

Appendix G

BG1LUC4E2 ER TA Agonist Protocol 02 August 2007

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**BG1LUC4E2 ER TA ASSAY
AGONIST PROTOCOL**

**National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative
Toxicological Methods (NICEATM)**

**Developed by:
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02 August 2007

LIST OF ACRONYMS AND ABBREVIATIONS

13 mm test tube	13 x 100 mm glass test tubes
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl sulfoxide
DMSO control	1% v/v dilution of DMSO in tissue culture media used as a vehicle control
E2	17 β -estradiol
E2 reference standard	10-point serial dilution of 17 β -estradiol reference standard for the LUMI-CELL [®] ER agonist assay
EC ₅₀ value	Concentration that produces a half-maximal response as calculated using the four parameter Hill function.
ER	Estrogen receptor
Estrogen-free DMEM	DMEM (phenol red free) supplemented with 1% penicillin/streptomycin, 2% L-glutamine, and 5% charcoal/dextran-treated FBS
FBS	Fetal bovine serum
G418	Gentamycin
Methoxychlor	<i>p,p'</i> -Methoxychlor
Methoxychlor control	3.13 μ g/mL methoxychlor positive control for the BG1LUC4E2 ER TA agonist assay
RPMI	RPMI 1640 growth medium
TA	Transcriptional activation
T25	25 cm ² tissue culture flask
T75	75 cm ² tissue culture flask
T150	150 cm ² tissue culture flask

1.0 Purpose

This protocol is designed to evaluate coded test substances for potential estrogen receptor (ER) agonist activity using the LUMI-CELL[®] ER assay.

2.0 Sponsor

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2.1 Substance Inventory and Distribution Management

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3.0 Definitions

- **Dosing Solution:** The test substance, control substance, or reference standard solution that is to be placed into the tissue culture wells for experimentation.
- **Raw Data:** Raw data includes information that has been collected but not formatted or analyzed, and consists of the following:
 - Data recorded in the Study Notebook
 - Computer printout of initial luminometer data
 - Other data collected as part of GLP compliance, e.g.:
 - Equipment logs and calibration records
 - Test substance and tissue culture media preparation logs
 - Cryogenic freezer inventory logs
- **Soluble:** Test substance exists in a clear solution without visible cloudiness or precipitate.
- **Study Notebook:** The study notebook contains recordings of all activities related to the conduct of the LUMI-CELL[®] agonist assay.
- **Test Substances:** Substances supplied to the testing laboratories that are coded and distributed such that only the Project Officer, Study Management Team (SMT), and the Substance Inventory and Distribution Management have knowledge of their true identity. The test substances will be purchased, aliquoted, coded, and distributed by the Supplier under the guidance of the NIEHS/NTP Project Officer and the SMT.

4.0 Testing Facility and Key Personnel

4.1 Testing Facility

Name of participating laboratory

4.2 Key Personnel

- Study Director:
- Laboratory Technician(s):
- Scientific Advisor:
- Quality Assurance Director:
- Safety Manager:

- Facility Management:
- Laboratory Director:

5.0 Identification of Test and Control Substances

5.1 Test Substances

Test substances are coded and will be provided to participating laboratories by the Substance Inventory and Distribution Management team.

5.2 Controls

Controls for the ER agonist protocol are as follows:

Vehicle control (dimethyl sulfoxide [DMSO]): 1% (v/v) DMSO (CASRN 67-68-5) diluted in tissue culture media.

Reference standard (17β-estradiol [E2]): Four concentrations of E2 (CASRN 50-28-2) in duplicate for range finder testing and a serial dilution consisting of 11 concentrations of E2 in duplicate for comprehensive testing

Positive control (p,p'-Methoxychlor [methoxychlor]): Methoxychlor (CASRN 72-43-5), 3.13 µg/mL in tissue culture media, used as a weak positive control.

6.0 Overview of General Procedures for Agonist Testing

All experimental procedures are to be carried out under aseptic conditions and all solutions, glassware, plasticware, pipettes, etc., shall be sterile. All methods and procedures shall be documented in the study notebook.

Agonist range finder testing is conducted on 96-well plates using four concentrations of E2 (5.00×10^{-5} , 1.25×10^{-5} , 3.13×10^{-6} and 7.83×10^{-7} µg/mL) in duplicate as the reference standard and four replicate wells for the DMSO control. Range finder testing uses all wells of the 96-well plate to test six substances as seven-point logarithmic serial dilutions in duplicate.

Comprehensive testing is conducted on 96-well plates using 11 concentrations of E2 in duplicate as the reference standard (**Table 6-1**). Four replicate wells for the DMSO control and four replicate wells for the methoxychlor control are included on each plate. Comprehensive testing uses all wells of the 96-well plate to test 2 substances as 11 point double serial dilutions in triplicate.

Table 6-1 Concentrations of E2 Reference Standard Used in Comprehensive Testing

E2 Concentrations ¹		
1.00×10^{-4}	6.25×10^{-6}	3.92×10^{-7}
5.00×10^{-5}	3.13×10^{-6}	1.95×10^{-7}
2.50×10^{-5}	1.56×10^{-6}	9.78×10^{-8}
1.25×10^{-5}	7.83×10^{-7}	

¹ Concentrations are presented in µg/mL.

