

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW TEST GUIDELINE

BG1Luc Estrogen Receptor Transcriptional Activation Test Method for Identifying Estrogen Receptor Agonists and Antagonists

INTRODUCTION

1. In 1998, the Organisation for Economic Co-operation and Development (OECD) initiated the revision of existing and the development of new Test Guidelines for the screening and testing of Endocrine Disrupting Chemicals. Since that time, several potential assays have been developed into Test Guidelines (TG), with additional assays still under development. These assays are contained within the “OECD Conceptual Framework for the Screening and Testing of Endocrine Disrupting Chemicals” (CF), which was revised in 2011 by the OECD Endocrine Disruptors Testing and Assessment Task Force. The original and revised CFs are included as Annexes in the Draft Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption [1]. The revised CF comprises five levels, each level corresponding to a difference level of biological complexity [1]. The BG1Luc Estrogen Receptor Transcriptional Activation (BG1Luc ER TA) Test Method for Identifying Estrogen Receptor Agonists and Antagonists is proposed for inclusion in level 2, which includes "*in vitro assays providing data about selected endocrine mechanism(s)/pathway(s) (Mammalian and non mammalian methods)*" [1].

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

3. There are at least two subtypes of nuclear ERs, termed α and β , which are encoded by distinct genes and with different tissue distributions, relative ligand binding affinities and biological functions [9-11]. Nuclear ER α mediates the classic estrogenic response [12-15], and therefore most models currently being developed to measure ER activation are specific to ER α . The BG1Luc ER TA test method offers the advantage of evaluating both ER α and ER β ligand mediated responses. This method is being proposed for screening and prioritisation purposes, but can also provide mechanistic information that can be used in a weight of evidence approach.

4. The BG1Luc ER TA test method, which has been validated by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [16], utilizes an ER responsive reporter gene (*luc*) in the human ovarian adenocarcinoma cell line, BG-1, to provide concentration-response data for substances with *in vitro* ER agonist or antagonist activity. BG-1 cells endogenously express both ER α and ER β .

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

INITIAL CONSIDERATIONS AND LIMITATIONS

6. The interaction of estrogens with ERs can affect transcription of estrogen-controlled genes, which could lead to the initiation or inhibition of cellular processes, including those necessary for cell proliferation, normal fetal development, and adult homeostasis [17-19]. Perturbation of normal estrogenic systems may have the potential to trigger adverse health effects.

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

8. This TG is applicable to a wide range of substances, provided they can be dissolved in dimethyl sulfoxide (DMSO; CASRN 67-68-5), do not react with DMSO or the cell culture medium, and are not cytotoxic. The demonstrated performance of the BG1Luc ER TA agonist test method suggests that data generated with this test method could be routinely considered for prioritization of substances for further testing.

PRINCIPLE OF THE TEST

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

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

11. Criteria for data interpretation are described in detail in paragraph #52. Briefly, a positive response is identified by a concentration-response curve containing at least three points with nonoverlapping error bars (mean \pm SD), as well as a change in amplitude of at least 20% of the maximal value for the reference substance (17 β -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

12. BG1Luc4E2 Cells: Human ovarian cancer cell line stably transfected with a plasmid containing an estrogen response element, pGudLuc7.0. The cell line is available with a technical licensing agreement from the University of California, Davis, California, USA, and from Xenobiotic Detection Systems Inc., Durham, North Carolina, USA.

Stability of the Cell Line

13. To maintain the stability and integrity of the cell line, 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

14. BG1Luc4E2 cells are based on a continuous ovarian carcinoma cell line (BG-1 cells) that endogenously express ER α and ER β and have been stably transfected with an ER responsive reporter gene (*luc*). Procedures specified in the Guidance on Good Cell Culture Practice [20, 21] 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.

15. 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 37°C \pm 1°C, 90% \pm 5% humidity, and 5.0% \pm 1% CO₂/air.

16. Upon reaching ~80% confluence, BG1Luc4E2 cells are subcultured and conditioned to an estrogen-free environment for 48 hours prior to plating the cells in 96-well plates for exposure to test substances and analysis of estrogen dependent induction of luciferase activity. The estrogen-free medium (EFM) contains Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 4.5% charcoal/dextran-treated FBS, 1.9% L-glutamine, and 0.9% Pen-Strep. All plasticware should be free of estrogenic activity (**Appendices 2 and 3**).

