-response data. For test chemicals that are positive at one or more concentrations, the concentration of test chemical 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 test chemical concentration to the response (typically following a sigmoidal curve) using the equation below:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\lg EC_{50} - X)\text{HillSlope}}}$$

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^{R} statistical software).

Determination of Outliers

37. Good statistical judgment could be facilitated by including (but not limited to) the Q-test (see agonist and antagonist protocols (7) for determining "unusable" wells that will be excluded from the data analysis.

38. 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

39. Raw data from the luminometer should be 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 should be 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 normalise 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 chemicals.

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 normalise 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 chemicals.

Collection and Adjustment of Luminometer Data for Comprehensive Testing

40. Raw data from the luminometer should be 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 VC.
Step 2 Subtract the mean value of the DMSO VC from each well value to normalise 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 chemicals.
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 normalise 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 chemicals.
Step 5 Calculate the mean adjusted RLU value for tamoxifen.
Step 6 Calculate the mean value for the E2 reference standard.

Data Interpretation Criteria

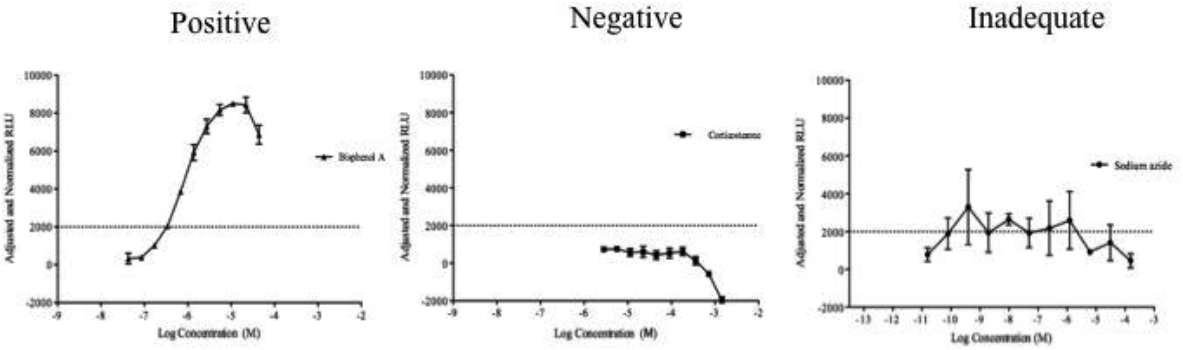
41. The BG1Luc ER TA is intended as part of a weight of evidence approach to help prioritise substances for ED testing *in vivo*. Part of this prioritisation procedure will be the classification of the test chemical as positive or negative for either ER agonist or antagonist activity. The positive and negative decision criteria used in the BG1Luc ER TA validation study are described in Table 1.

Table 1: **Positive and Negative Decision Criteria**

AGONIST ACTIVITY	
Positive	• All test chemicals 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 test chemical.
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 chemical is positive or negative. Chemicals should be retested.
ANTAGONIST ACTIVITY	
Positive	• Test chemical 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 chemical should be less than or equal to $1x10^{-5}$ M. • If possible, an IC_{50} value should be calculated for each positive test chemical.
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 chemical is positive or negative. Chemical should be retested.

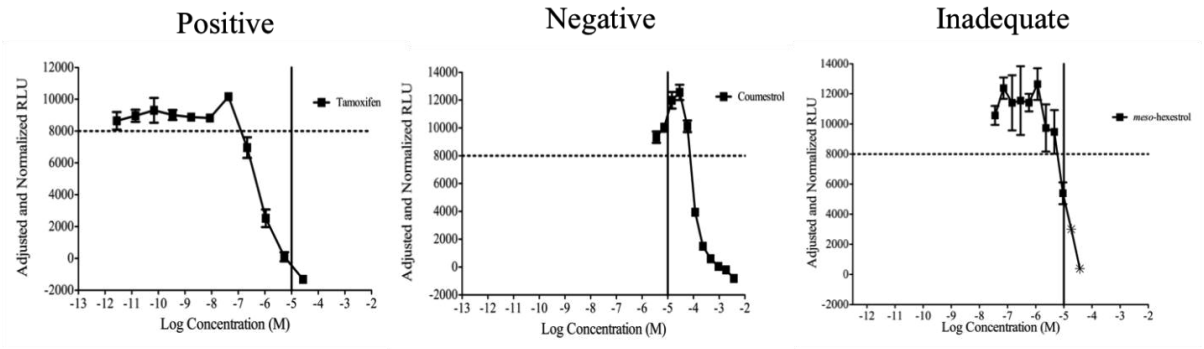
42. Positive results will be characterised by both the magnitude of the effect and the concentration at which the effect occurs, where possible. Examples of positive, negative and inadequate data are shown in Figures 5 and 6.

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



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

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



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

Asterisked 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.

43. The calculations of EC_{50} and IC_{50} can be made using a four-parameter Hill Function (see agonist protocol and antagonist protocol for more details (7)). Meeting the acceptability criteria indicates the 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

44. See paragraph 20 of "**ER TA TEST METHOD COMPONENTS**" (Page 14-15 of this Test Guideline)

LITERATURE (3)

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(2) Monje P., Boland R. (2001). Subcellular Distribution of Native Estrogen Receptor α and β Isoforms in Rabbit Uterus and Ovary, J. Cell Biochem., 82(3): 467-479.

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(9) Escande A., et al. (2006). Evaluation of Ligand Selectivity Using Reporter Cell Lines Stably Expressing Estrogen Receptor Alpha or Beta, Biochem. *Pharmacol*., 71(10):1459-69.

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