Visual observations for cell viability are conducted for all experimental plates just prior to BG1LUC4E2 ER TA evaluation, as outlined in **Section 11.2**.

Luminescence data, measured in relative light units (RLUs), is corrected for background luminescence by subtracting the mean RLU value of the vehicle control (DMSO) wells from the RLU measurements for

each of the other wells of the 96-well plate. Data is then transferred into Excel[®] data management spreadsheets and GraphPad PRISM[®] 4.0 statistical software, graphed, and evaluated as follows:

- A response is considered positive for agonist activity when the average adjusted RLU for a given concentration is greater than the mean RLU value plus three times the standard deviation for the vehicle control.
 - Any response below this threshold is considered negative for agonist activity.

For substances that are positive at one or more concentrations, the concentration that causes a half-maximal response (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:

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

where Y = response (i.e., relative light units); X = the logarithm of concentration; Bottom = the minimum response; Top = the maximum response; log EC₅₀ = 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 EC₅₀ parameters. See **Section 11.6.5** for more details.

Acceptance or rejection of a test is based on evaluation of reference standard and control results from each experiment conducted on a 96-well plate. Results for these controls are compared to historical results compiled in the historical database, as seen in **Section 14.0**.

6.1 Range Finder Testing

Agonist range finding for coded substances consists of a seven-point logarithmic serial dilution using duplicate wells per concentration. Concentrations for comprehensive testing are selected based on the response observed in range finder testing. If necessary, a second range finder test can be conducted to clarify the optimal concentration range to test (see **Section 12.0**).

6.2 Comprehensive Testing

Comprehensive agonist testing for coded substances consists of 11-point double serial dilutions, with each concentration tested in triplicate wells of the 96-well plate. Three separate experiments are conducted for comprehensive testing on three separate days, except during Phases III and IV of the validation effort, in which comprehensive testing experiments are conducted once (see **Section 13.0**).

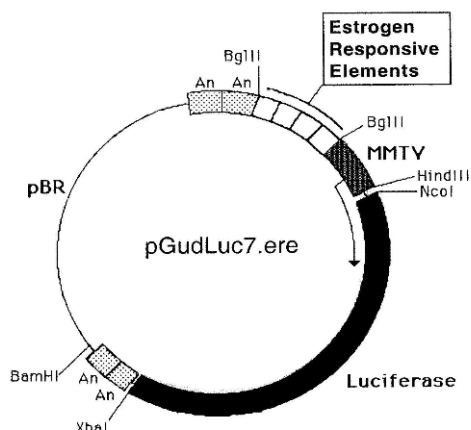
7.0 Materials for BG1LUC4E2 ER TA Agonist Testing

This section provides the materials needed to conduct BG1LUC4E2 ER TA testing, with associated brand names/vendors¹ in brackets.

7.1 BG1Luc4E2 Cells

BG1Luc4E2 cells are a human ovarian cancer cell line stably transfected with a plasmid containing an estrogen response element pGudLuc7.0 (**Figure 7-1**) [XDS].

¹ Brand names and vendors should not be considered an endorsement by the U.S. Government or any member of the U.S. Government; such information is provided as examples.

Figure 7-1 pGudLuc7.ERE Plasmid

7.2 Technical Equipment

All technical equipment may be obtained from Fisher Scientific International, Inc. (Liberty Lane, Hampton, NH, USA 03842). Equivalent technical equipment from another commercial source can be used.

- Analytical balance (Cat. No. 01-910-320)
- Berthold Orion 1 Microplate Luminometer [Berthold CatNo.: Orion 1 MPL3] or equivalent and dedicated computer
- Biological safety hood, class II, and stand (Cat. No. 16-108-99)
- Centrifuge (low speed, tabletop with swinging bucket rotor) (Cat. No. 04-978-50 centrifuge, and 05-103B rotor)
- Combustion test kit (CO₂ monitoring) (Cat. No. 10-884-1)
- Drummond diaphragm pipetter (Cat. No. 13-681-15)
- Freezers, –20°C (Cat. No. 13-986-150), and –70°C (Cat. No. 13-990-86)
- Hand tally counter (Cat. No. 07905-6)
- Hemocytometer, cell counter (Cat. No. 02-671-5)
- Light microscope, inverted (Cat. No. 12-561-INV)
- Light microscope, upright (Cat. No. 12-561-3M)
- Liquid nitrogen flask (Cat. No. 11-675-92)
- Micropipetter, repeating (Cat. No. 21-380-9)
- Pipettors, air displacement, single channel (0.5 –10µl (Cat. No. 21-377-191), 2 –20 µl (Cat. No. 21-377-287), 20 – 200 µl (Cat. No. 21-377-298), 200 - 1000 µl (Cat. No. 21-377-195))
- Refrigerator/freezer (Cat. No. 13-986-106A)
- Shaker for 96-well plates (Cat. No. 14-271-9)
- Sodium hydroxide (Cat. No. 5318-500)
- Sonicating water bath (Cat. No. 15-335-30)
- Tissue culture incubator with CO₂ and temperature control (Cat. No. 11-689-4)
- Vacuum pump with liquid trap (side arm Erlenmeyer) (Cat. No. 01-092-29)
- Vortex mixer (Cat. No. 12-814)

Equipment should be maintained and calibrated as per GLP guidelines and individual laboratory SOPs.