Acceptability Criteria

17. 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. 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.

Agonist Test

Range Finder Test

- Induction: Plate induction is measured by dividing the average highest E2 reference standard relative light unit (RLU) value by the average DMSO control RLU value, and must be greater than three-fold.

- DMSO control results: Solvent control RLU values must be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- An experiment that fails either acceptance criterion will be discarded and repeated.

Comprehensive Test

Includes acceptance criteria from the agonist range finder test and the following:

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

Antagonist Test

Range Finder Test

- Reduction: Plate reduction is measured by dividing the average highest Ral/E2 reference standard RLU value by the average DMSO control RLU value, and must be greater than three-fold.
- E2 control results: E2 control RLU values must be within 2.5 times the standard deviation of the historical E2 control mean RLU value.
- DMSO control results: DMSO control RLU values must be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- An experiment that fails either acceptance criterion will be discarded and repeated.

Comprehensive Test

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 must 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)

18. The vehicle control is a 1% (v/v) DMSO diluted in EFM.

Reference Standard (Agonist Range Finder)

19. The reference standard is E2 and for range finder testing, is comprised of four concentrations of E2 (**Table 1**), with each concentration tested in duplicate wells.

Table 1 Agonist Range Finder Reference Standard Concentrations

| | Concentration (µg/mL) | Concentration (M) |
|----------|-----------------------|------------------------|
| 1 | 5.00×10^{-5} | 1.84×10^{-10} |
| 2 | 1.25×10^{-5} | 4.59×10^{-11} |
| 3 | 3.13×10^{-6} | 1.15×10^{-11} |
| 4 | 7.83×10^{-7} | 2.87×10^{-12} |

Abbreviations: µg/mL = micrograms per milliliter; M = Molar

Reference Standard (Agonist Comprehensive)

20. E2 for comprehensive testing is comprised of a serial dilution consisting of 11 concentrations (**Table 2**) of E2 in duplicate wells.

Table 2 Agonist Comprehensive Reference Standard Concentrations

| | Concentration (µg/mL) | Concentration (M) |
|-----------|-----------------------|------------------------|
| 1 | 1.00×10^{-4} | 3.67×10^{-10} |
| 2 | 5.00×10^{-5} | 1.84×10^{-10} |
| 3 | 2.50×10^{-5} | 9.18×10^{-11} |
| 4 | 1.25×10^{-5} | 4.59×10^{-11} |
| 5 | 6.25×10^{-6} | 2.29×10^{-11} |
| 6 | 3.13×10^{-6} | 1.15×10^{-11} |
| 7 | 1.56×10^{-6} | 5.73×10^{-12} |
| 8 | 7.83×10^{-7} | 2.87×10^{-12} |
| 9 | 3.92×10^{-7} | 1.44×10^{-12} |
| 10 | 1.95×10^{-7} | 7.16×10^{-13} |
| 11 | 9.78×10^{-8} | 3.59×10^{-13} |

Abbreviations: µg/mL = micrograms per milliliter; M = Molar

Reference Standard (Antagonist Range Finder)

21. The reference standard is a combination of Ral and E2. Ral/E2 for range finder testing is comprised of three concentrations of Ral (**Table 3**) plus a fixed concentration (2.5×10^{-5} µg/mL [9.18×10^{-11} M]) of E2 in duplicate wells.

Table 3 Antagonist Range Finder Reference Standard Concentrations

| | Concentration (µg/mL) | Concentration (M) |
|----------|-----------------------|------------------------|
| 1 | 1.56×10^{-3} | 3.06×10^{-9} |
| 2 | 3.91×10^{-4} | 7.67×10^{-10} |
| 3 | 9.77×10^{-5} | 1.92×10^{-10} |

Abbreviations: µg/mL = micrograms per milliliter; M = Molar

Reference Standard (Antagonist Comprehensive)

22. Ral/E2 for comprehensive testing is comprised of a serial dilution of Ral plus a fixed concentration (2.5×10^{-5} µg/mL [9.18×10^{-11} M]) of E2 consisting of nine concentrations of Ral/E2 in duplicate wells (**Table 4**).

