OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW TEST GUIDELINE

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

INTRODUCTION

1. In 1998, the Organisation for Economic Co-operation and Development (OECD) initiated the revision of existing and the development of new Test Guidelines for the screening and testing of Endocrine Disrupting Chemicals. Since that time, several potential assays have been developed into Test Guidelines (TG), with additional assays still under development. These assays are contained within the "OECD Conceptual Framework for the Screening and Testing of Endocrine Disrupting Chemicals" (CF), which was revised in 2011. The original and revised CFs are included as Annexes in the Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (1). The revised CF comprises five levels, each level corresponding to a difference level of biological complexity (1). The BG1Luc Estrogen Receptor Transactivation (BG1Luc ER TA) Test Method for Identifying Estrogen Receptor Agonists and Antagonists is 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 transactivation. TA assays using activation of reporter genes are screening assays that have long been used to evaluate the specific gene expression regulated by specific nuclear receptors, such as the estrogen receptors (ERs) (2) (3) (4) (5). They have been proposed for detection of estrogenic transactivation regulated by the ER (6) (7) (8).

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

4. The BG1Luc ER TA test method, 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 a stably transfected ER responsive luciferase reporter gene in the human ovarian adenocarcinoma cell line, BG-1, to provide concentration-response data for substances with *in vitro* ER agonist or antagonist activity (17).

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

INITIAL CONSIDERATIONS AND LIMITATIONS

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

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

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

PRINCIPLE OF THE TEST

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

11. The BG1Luc ER TA utilizes an ER responsive human ovarian adenocarcinoma cell line, BG-1, which has been stably transfected with a firefly *luc* reporter construct under control of four estrogen response elements placed upstream of the mouse mammary tumor virus promoter (MMTV), to detect substances with *in vitro* ER agonist or antagonist activity. This MMTV promoter exhibits only minor cross-reactivity with other steroid and non-steroid hormones (Rogers and Denison 2000). The protocols (agonist and antagonist) for this TG incorporate essential test method components for *in vitro* ER TA assays that were recommended by ICCVAM (8).

12. Criteria for data interpretation are described in detail in paragraphs 54 through 56. Briefly, a positive response is identified by a concentration-response curve containing at least three points with nonoverlapping error bars (mean ± SD), as well as a change in amplitude (normalized relative light unit [RLU]) of at least 20% of the maximal value for the reference substance (17β-estradiol [E2; CASRN 50-28-2] for the agonist assay, raloxifene HCl [Ral; CASRN 84449-90-1]/E2 for the antagonist assay).

PROCEDURE

Cell Line

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

Stability of the Cell Line

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

Cell Culture and Plating Conditions

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

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

17. Upon reaching ~80% confluence, BG1Luc4E2 cells are subcultured and conditioned to an estrogen-free environment for 48 hours prior to plating the cells in 96-well plates for exposure to test substances and analysis of estrogen dependent induction of luciferase activity. The estrogen-free medium (EFM) contains Dulbecco's Modification of Eagle's Medium (DMEM) without phenol red, supplemented with 4.5% charcoal/dextran-treated FBS, 1.9% L-glutamine, and 0.9% Pen-Strep. All plasticware should be free of estrogenic activity.

Acceptability Criteria

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

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[2] Xenobiotic Detection Systems, Inc. 1601 E Geer St # S Durham, NC 27704. (919) 688-4804

databases are updated with reference standard and control values on a continuous basis. Changes in equipment or laboratory conditions may necessitate generation of updated historical databases.

Agonist Test

Range Finder Test

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

Comprehensive Test

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

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

Antagonist Test

Range Finder Test

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

Comprehensive Test

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

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

Reference Standards, Positive, and Vehicle Controls

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

Vehicle Control (Agonist and Antagonist Assays)

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

Reference Standard (Agonist Range Finder)

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

Reference Standard (Agonist Comprehensive)

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

Reference Standard (Antagonist Range Finder)

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

Reference Standard (Antagonist Comprehensive)

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

Weak Positive Control (Agonist)

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

Weak Positive Control (Antagonist)

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

E2 Control (Antagonist Assay Only)

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

Fold-Induction (Agonist)

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

Fold-Reduction (Antagonist)