7.3 Reference Standard, Controls, and Tissue Culture Supplies

All tissue culture reagents must be labeled to indicate source, identity, storage conditions and expiration dates. Tissue culture solutions must be labeled to indicate concentration, stability (where known), and preparation and expiration dates.

Equivalent tissue culture media and sera from another commercial source can be used, but must first be tested as described in **Section 15.0** to determine suitability for use in this test method.

The following are the necessary tissue culture reagents and possible commercial sources (in brackets) based on their use in the pre-validation studies:

- BackSeal-96/384, white adhesive bottom seal for 96-well and 384-well microplate [Perkin-Elmer, Cat. No. 6005199]
- 17 β -estradiol (CAS RN: 50-28-2) [Sigma-Aldrich, Cat. No. E8875]
- Cryovial, 2 mL (Corning Costar) [Fisher Scientific Cat. No. 03-374-21]
- Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 1000 9186R38]²
- Culture tube, 50 mL conical (Corning Costar) [Fisher Scientific Cat. No. 05-526C]
- DMSO, U.S.P. analytical grade. [Sigma-Aldrich, Cat. No. 34869-100ML]
- Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L glucose, with sodium pyruvate, without phenol red or L-glutamine [Mediatech/Cellgro, Cat. No. 17-205-CV]
- Fetal bovine serum [Mediatech/Cellgro Cat. No. MT 35-010-CV]
- Fetal bovine serum, charcoal/dextran-treated, triple 0.1 μ m sterile filtered [Hyclone, Cat. No. SH30068.03]
- Gentamycin sulfate (G418), 50 mg/mL [Mediatech/Cellgro Cat. No. 30-234-CR]
- L-glutamine, 29.2 mg/mL [Cellgro, Cat. No. 25005-CI]
- Luciferase Assay System (10-Pack) [Promega, Cat. No. E1501]
- Lysis Solution 5X [Promega, Cat. No. E1531]
- Methoxychlor (CAS RN: 72-43-5) [Sigma-Aldrich, Cat. No. 49054]
- Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 μ g/mL streptomycin [Cellgro, Cat. No. 30-001-CI].
- Phosphate buffered saline (PBS, 1X) without calcium and magnesium [Cellgro, Cat. No. 21-040-CV]
- Pipettes, serological: 2.0 mL [Sigma-Aldrich, Cat. No. P1736], 5.0 mL [Sigma-Aldrich, Cat. No. P1986], 25 mL [Sigma-Aldrich, Cat. No. P2486]
- RPMI 1640 medium, containing L-glutamine [Mediatech, Cat. No. 10-040-CV]
- Tissue culture flasks (Corning-Costar): 25 cm² (T25) [Fisher Cat. No. 10-126-28]; 75 cm² (T75) [Fisher Cat. No. 10-126-37]; and 150 cm² (T150) [Fisher Cat. No. 10-126-34]
- Tissue culture plates (Corning-Costar): 96-well [Thomas Scientific Cat. No. 6916A05]
- Trypsin (10X), 2.5% in Hank's balanced salt solution (HBSS), without calcium and magnesium, without phenol red [Cellgro, Cat. No. 25-054-CI].

All reagent lot numbers and expiration dates must be recorded in the study notebook.

8.0 Preparation of Tissue Culture Media and Solutions

All tissue culture media and media supplements must be quality tested before use in experiment (see Section 15.0).

8.1 RPMI 1640 Growth Medium (RPMI)

RPMI 1640 is supplemented with 0.9% Pen-Strep and 8.0% FBS to make RPMI growth medium (RPMI).

² If glass tubes can not be obtained from Thomas Scientific, the preference is for flint glass, then lime glass, then borosilicate glass.

Procedure for one 549 mL bottle:

1. Remove FBS from -70°C freezer and Pen-Strep from -20°C freezer. Allow to equilibrate to room temperature.
2. Add 44 mL of FBS and 5 mL Pen-Strep to the bottle of RPMI 1640.
3. Label RPMI bottle as indicated in **Section 7.3**

Store at 2-8 °C for no longer than six months or until the shortest expiration date of any media component.

8.2 Estrogen-Free DMEM Medium

DMEM is supplemented to contain 4.5% charcoal/dextran treated FBS, 1.9% L-glutamine, 0.9% Pen-Strep.

Procedure for one 539 mL bottle:

1. Remove charcoal/dextran treated FBS from -70°C freezer, and L-glutamine and Pen-Strep from -20°C freezer and allow to equilibrate to room temperature.
2. Add 24 mL of charcoal/dextran treated FBS, 10 mL L-glutamine, and 5 mL Pen-Strep to one 500 mL bottle of DMEM.
3. Label estrogen-free DMEM bottle as indicated in **Section 7.3**

Store at 2-8 °C for no longer than six months or until the shortest expiration date of any media component.

8.3 1X Trypsin Solution

1X Trypsin solution is prepared by dilution from a 10X premixed stock solution. The 10X stock solution should be stored in 10 mL aliquots in a -20°C freezer.

Procedure for making 100 mL of 1X trypsin:

1. Remove a 10 mL aliquot of 10X trypsin from -20°C freezer and allow to equilibrate to room temperature.
2. Aliquot 1 mL trypsin (10X) along with 9 mL of 1X PBS into ten 15 mL sterile centrifuge tubes.
3. Label 1X trypsin aliquots as indicated in **Section 7.3**

1X Trypsin should be stored at -20°C.