Table 4 Antagonist Comprehensive Reference Standard Concentrations

| | Concentration (µg/mL) | Concentration (M) |
|----------|-----------------------|------------------------|
| 1 | 1.25×10^{-2} | 2.45×10^{-8} |
| 2 | 6.25×10^{-3} | 1.23×10^{-8} |
| 3 | 3.13×10^{-3} | 6.14×10^{-9} |
| 4 | 1.56×10^{-3} | 3.06×10^{-9} |
| 5 | 7.81×10^{-4} | 1.53×10^{-9} |
| 6 | 3.91×10^{-4} | 7.67×10^{-10} |
| 7 | 1.95×10^{-4} | 3.82×10^{-10} |
| 8 | 9.77×10^{-5} | 1.92×10^{-10} |
| 9 | 4.88×10^{-5} | 9.57×10^{-11} |

Abbreviations: µg/mL = micrograms per milliliter; M = Molar

Weak Positive Control (Agonist)

23. The weak positive control is 3.13 µg/mL (9.06×10^{-6} M) *p,p'*-methoxychlor (methoxychlor; CASRN 72-43-5) in EFM.

Weak Positive Control (Antagonist)

24. The weak positive control consists of tamoxifen (CASRN 10540-29-1) 1.26 µg/mL (3.36×10^{-6} M) with 2.5×10^{-5} µg/mL (9.18×10^{-11} M) E2 in EFM.

E2 Control (Antagonist Assay Only)

25. The E2 control is 2.5×10^{-5} $\mu\text{g/mL}$ (9.18×10^{-11} M) E2 in EFM and used as a base line negative control.

Fold-Induction (Agonist)

26. 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 must be greater than three-fold.

Fold-Reduction (Antagonist)

27. 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 must be greater than three-fold.

Demonstration of Laboratory Proficiency

28. 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 separate agonist and 10 separate antagonist experiments, conducted on different days.

29. Once the historical databases are compiled, the agonist and antagonist proficiency substances listed in **Tables 5** and **6**, respectively, should be tested. For each test substance, starting concentrations should first be selected based on range finder test results, and then at least two comprehensive tests conducted. Proficiency is demonstrated by correct classification of each proficiency substance. Proficiency testing should be repeated by each technician learning the test methods, but does not have to be repeated by laboratory personnel more than once.

Table 5 Agonist Substances for Demonstration of Laboratory Proficiency

| Substance | CASRN | Expected Response ^a | BG1Luc ER TA Mean EC ₅₀ (M) ^{b,c} | MeSH Chemical Class ^d | Product Class ^e |
|-----------------------|----------|--------------------------------|---|---------------------------------------|---|
| Ethyl paraben | 120-47-8 | POS | 2.48×10^{-5} | Carboxylic Acid, Phenol | Pharmaceutical, Preservative |
| Kaempferol | 520-18-3 | POS | 3.99×10^{-6} | Flavonoid, Heterocyclic Compound | Natural Product |
| Butylbenzyl phthalate | 85-68-7 | POS | 1.98×10^{-6} | Carboxylic Acid, Ester, Phthalic Acid | Plasticizer, Industrial Chemical |
| Apigenin | 520-36-5 | POS | 1.85×10^{-6} | Heterocyclic Compound | Dye, Natural Product, Pharmaceutical Intermediate |
| Daidzein | 486-66-8 | POS | 8.71×10^{-7} | Flavonoid, Heterocyclic Compound | Natural Product |

| Substance | CASRN | Expected Response ^a | BG1Luc ER TA Mean EC ₅₀ (M) ^{b,c} | MeSH Chemical Class ^d | Product Class ^e |
|--------------------------------|-----------|--------------------------------|---|----------------------------------|---|
| Bisphenol A | 80-05-7 | POS | 5.33×10^{-7} | Phenol | Chemical Intermediate, Flame Retardant, Fungicide |
| Genistein | 446-72-0 | POS | 2.71×10^{-7} | Flavonoid, Heterocyclic Compound | Natural Product, Pharmaceutical |
| Coumestrol | 479-13-0 | POS | 8.77×10^{-8} | Heterocyclic Compound | Natural Product |
| 17 α -Estradiol | 57-91-0 | POS | 1.54×10^{-9} | Steroid | Pharmaceutical, Veterinary Agent |
| Estrone | 53-16-7 | POS | 2.57×10^{-10} | Steroid | Pharmaceutical, Veterinary Agent |
| Diethylstilbestrol | 56-53-1 | POS | 3.34×10^{-11} | Hydrocarbon (Cyclic) | Pharmaceutical, Veterinary Agent |
| 17 α -Ethinyl estradiol | 57-63-6 | POS | 7.31×10^{-12} | Steroid | Pharmaceutical, Veterinary Agent |
| Atrazine | 1912-24-9 | NEG | - | Heterocyclic Compound | Herbicide |
| Corticosterone | 50-22-6 | NEG | - | Steroid | Pharmaceutical |
| Linuron | 330-55-2 | NEG | - | Urea | Herbicide |
| Spirolactone | 52-01-7 | NEG | - | Lactone, Steroid | Pharmaceutical |