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

Demonstration of Laboratory Proficiency

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

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

Table 1 Agonist Substances for Demonstration of Laboratory Proficiency

Substance	CASRN	Expected Response[a]	BG1Luc ER TA Mean EC_{50} (M)[b,c]	MeSH Chemical Class[d]	Product Class[e]
Ethyl paraben	120-47-8	POS	2.48×10^{-5}	Carboxylic Acid, Phenol	Pharmaceutical, Preservative
Kaempferol	520-18-3	POS	3.99×10^{-6}	Flavonoid, Heterocyclic Compound	Natural Product
Butylbenzyl phthalate	85-68-7	POS	1.98×10^{-6}	Carboxylic Acid, Ester, Phthalic Acid	Plasticizer, Industrial Chemical
Apigenin	520-36-5	POS	1.85×10^{-6}	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
Daidzein	486-66-8	POS	8.71×10^{-7}	Flavonoid, Heterocyclic Compound	Natural Product
Bisphenol A	80-05-7	POS	5.33×10^{-7}	Phenol	Chemical Intermediate, Flame Retardant, Fungicide

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

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

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

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

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

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

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

Table 2 Antagonist Substances for Demonstration of Laboratory Proficiency

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

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

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

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

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

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

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

32. For each proficiency substance, starting concentrations should first be selected based on range finder test results (paragraphs 44 and 45), and then at least two comprehensive tests conducted. Each comprehensive test should be conducted on a separate experimental day. If the results of the tests contradict each other (e.g., one test is positive, the other negative), or if one of the tests is inadequate, a third additional test should be conducted. Proficiency is demonstrated by correct classification (positive/negative) of each proficiency substance (see **Tables 1,2,** and **4**). Proficiency testing should be repeated by each technician learning the test methods.

Vehicle

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

Preparation of Test Substances

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

Solubility and Cytotoxicity: Considerations for Range Finding

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

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

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

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

Test Substance Exposure and Assay Plate Organization

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

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

Range Finder Tests

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

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

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

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

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

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

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

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

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

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

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

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

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

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

- If there are no points on the test substance concentration curve that are less than the mean minus three times the standard deviation of the E2 control comprehensive testing will be conducted using an 11-point 1:2 serial dilution starting at the maximum soluble concentration.
- If there are points on the test substance concentration curve that are less than the mean minus three times the standard deviation of the E2 control, the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one of the following:
  - The concentration giving the lowest adjusted RLU value in the range finder
  - The maximum soluble concentration (See antagonist protocol (30), Figure 14-2)
  - The lowest cytotoxic concentration (See antagonist protocol (30), Figure 14-3 for a related example).
- The 11-point dilution scheme will be based on either a 1:2 or 1:5 serial or dilution according to the following criteria:

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

Comprehensive Tests

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

- *Agonist* comprehensive testing uses 11 concentrations of E2 in duplicate as the reference standard. Four replicate wells for the DMSO control and three 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 three replicate wells for the E2 9.18×10^{-11} M control, three 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	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
B	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
C	TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
D	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	VC
E	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Meth
F	TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Meth
G	E2-1	E2-2	E2-3	E2-4	E2-5	E2-6	E2-7	E2-8	E2-9	E2-10	E2-11	Meth
H	E2-1	E2-2	E2-3	E2-4	E2-5	E2-6	E2-7	E2-8	E2-9	E2-10	E2-11	Meth

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

Figure 4: Antagonist Comprehensive Test 96-well Plate Layout

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

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

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

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

EC_{50}/IC_{50} Determination

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

mathematical model relating the substance concentration to the response (typically following a sigmoidal curve) using the equation below:

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

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

Determination of Outliers

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

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 EC_{50} 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 IC_{50} 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 EC_{50} 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 IC_{50} 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 positive and negative decision criteria used in the BG1Luc ER TA validation study are described in Table 4.

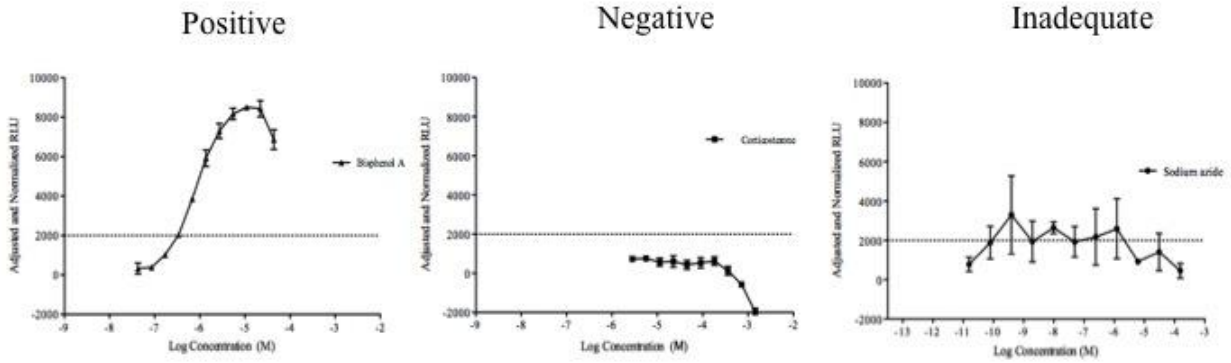
Table 4: Positive and Negative Decision Criteria