8.4 1X Lysis Solution

Lysis solution is prepared by dilution from a 5X premixed stock solution. Both the 5X and 1X solutions can be repeatedly freeze-thawed.

The procedure for making 10 mL of 1X lysis solution:

1. Thaw the 5X Promega Lysis Solution and allow it to reach room temperature.
2. Remove 2 mL of 5X solution and place it in a 15 mL conical centrifuge tube.
3. Add 8 mL of distilled, deionized water to the conical tube.
4. Cap and shake gently until solutions are mixed.

Store at -20 °C for no longer than 1 year from receipt.

8.5 Reconstituted Luciferase Reagent

Luciferase reagent consists of two components, luciferase buffer and lyophilized luciferase substrate.

For long term storage, unopened containers of the luciferase buffer and lyophilized luciferase substrate can be stored at -70°C for up to one year.

To reconstitute luciferase reagent:

1. Remove luciferase buffer and luciferase substrate from -70°C freezer and allow them to equilibrate to room temperature.
2. Add 10 mL of luciferase buffer solution to luciferase substrate container and swirl or vortex gently to mix; the luciferase substrate should readily go into solution.
3. After solutions are mixed, aliquot to a 15mL centrifuge tube.
4. Store complete solution at -20°C .

Reconstituted luciferase reagent is stable for up to 1 month at -20°C .

9.0 Overview of Propagation and Experimental Plating of BG1LUC4E2 Cells

The BG1Luc4E2 (BG-1) cells are stored in liquid nitrogen in 2 mL cryovials. BG-1 cells are grown as a monolayer in tissue culture flasks in a dedicated tissue culture incubator at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90\% \pm 5\%$ humidity, and $5.0\% \pm 1\%$ CO_2/air . The cells should be examined on a daily basis during working days under an inverted phase contrast microscope and any changes in morphology and/or adhesive properties must be noted in the study notebook.

Two T150 flasks containing cells at 80 to 90% confluence will usually yield a sufficient number of cells to fill three 96-well plates for use in experiments.

9.1 Procedures for Thawing Cells and Establishing Tissue Cultures

Warm all of the tissue culture media and solutions to room temperature by placing them under the tissue culture hood several hours before use.

All tissue culture media, media supplements, and tissue culture plasticware must be quality tested before use in experiments (**Section 15.0**).

9.1.1 Thawing Cells

1. Remove a cryovial of frozen BG-1 cells from the liquid nitrogen flask.
2. Facilitate rapid thawing by loosening the top slightly (do not remove top) to release trapped gasses and retightening it. Roll vial between palms.
3. Use a micropipette to transfer cells to a 50 mL conical centrifuge tube.
4. Rinse cryovial twice with 1X PBS and add PBS rinse material to the conical tube.
5. Add 20 mL of RPMI to the conical tube.
6. Centrifuge at $1000 \times g$ for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
7. Aspirate media from pellet and resuspend it in 5 mL RPMI, drawing the pellet repeatedly through a 1.0 mL serological pipette to break up any clumps of cells.
8. Transfer cells to a T25 flask, place them in an incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

9.1.2 Establishing Tissue Cultures

Once cells have reached 80 to 90% confluence, transfer the cells to a T75 flask by performing, for example, the following steps:

1. Remove the T25 flask from the incubator.
2. Aspirate the RPMI, then add 5 mL 1X PBS, making sure that the cells are coated with PBS.
3. Aspirate 1X PBS, then add 1 to 2 mL 1X trypsin to the T25 flask, gently swirling the flask to coat all cells with the trypsin.
4. Place the flask in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
5. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.

6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, then hit the flask again.
7. After cells have detached, add 5 mL PBS, and transfer the suspended cells to a 50 mL centrifuge tube. Wash the flask one additional time with 5 mL PBS.
8. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular digestion by residual trypsin.
9. Pellet the cells by centrifugation, as described in **Section 9.1.1**, and resuspend the cells in 10 mL RPMI medium.
10. Draw the pellet repeatedly through a 25 mL serological pipette to break up clumps of cells.
11. Transfer cells to a T75 flask, then place the flask in an incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

When cells have reached 80% to 90% confluency, transfer them into a T150 flask by performing, for example, the following steps:

12. Remove the T75 flask from the incubator, aspirate the old media and add 5 mL 1X PBS.
13. Aspirate 1X PBS, add 2 mL of 1X trypsin to the flask, and place it in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
14. Repeat steps **5** through **11** in **Section 9.1.2**, resuspending the pellet in 20 mL of RPMI.
15. Transfer cells to a T150 flask and place it in the incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
16. Remove the T150 flask from the incubator.
17. Aspirate the RPMI and add 5 mL 1X PBS.
18. Aspirate 1X PBS and add 3 mL 1X trypsin to the T150 flask, making sure that the cells are coated with the trypsin.
19. Incubate cells in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
20. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.
21. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, then hit the flask again.
22. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the flask, swirl around the flask, and then transfer the PBS to the 50 mL conical tube.
23. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular digestion by residual trypsin.
24. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
25. Aspirate the media from the pellet and resuspend it in 40 mL RPMI, drawing the pellet repeatedly through a 25 mL serological pipette to break up any clumps of cells.
26. Transfer 20 mL of cell suspension to each of two T150 flasks, place them in an incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

9.2 Ongoing Tissue Culture Maintenance, Conditioning in Estrogen-free Medium, and Plating Cells for Experimentation

The following procedure is used to condition the BG1Luc4E2 cells to an estrogen-free environment prior to plating the cells in 96-well plates for analysis of estrogen dependent induction of luciferase activity.