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; EC₅₀ = half maximal effective concentration of a test substance; MeSH = U.S. National Library of Medicine's Medical Subject Headings; NEG = negative; POS = positive.

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

^bMean EC₅₀ calculated from values reported by the laboratories of the BG1Luc ER TA validation study [22].

^cTable is sorted in the order of expected EC₅₀ (M) of response in the BG1Luc assay.

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

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

Table 6 Antagonist Substances for Demonstration of Laboratory Proficiency

| Substance | CASRN | Expected Response ^a | BG1Luc ER TA Mean IC ₅₀ (M) ^{b,c} | MeSH Chemical Class ^d | Product Class ^e |
|--------------------|------------|--------------------------------|---|----------------------------------|----------------------------|
| Tamoxifen | 10540-29-1 | POS | 8.17×10^{-7} | Hydrocarbon (Cyclic) | Pharmaceutical |
| 4-Hydroxytamoxifen | 68047-06-3 | POS | 2.08×10^{-7} | Hydrocarbon (Cyclic) | Pharmaceutical |

| | | | | | |
|---------------------------------|------------|-----|-----------------------|----------------------------------|---|
| Raloxifene HCl | 82640-04-8 | POS | 1.19×10^{-9} | Hydrocarbon (Cyclic) | Pharmaceutical |
| 17 α - Ethinyl estradiol | 57-63-6 | NEG | - | Steroid | Pharmaceutical, Veterinary Agent |
| Apigenin | 520-36-5 | NEG | - | Heterocyclic Compound | Dye, Natural Product, Pharmaceutical Intermediate |
| Chrysin | 480-40-0 | NEG | - | Flavonoid, Heterocyclic Compound | Natural Product |
| Coumestrol | 479-13-0 | NEG | - | Heterocyclic Compound | Natural Product |
| Genistein | 446-72-0 | NEG | - | Flavonoid, Heterocyclic Compound | Natural Product, Pharmaceutical |
| Kaempferol | 520-18-3 | NEG | - | Flavonoid, Heterocyclic Compound | Natural Product |
| Resveratrol | 501-36-0 | NEG | - | Hydrocarbon (Cyclic) | Natural Product |

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; IC₅₀ = half maximal inhibitory concentration; MeSH = U.S. National Library of Medicine's Medical Subject Headings; NEG = negative; POS = positive.

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

^bMean IC₅₀ calculated from values reported by the laboratories of the BG1Luc ER TA validation study.

^cTable is sorted in the order of expected IC₅₀ (M) of response in the BG1Luc assay.

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

^eSubstances 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>)

Vehicle

30. The vehicle is DMSO. Test substances are dissolved in 100% DMSO and then diluted to 1% v/v in EFM.

31. 1% v/v DMSO is used as the solvent for the positive and negative controls, and is also used as the concurrent vehicle control.

Preparation of Test Substances

32. The solvent used for dissolution of test substances is 100% DMSO. All test substances should be allowed to equilibrate to room temperature before being dissolved and diluted. Test substance solutions (except for reference standards and controls) should not be prepared in bulk for use in subsequent tests. Test substances are to be used within 24 hours of preparation. Solutions should not have noticeable precipitate or cloudiness.

Solubility and Cytotoxicity: Considerations for Range Finding

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

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

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

35. An assessment of cell viability/cytotoxicity is included in the agonist and antagonist test method protocols and is incorporated into range finder and comprehensive testing. The cytotoxicity method that was used to assess cell viability during the validation of the BG1Luc ER TA [16] was a qualitative visual observation method that assesses viability on a scale of 1 (normal) to 4 (significant loss of viability; **Table 7**).