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

Positive	– Test substance data produce a concentration-response curve consisting of a baseline, which is followed by a negative slope. – The line defining the negative slope should contain at least three points with non-overlapping error bars; points forming the baseline are excluded but the linear portion of the curve may include the first point of the plateau. – There should be 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. – If possible, an IC50 value should be calculated for each positive substance.
Negative	All data points are above the ED_{80} value (80% of the E2 response, or 8000 RLUs), at concentrations less than 1.0 × 10-5M.
Inadequate	Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test substance is positive or negative. Substance should be retested.

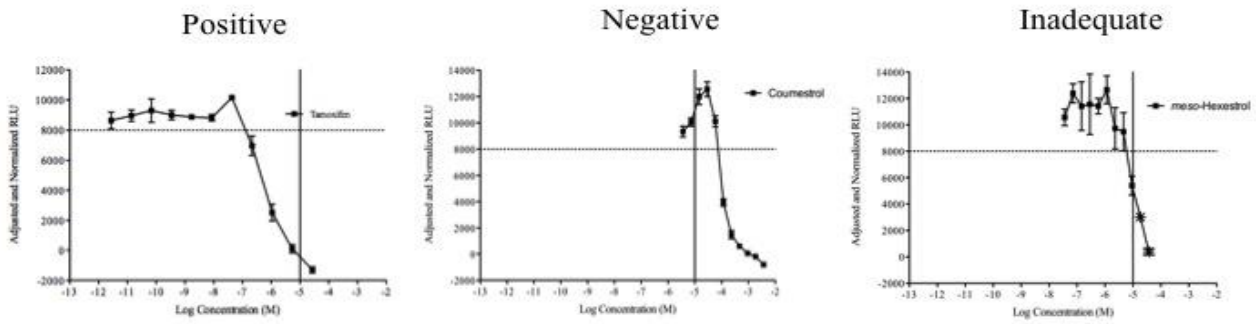
53. Data interpretation criteria are shown in Table 4. Positive results will be characterized by both the magnitude of the effect and the concentration at which the effect occurs, where possible. Examples of positive, negative, and inadequate data are shown in Figures 5 and 6.

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



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

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



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

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

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

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

54. The calculations of EC_{50} and IC_{50} can be made using a four-parameter Hill Function (See agonist protocol and antagonist protocol (30) for more details).
Meeting the 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 substances:

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

Cells:

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

Test conditions:

- cytotoxicity data and solubility limitations;
- concentration of test substance;
- volume of vehicle and test substance added;
- incubation temperature, humidity, and CO_2 concentration;
- duration of treatment;
- cell density during treatment.

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

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

For comprehensive experiments:

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

Results:

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

Discussion of results:

Conclusion:

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APPENDIX 1

DEFINITIONS AND ABBREVIATIONS

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

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

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

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

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

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

Cell morphology: The shape and appearance of cells grown in a monolayer in a single well of a tissue culture plate. Cells that are dying often exhibit abnormal 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 alternations in "basal cell functions" (i.e., via mitochondria, plasma membrane integrity, etc.).

DMEM: Dulbecco's Modification of Eagle's Medium

DMSO: Dimethyl sulfoxide

E2: 17β-estradiol

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

ED: Endocrine disruption

EE: 17α-ethynyl estradiol

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

ER: Estrogen receptor

ERE: Estrogen response element

FBS: Fetal bovine serum

hERα: Human estrogen receptor alpha

hERß: Human estrogen receptor beta

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

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

MMTV: Mouse Mammary Tumor Virus

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

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

Ral: raloxifene HCl

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

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

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

RLU: Relative Light Units

RNA: Ribonucleic Acid

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

SD: Standard deviation

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

TG: Test Guideline

Transcription: mRNA synthesis

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

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

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