To start the tissue culture maintenance and estrogen-free conditioning, split the two T150 culture flasks into four T150 flasks. Two of these flasks will be used for continuing tissue culture and will use the RPMI media mentioned above. The other two flasks will be cultured in estrogen-free DMEM for experimental use. Extra care must be taken to avoid contaminating the estrogen-free cells with RPMI.

1. Remove both T150 flasks from the incubator.
2. Aspirate the medium and rinse the cells with 5 mL 1X PBS.

3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask to coat all cells with the trypsin.
4. Incubate cells in the incubator (see conditions in **Section 9.0**) for 5 to 10 min.
5. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.
6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, then hit the flask again.
7. After cells have detached, add 5 mL 1X PBS to the first T150 flask and transfer the suspended cells to the second T150 flask.
8. Transfer the contents of both flasks to a 50 mL conical tube. Repeat step 7 with an additional 5 mL 1X PBS and transfer to the 50 mL conical tube.
9. Immediately add 20 mL estrogen-free DMEM to the 50 mL conical tube to inhibit further cellular digestion by residual trypsin.
10. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
11. Aspirate media from pellet and resuspend it in 4 mL estrogen-free DMEM, drawing the pellet repeatedly through a 1 mL serological pipette to break up clumps of cells.

At this point, cells are ready to be divided into the ongoing tissue culture and estrogen-free conditioning groups.

9.2.1 Ongoing Tissue Culture Maintenance

1. Add 20 mL RPMI to two T150 flasks.
2. Add 220 µL G418 to the RPMI in the T150 flasks
3. Add 1 mL of cell suspension from **9.2** step **11** to each flask.
4. Place T150 flasks in tissue culture incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
5. Tissue culture medium may need to be changed 24 hours after addition of G418 to remove cells that have died because they do not express reporter plasmid.
6. G418 does not need to be added to the flasks a second time.
7. Repeat **Section 9.2 steps 1-11** for ongoing tissue culture maintenance.

9.2.2 Conditioning in Estrogen-free Medium

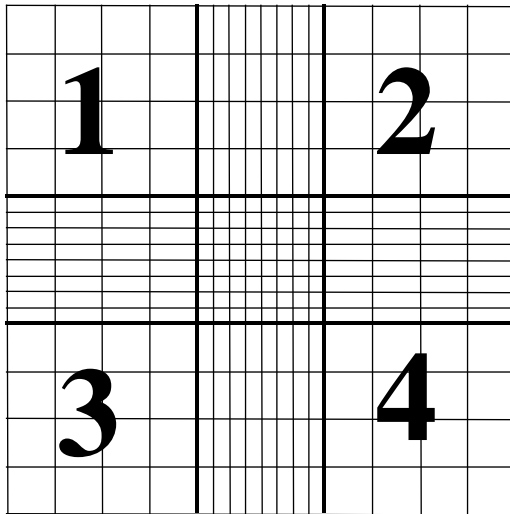
1. Add 20 mL estrogen-free DMEM to two T150 flasks.
2. Add 150 µL G418 to the estrogen-free DMEM in the T150 flasks.
3. Add 1 mL of cell suspension from **Section 9.2 step 11** to each flask.
4. Tissue culture medium may need to be changed 24 hours after addition of G418 to remove cells that have died because they do not express reporter plasmid.
5. G418 does not need to be added to the flasks a second time.
6. Place the T150 flasks in the incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

9.2.3 Plating Cells Grown in Estrogen-free DMEM for Experimentation

1. Remove the T150 flasks that have been conditioned in estrogen-free DMEM for 48 to 72 hours from the incubator.
2. Aspirate the medium, then rinse the cells with 5 mL 1X PBS.
3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask to coat all cells with the trypsin.
4. Place the flasks in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
5. Detach cells by hitting the side of the flask sharply against the palm or the heel of the hand.
6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for 2 additional minutes, then hit the flask again.

7. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the flask, gently swirl around the flask, and then transfer to the 50 mL conical tube.
8. Immediately add 20 mL estrogen-free DMEM to each conical tube to inhibit further cellular digestion by residual trypsin.
9. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
10. Aspirate the media from the pellet and re-suspend it in 20 mL DMEM, drawing the pellet repeatedly through a 25 mL serological pipette to break up any clumps of cells.
11. Pipette 15 μ L of the cell suspension into the “v” shaped slot on the hemocytometer. Ensure that the solution covers the entire surface area of the hemocytometer grid, and allow cells to settle before counting.
12. Using 100x magnification, view the counting grid.
13. The counting grid on the hemocytometer consists of nine sections, four of which are counted (upper left, upper right, lower left, and lower right, see **Figure 9-1**). Each section counted consists of four by four grids. Starting at the top left and moving clockwise, count all cells in each of the four by four grids. Some cells will be touching the outside borders of the square, but only count those that touch the top and right borders of the square. This value is then used in the calculation below to get to the desired concentration of 200,000 cells/mL.