Table 7 Visual Observation Scoring Table for Cell Viability

| Viability Score | Brief Description |
|------------------------|---|
| 1 | Normal Cell Morphology and Cell Density |
| 2 | Altered Cell Morphology and/or Small Gaps between Cells |
| 3 | Altered Cell Morphology and/or Large Gaps between Cells |
| 4 | Few (or no) Visible Cells |
| P | Wells containing precipitation are to be noted with “P” |

36. Test substance concentrations with a viability score of 2 or greater should be excluded from data evaluation.

37. If desired, a more quantitative method for the determination of cytotoxicity can be used.

Test Substance Exposure and Assay Plate Organization

38. Cells are counted and plated into 96-well tissue culture plates (2 x 10⁵ 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 and incubated for 19-24 hours.

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

Range Finder Tests

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

- *Agonist* range finder testing uses four concentrations of E2 (**Table 1**) in duplicate as the reference standard and four replicate wells for the DMSO control.
- *Antagonist* range finder testing uses three concentrations of Ral/E2 (**Table 3**) with 2.50×10^{-5} $\mu\text{g/mL}$ (9.18×10^{-11} M) E2 in duplicate as the reference standard, with three replicate wells for the E2 and DMSO controls.

Figure 1 Agonist Range Finder Test 96-well Plate Layout

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

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

Figure 2 Antagonist Range Finder Test 96-well Plate Layout

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

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

41. The recommended final volume of media required for each well is 200 μL . Only use test plates in which the cells in all wells give a viability score of 1 according to **Table 7**.

42. To determine starting concentrations for comprehensive *agonist* testing use the following criteria:

- If results in the range finder test suggest that the test substance is negative for agonist activity (i.e., if there are no points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control, see **APPENDIX 2, Figure 12-2**), comprehensive testing will be conducted using an 11-point 1:2 serial dilution starting at the maximum soluble concentration.
- If results in the range finder test suggest that the test substance is negative for agonist activity (i.e., if there are no points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control), and the higher concentrations in the range finder are cytotoxic, comprehensive testing will be conducted using an 11-point 1:2 serial dilution with the lowest cytotoxic concentration as the starting concentration (see **APPENDIX 2, Figure 12-3**).
- If results in the range finder test suggest that the test substance is positive for agonist activity (i.e., if there are points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control), the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one log higher than the concentration giving the highest adjusted RLU value in the range finder. The 11-point dilution scheme will be based on either 1:2 or 1:5 dilutions according to the following criteria:
 - An 11-point 1:2 serial dilution should be used if the resulting concentration range (note: an 11-point 1:2 serial dilution will cover a range of concentrations over approximately three orders of magnitude [three logs]) will encompass the full range of responses based on the concentration response curve generated in the range finder test (see **APPENDIX 2, Figure 12-4**).
 - If the concentration range that would be generated with the 1:2 serial dilution will not encompass the full range of responses based on the concentration response curve in the range finder test (see **APPENDIX 2, Figures 12-5 and 12-6**), an 11-point 1:5 serial dilution should be used instead.
- If a substance exhibits a biphasic concentration response curve in the range finder test, both phases should also be resolved in comprehensive testing. In order to resolve both curves, the starting concentration should be based on the peak associated with the higher concentration and should be one log higher than the concentration giving the highest adjusted RLU value in the range finder. As an example, an 11-point 1:5 serial dilution should be used based on the range finder results presented in **APPENDIX 2, Figure 12-7**.

43. To determine starting concentrations for comprehensive *antagonist* testing use the following criteria:

- If results in the range finder test suggest that the test substance is negative for antagonist activity (i.e., if there are no points on the test substance concentration curve that are less than the mean minus three times the standard deviation of the E2 control, see **APPENDIX 3, Figure 14-2**), comprehensive testing will be conducted using an 11-point 1:2 serial dilution using the maximum soluble concentration of test substance as the with the limit dose as the starting concentration.

- If results in the range finder test suggest that the test substance is negative for agonist activity (i.e., if there are no points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control), and the higher concentrations in the range finder are cytotoxic, comprehensive testing will be conducted using an 11-point 1:2 serial dilution with the lowest cytotoxic concentration as the starting concentration (see **APPENDIX 3, Figure 14-3**).
- If results in the range finder test suggest that the test substance is positive for antagonist activity (i.e., if there are points on the test substance concentration curve that are less than the mean minus three times the standard deviation of the E2 control), the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one of the following:
 - The concentration giving the lowest adjusted RLU value in the range finder
 - The maximum soluble concentration (See **APPENDIX 3, Figure 14-2**)
 - The lowest cytotoxic concentration (See **APPENDIX 3, 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 (note: an 11-point 1:2 serial dilution will cover a range of concentrations over approximately three orders of magnitude [three logs]) will encompass the full range of responses based on the concentration response curve generated in the range finder test (see **APPENDIX 3, Figure 14-4**).
 - If the concentration range that would be generated with the 1:2 serial dilution will not encompass the full range of responses based on the concentration response curve in the range finder test (see **APPENDIX 3, Figure 14-5**), an 11-point 1:5 serial dilution should be used instead.
- If a substance exhibits a biphasic concentration response curve in the range finder test (see **APPENDIX 3, Figure 14-6**), both phases should also be resolved in comprehensive testing. In this case, two peaks could potentially be used to identify the top concentration to be used for the 11-point dilution scheme in comprehensive testing. In order to resolve both curves, the top concentration should be based on the peak associated with the higher concentration and the top dose one log concentration higher than the concentration giving the lowest adjusted RLU value in the range finder. An 11-point 1:5 serial dilution should be used.

Comprehensive Tests

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

- *Agonist* comprehensive testing uses 11 concentrations of E2 (**Table 2**) in duplicate as the reference standard. Four replicate wells for the DMSO control and three replicate wells for the methoxychlor control (3.13 µg/mL) are included on each plate.
- *Antagonist* comprehensive testing uses nine concentrations of Ral/E2 (**Table 4**) with 2.50×10^{-5} µg/mL (9.18×10^{-11} M) E2 in duplicate as the reference standard, with

three replicate wells for the E2 2.50×10^{-5} $\mu\text{g/mL}$ (9.18×10^{-11} M) control, three replicate wells for DMSO controls, and four replicate wells for tamoxifen $1.26 \mu\text{g/mL}$ (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 | TS1-1 | TS1-2 | TS1-3 | TS1-4 | TS1-5 | TS1-6 | TS1-7 | TS1-8 | TS1-9 | TS1-10 | TS1-11 | VC |
| B | TS1-1 | TS1-2 | TS1-3 | TS1-4 | TS1-5 | TS1-6 | TS1-7 | TS1-8 | TS1-9 | TS1-10 | TS1-11 | VC |
| C | TS1-1 | TS1-2 | TS1-3 | TS1-4 | TS1-5 | TS1-6 | TS1-7 | TS1-8 | TS1-9 | TS1-10 | TS1-11 | VC |
| D | TS2-1 | TS2-2 | TS2-3 | TS2-4 | TS2-5 | TS2-6 | TS2-7 | TS2-8 | TS2-9 | TS2-10 | TS2-11 | VC |
| E | TS2-1 | TS2-2 | TS2-3 | TS2-4 | TS2-5 | TS2-6 | TS2-7 | TS2-8 | TS2-9 | TS2-10 | TS2-11 | Meth |
| F | TS2-1 | TS2-2 | TS2-3 | TS2-4 | TS2-5 | TS2-6 | TS2-7 | TS2-8 | TS2-9 | TS2-10 | TS2-11 | Meth |
| G | E2-1 | E2-2 | E2-3 | E2-4 | E2-5 | E2-6 | E2-7 | E2-8 | E2-9 | E2-10 | E2-11 | Meth |
| H | E2-1 | E2-2 | E2-3 | E2-4 | E2-5 | E2-6 | E2-7 | E2-8 | E2-9 | E2-10 | E2-11 | Meth |

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

Figure 4 Antagonist Comprehensive Test 96-well Plate Layout

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

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

Note: As noted, all reference and test wells contain a fixed concentration of E2 (4.90×10^{-11} M)

45. 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

46. 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. Light emission from each well is expressed as RLU per well.