Figure 9-1 Hemocytometer Counting Grid.



The volume of each square is 10^{-4} mL, therefore:

$$\text{Cells/mL} = (\text{average number per grid}) \times 10^{-4} \text{ mL} \times 1/(\text{starting dilution}).$$

Starting dilution: 20 mL (for T150 flasks)

Harvested cells for a T150 flask are suspended in 20 mL of estrogen-free DMEM and sampled for determination of concentration of cells/mL.

Example Calculation:

- Grids 1, 2, 3, and 4 are counted and provide the following data:

- 50, 51, 49, and 50: average number of cells per grid is equal to 50.

Cells/mL = 50 cells per grid ÷ 10^{-4} mL volume of grid = 50×10^4 cells/mL (or 500,000 cells/mL)

Total # of Cells Harvested = 500,000 cells/mL x 20 mL

Desired Concentration (or Concentration_{Final}) = 200,000 cells/mL

Formula: (Concentration_{Final} x Volume_{Final} = Concentration_{Initial} x Volume_{Initial})

Concentration_{Final} = 200,000 cells/mL

Concentration_{Initial} = 500,000 cells/mL

Volume_{Initial} = 20 mL

Volume_{Final} – to be solved for.

Therefore: 200,000 cells/mL x Volume_{Final} = 500,000 cells/mL x 20 mL

Solving for Volume_{Final} we find = 50 mL

Therefore, add 30 mL of estrogen-free DMEM to the cell suspension for a total volume of 50 mL, which will yield the desired concentration of 200,000 cells/mL for plating.

14. This dilution scheme will give a concentration of 200,000 cells/mL. 200 µL of this cell suspension is used for each well of a 96-well plate (i.e., 40,000 cells per well).
15. Remove a 96-well plate from its sterile packaging. Use a repeater pipetter to pipette:
 - 200 µL of cell suspension into each well for range finder testing
 - 200 µL of cell suspension into each well except the outside ring of wells for comprehensive testing and add 200 µL of estrogen-free DMEM to the outside ring of wells
16. Incubate plate(s) in an incubator (see conditions in **Section 9.0**) for a minimum of 24 hours, but no longer than 48 hours before dosing.

Two T150 flasks containing cells at 80% to 90% confluence will typically yield sufficient cells to fill four 96-well plates (not including the perimeter wells).

10.0 Preparation of Test Substances

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.

All information on weighing, solubility testing, and calculation of final concentrations for test substances, reference standards and controls is to be recorded in the study notebook.

10.1 Determination of Test Substance Solubility

1. Prepare a 100 mg/mL solution of the test substance in 100% DMSO in a 4 mL conical tube.
2. Vortex to mix.
3. If the test substance does not dissolve at 100 mg/mL, prepare a 10 mg/mL solution and vortex as above.
4. If the test substance does not dissolve at 10 mg/mL solution, prepare a 1 mg/mL solution in a 4 mL conical tube and vortex as above.
5. If the test substance does not dissolve at 1 mg/mL, prepare a 0.1 mg/mL solution in a 4 mL conical tube and vortex as above.

- Continue testing, using 1/10 less substance in each subsequent attempt until test substance is solubilized in DMSO.

Once the test substance has fully dissolved in 100% DMSO, the solubility of the test substance must be determined in the 1% DMSO/99% estrogen-free DMEM mixture used for BG1LUC4E2 ER TA testing.

- Add 4 µL of the highest concentration of the test substance/DMSO solution to a 13 mm test tube.
- Add 400 µL estrogen-free DMEM to the test tube and vortex gently,
- If cloudiness or precipitate develop, vortex for up to 10 minutes.
- If test substance has visible precipitate or is cloudy return to **10.1 step 7** to try the next lower concentration for the test substance.

The Testing Facility shall forward the results from the solubility tests assay to the SMT through the designated contacts in electronic format and hard copy upon completion of testing.

10.2 Preparation of Reference Standards, Control and Test Substances

All “dosing solutions” of test substance concentrations are to be expressed as µg/mL in the study notebook and in all laboratory reports.

All information on preparation of test substances, reference standards and controls is to be recorded in the study notebook.

10.2.1 Preparation of Reference Standard and Positive Control Stock Solutions

Stock solutions of E2 and methoxychlor are prepared in 100% DMSO and stored at room temperature for up to three years or until the expiration date listed in the certificate of analysis for that substance.

10.2.1.1 E2 Stock Solution

The final concentration of the E2 stock solution is 1.0×10^{-2} µg/mL. Prepare the E2 stock as shown in **Table 10-1**.

Table 10-1 Preparation of E2 Stock Solution

Step #	Action	DMSO	E2 Concentration
1	Make a 10 mg/mL stock solution in 100% DMSO in a 4mL vial.	-	10 mg/mL
2	Transfer 10 µL E2 solution from Step #1 to a new 4 mL vial.	Add 990 µL of 100% DMSO. Vortex to mix.	100 µg/mL
3	Transfer 10 µL E2 solution from Step #2 to a new 4mL vial.	Add 990 µL of 100% DMSO. Vortex to mix.	1 µg/mL
4	Transfer 10 µL E2 solution from Step #3 to a 13 mm test tube to create the working solution.	Add 990 µL of 100% DMSO. Vortex to mix.	1.0×10^{-2} µg/mL

10.2.1.2 Methoxychlor Stock Solution

The final concentration of the methoxychlor stock solution is 313 µg/mL.

To prepare the methoxychlor stock solution, proceed as follows:

- Make a 10 mg/mL stock solution of Methoxychlor in 100% DMSO in a 4 mL vial.
- Remove 94 µL of the methoxychlor solution and place it in a new 4 mL vial.
- Add 2.906 mL of 100% DMSO to the 4mL vial and gently vortex to mix.

10.2.2 Preparation of Reference Standard and DMSO Control for Range Finder Testing

Range finder testing is conducted on 96-well plates using four concentrations of E2 in duplicate as the reference standard. Four replicate wells are used for the DMSO control. All wells on the 96-well plate are used during range finder testing.

Store dosing solutions at room temperature. Use within 24 hours of preparation.

10.2.2.1 Preparation of E2 Reference Standard for Range Finder Testing

To make E2 dosing solutions:

1. Label four 4 mL conical tubes with numbers 1 through 4 and place them in a tube rack.
2. Label four 13 mm glass test tubes with numbers 1 through 4, place them in a tube rack and add 600 µL of estrogen-free DMEM to each tube.

Prepare dilutions to give final concentrations of the E2 as shown in **Table 10-2**.

Table 10-2 Preparation of E2 Reference Standard Dosing Solution for Range Finder Testing

Tube Number	100% DMSO	E2 ¹	Estrogen-free DMEM ²	Final Volume	E2 Concentration
1	6 µL	6 µl of 1.0 x 10 ⁻² µg/mL working solution	600 µL	606 µL	5.00 x 10 ⁻⁵ µg/mL
2	18 µL	6 µL of 1.0 x 10 ⁻² µg/mL working solution	600 µL	606 µL	1.25 x 10 ⁻⁵ µg/mL
3	18 µL	6 µL from conical tube #2	600 µL	606 µL	3.13 x 10 ⁻⁶ µg/mL
4	18 µL	6 µL from conical tube #3	600 µL	606 µL	7.83 x 10 ⁻⁷ µg/mL

¹ Add specified volume of 100% DMSO and 6 µl of the specified E2 solution to labeled 4 mL conical tubes and vortex.

² Transfer 6 µL of DMSO/E2 solution from 4 mL conical tube to labeled 13 mL glass tubes containing DMEM and vortex.

10.2.2.2 Preparation of DMSO Control for Range Finder Testing

1. Add 10 µL of 100% DMSO to a 13 mm glass tube.
2. Add 1000 µL of estrogen-free DMEM to tube and vortex.

10.2.3 Preparation of Test Substance Dosing Solutions for Range Finder Testing

Range finder experiments are used to determine the concentrations of test substance to be used during comprehensive testing. Agonist range finding for coded substances consists of seven-point logarithmic serial dilutions run in duplicate.

To make dosing solutions for coded substances:

1. Label seven 4 mL conical tubes with numbers 1 through 7 and place them in a tube rack
2. Label seven 13 mm glass test tubes with numbers 1 through 7, place them in a tube rack and add 600 µL of estrogen-free DMEM to each tube

Prepare dilutions as shown in **Table 10-3**.

Table 10-3 Preparation of Test Substance Dosing Solutions for Range Finder Testing

Tube Number	100% DMSO	Test Substance ¹	Transfer	Estrogen-free DMEM	Final Volume
1	-	6 µL of test substance solution from Section 10.1 step 10	6 µL	600 µL	606 µL
2	90 µL	10 µL of test substance solution from Section 10.1 step 10	6 µL	600 µL	606 µL
3	90 µL	10 µL from conical tube #2	6 µL	600 µL	606 µL
4	90 µL	10 µL from conical tube #3	6 µL	600 µL	606 µL
5	90 µL	10 µL from conical tube #4	6 µL	600 µL	606 µL
6	90 µL	10 µL from conical tube #5	6 µL	600 µL	606 µL
7	90 µL	10 µL from conical tube #6	6 µL	600 µL	606 µL

¹ Add specified volume of 100% DMSO and test substance solution to labeled 4 mL conical tubes and vortex.

² Transfer 6 µL of DMSO/E2 solution from 4 mL conical tube to labeled 13 mL glass tubes containing DMEM and vortex.

Determination of whether a substance is positive in range finder testing and selection of starting concentrations for comprehensive testing will be discussed in **Section 12.0**.

10.2.4 Preparation of Reference Standard and Positive Control Dosing Solutions for Comprehensive Testing

Comprehensive testing is conducted on 96-well plates using 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 are included on each plate.

Store dosing solutions at room temperature. Use within 24 hours of preparation.

10.2.4.1 Preparation of E2 Reference Standard for Comprehensive Testing

To make E2 dosing solutions:

1. Label 11 4 mL conical tubes with numbers 1 through 11 and place them in a tube rack.
2. Label 11 13 mm glass test tubes with numbers 1 through 11, place them in a tube rack and add 600 µL of DMEM to each tube.

Prepare dilutions to give final concentrations of E2 as shown in **Table 10-4**.