ANALYSIS OF DATA

EC₅₀/IC₅₀ Determination

47. The EC₅₀ value (half maximal effective concentration of a test substance [agonists]) and the IC₅₀ value (half maximal inhibitory concentration of a test substance [antagonists]) are determined from the concentration-response data. For substances that are positive at one or more concentrations, the concentration of test substance that causes a half-maximal response (IC₅₀ or EC₅₀) is calculated using a Hill function analysis. The Hill function is a four-parameter logistic mathematical model relating the substance concentration to the response (typically following a sigmoidal curve) using the equation below:

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

where Y = response (i.e., RLUs); X = the logarithm of concentration; Bottom = the minimum response; Top = the maximum response; log IC₅₀ (or log EC₅₀) = the logarithm of X as the response midway between Top and Bottom; and Hill slope describes the steepness of the curve. The model calculates the best fit for the Top, Bottom, Hill slope, and IC₅₀ and EC₅₀ parameters.

Determination of Outliers

48. The Study Director will use good statistical judgment for determining “unusable” wells that will be excluded from the data analysis.

49. 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

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

Agonist

- Step 1 Calculate mean value for the DMSO vehicle control (VC).
- Step 2 Subtract the mean value of the DMSO VC from each well value to normalize the data.
- Step 3 Calculate the mean fold induction for the reference standard (E2).
- Step 4 Calculate the mean EC₅₀ value for the test substances.

Antagonist

- Step 1 Calculate mean value for the DMSO VC.
- Step 2 Subtract the mean value of the DMSO VC from each well value to normalize the data.
- Step 3 Calculate the mean fold reduction for the reference standard (Ral/E2).
- Step 4 Calculate mean value for the E2 reference standard.
- Step 5 Calculate the mean IC₅₀ value for the test substances.

Collection and Adjustment of Luminometer Data for Comprehensive Testing

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

Agonist

- Step 1 Calculate mean value for the DMSO VC.
- Step 2 Subtract the mean value of the DMSO VC from each well value to normalize the data.
- Step 3 Calculate the mean fold induction for the reference standard (E2).
- Step 4 Calculate the mean EC₅₀ value for E2 and the test substances.
- Step 5 Calculate the mean adjusted RLU value for methoxychlor.

Antagonist

- Step 1 Calculate mean value for the DMSO VC.
- Step 2 Subtract the mean value of the DMSO VC from each well value to normalize the data.
- Step 3 Calculate the mean fold induction for the reference standard (Ral/E2).
- Step 4 Calculate the mean IC₅₀ value for Ral/E2 and the test substances.
- Step 5 Calculate the mean adjusted RLU value for tamoxifen.
- Step 6 Calculate mean value for the E2 reference standard.

Data Interpretation Criteria

52. The BG1Luc ER TA is intended as part of a weight of evidence approach to help prioritize substances for ED testing *in vivo*. Part of this prioritization procedure will be the classification of the test substance as positive or negative for either ER agonist or antagonist activity. The classification system used in the BG1Luc ER TA validation study is described in **Table 8**.

Table 8 Positive and Negative Decision Criteria

| AGONIST ACTIVITY | |
|----------------------------|--|
| Positive | <ul style="list-style-type: none"> – All test substances classified as positive for ER agonist activity should have a concentration–response curve consisting of a baseline, followed by a positive slope, and concluding in a plateau or peak. In some cases, only two of these characteristics (baseline–slope or slope–peak) may be defined. – The line defining the positive slope must contain at least three points with nonoverlapping error bars (mean ± SD). Points forming the baseline are excluded, but the linear portion of the curve may include the peak or first point of the plateau. – A positive classification requires a response amplitude, the difference between baseline and peak, of at least 20% of the maximal value for the reference estrogen (i.e., 2000 RLUs when the maximal response value of the reference estrogen is adjusted to 10,000 RLUs). – If possible, an EC₅₀ value should be calculated for each positive substance. |
| Negative | The average adjusted RLU for a given concentration is at or below the mean DMSO control RLU value plus three times its standard deviation. |
| Inadequate | Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test substance is positive or negative. |
| ANTAGONIST ACTIVITY | |
| Positive | <ul style="list-style-type: none"> – Test substance data produce a concentration-response curve consisting of a baseline, which is followed by a negative slope. – The line defining the negative slope must 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 a response amplitude, the difference between baseline and bottom, of at least 80% of the maximal value for the reference estrogen (i.e., 8000 RLU when the maximal response value of the reference estrogen is adjusted to 10,000 RLUs). – The highest non-cytotoxic concentrations of the test substance should be less than or equal to 1x10⁻⁵ M. |
| Negative | All data points are above the ED ₈₀ value (80% of the E ₂ response, or 8000 RLUs) |
| Inadequate | Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test substance is positive or negative. |

53. Data interpretation criteria are shown in **Table 8**. Positive results will be characterized by both the magnitude of the effect and the concentration at which the effect occurs, where possible.

54. The calculations of EC₅₀ and IC₅₀ can be made using a-parameter Hill Function (**Appendix 1** and **Appendix 2**).

Meeting the performance standards indicates the assay system is operating properly, but it does not ensure that any particular run will produce accurate data. Duplicating the results of the first run is the best assurance that accurate data were produced.

Test Report

55. The test report should contain the following information:

Test substance and control test substance:

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

Solvent/vehicle:

- identification data (purity; concentration, where appropriate; volume used);
- characterization (physical nature, supplier, and lot number);
- solubility and stability of test substance in solvent/vehicle, if known;
- justification for choice of solvent/vehicle.

Cells:

- type and source of cells;
- number of cell passages;
- methods for maintenance of cell cultures.

Test conditions:

- cytotoxicity data and solubility limitations;
- composition of media;
- concentration of test substance;
- volume of vehicle and test substance added;
- incubation temperature, humidity, and CO₂ concentration;
- duration of treatment;
- cell density during treatment;
- positive and negative reference chemicals;
- criteria for considering tests as positive, negative or equivocal.

Reliability check:

- Fold inductions for each assay plate.
- Actual logEC₅₀, logIC₅₀, and Hillslope values for concurrent reference chemicals.

Results:

- Raw and normalised data of luminescent signals;
- Concentration-response relationship, where possible;
- IC₅₀/EC₅₀ values, if appropriate;
- Statistical analyses, if any, together with a measure of error (*e.g.*, SEM, SD, CV or 95% CI) and a description of how these values were obtained.

Discussion of results

Conclusion

LITERATURE

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APPENDIX 1

DEFINITIONS AND ABBREVIATIONS

Acceptance criteria: Minimum standards for the performance of experimental controls and reference standards. All acceptance criteria must be met for an experiment to be considered valid.

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

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

Androgen: A class of steroid hormone, which includes testosterone and 5 α -dihydrotestosterone, responsible for the development and maintenance of the male reproductive system.

Androgen receptor: The receptor to which androgens bind.

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

BG-1: An immortalized adenocarcinoma cells that endogenously express estrogen receptor.

BG-1Luc4E2: The BG-1Luc4E2 cell line was derived from BG-1 immortalized adenocarcinoma cells that endogenously express both forms of the estrogen receptor (ER α and ER β) 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 cellular morphology.

CF: The OECD Conceptual Framework for the Screening and Testing of Endocrine Disrupting Chemicals.

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

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

DMEM: Dulbecco’s Modification of Eagle’s Medium

DMSO: Dimethyl sulfoxide

E2: 17 β -estradiol

EC₅₀: The half maximal effective concentration of a test substance.

EE: 17 α -ethynyl estradiol

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

ER: Estrogen receptor

ERE: Estrogen response element

FBS: Fetal bovine serum

hER α : Human estrogen receptor alpha

hER β : Human estrogen receptor beta

IC₅₀: The half maximal effective concentration of an inhibitory test substance.

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

Interlaboratory reproducibility: A measure of whether different qualified laboratories using the same protocol and test substances can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the validation process and indicates the extent to which a test method can be transferred successfully among laboratories.

Intralaboratory repeatability: The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period.

Intralaboratory reproducibility: The first stage of validation; a determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times.

MMTV: Mouse Mammary Tumor Virus

OECD: The Organisation for Economic Co-operation and Development

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

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

Ral: raloxifene HCl

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

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

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

RLU: Relative Light Units

RNA: Ribonucleic Acid

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

SD: Standard deviation

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

TG: Test Guideline

Transcription: mRNA synthesis

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

Validated test method: An accepted test method for which validation studies have been completed to determine the accuracy and reliability of the method for a specific proposed use.

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

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