Table 10-4 Preparation of E2 Reference Standard Dosing Solution for Comprehensive Testing

Tube Number	100% DMSO	E2 ¹	Estrogen-free DMEM ²	Final Volume	E2 Concentration
1	-	6 µl of 1.0 x 10 ⁻² µg/mL working solution	600 µL	606 µL	1.00 x 10 ⁻⁴ µg/mL
2	6 µL	6 µL of 1.0 x 10 ⁻² µg/mL working solution	600 µL	606 µL	5.00 x 10 ⁻⁵ µg/mL
3	6 µL	6 µL from conical tube #2	600 µL	606 µL	2.50 x 10 ⁻⁵ µg/mL
4	6 µL	6 µL from conical tube #3	600 µL	606 µL	1.25 x 10 ⁻⁵ µg/mL
5	6 µL	6 µL from conical tube #4	600 µL	606 µL	6.25 x 10 ⁻⁶ µg/mL
6	6 µL	6 µL from conical tube #5	600 µL	606 µL	3.13 x 10 ⁻⁶ µg/mL
7	6 µL	6 µL from conical tube #6	600 µL	606 µL	1.56 x 10 ⁻⁶ µg/mL
8	6 µL	6 µL from conical tube #7	600 µL	606 µL	7.83 x 10 ⁻⁷ µg/mL
9	6 µL	6 µL from conical tube #8	600 µL	606 µL	3.92 x 10 ⁻⁷ µg/mL
10	6 µL	6 µL from conical tube #9	600 µL	606 µL	1.95 x 10 ⁻⁷ µg/mL
11	6 µL	6 µL from conical tube #10	600 µL	606 µL	9.78 x 10 ⁻⁸ µg/mL

¹ Add specified volume of 100% DMSO and 6 µl of the specified E2 solution to labeled 4 mL conical tubes and vortex.

² Transfer 6 µL of DMSO/E2 solution from 4 mL conical tube to labeled 13 mL glass tubes containing DMEM and vortex.

10.2.4.2 Preparation of Methoxychlor Control Dosing Solution for Comprehensive Testing

1. Add 10 µL of the 313 µg/mL methoxychlor to a 13 mm glass tube.
2. Add 1000 µL of estrogen-free DMEM to the tube and vortex.

10.2.4.3 Preparation of DMSO Control Dosing Solution for Comprehensive Testing

1. Add 10 µL of 100% DMSO to four 13 mm tubes (solvent/negative controls).
2. Add 1000 µL of estrogen-free DMEM to the tube and vortex.

10.2.5 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

Comprehensive testing experiments are used to determine whether a substance possesses ER agonist activity in the BG1LUC4E2 ER TA test method. Agonist comprehensive testing for coded substances consists of 11-point double serial dilutions, with each concentration tested in triplicate wells of the 96-well plate.

Start the 11-point serial dilution series at a single log dilution higher than the concentration giving the highest adjusted RLU value during the range finder (e.g., if the highest adjusted RLU value occurred at a concentration of 0.01 mg/mL, start the serial dilution at 0.1 mg/mL).

To make dosing solutions for coded substances:

1. Label eleven 4 mL conical tubes with numbers 1 through 11 and place them in a tube rack.
2. Label eleven 13 mm glass test tubes with numbers 1 through 11, place them in a tube rack and add 800 µL of estrogen-free DMEM to each tube.

Prepare dilution of test substance as shown in **Table 10-5**.

Table 10-5 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

Tube Number	100% DMSO	Test Substance ¹	Transfer	Estrogen-free DMEM	Final Volume
1	-	8 µL of highest concentration of test substance solution	8 µL	800 µL	808 µL
2	8 µL	8 µL of highest concentration of test substance solution	8 µL	800 µL	808 µL
3	8 µL	8 µL from conical tube #2	8 µL	800 µL	808 µL
4	8 µL	8 µL from conical tube #3	8 µL	800 µL	808 µL
5	8 µL	8 µL from conical tube #4	8 µL	800 µL	808 µL
6	8 µL	8 µL from conical tube #5	8 µL	800 µL	808 µL
7	8 µL	8 µL from conical tube #6	8 µL	800 µL	808 µL
8	8 µL	8 µL from conical tube #7	8 µL	800 µL	808 µL
9	8 µL	8 µL from conical tube #8	8 µL	800 µL	808 µL
10	8 µL	8 µL from conical tube #9	8 µL	800 µL	808 µL
11	8 µL	8 µL from conical tube #10	8 µL	800 µL	808 µL

¹ Add specified volume of 100% DMSO and test substance solution to labeled 4 mL conical tubes, and vortex.

² Transfer 6 µL of DMSO/E2 solution from 4 mL conical tube to labeled 13 mL glass tubes containing DMEM and vortex.

11.0 General Procedures for the Testing of Coded Substances

Range finder experiments are used to determine the concentrations of test substance to be used during comprehensive testing. Comprehensive testing experiments are used to determine whether a substance possesses ER agonist activity in the BG1LUC4E2 ER TA assay.

General procedures for range finder and comprehensive are similar. For specific details (such as plate layout) of range finder testing see **Section 12.0**. For specific details of comprehensive testing, see **Section 13.0**.

11.1 Application of Reference Standard, Controls, and Test Substances

1. Remove seeded 96-well plates from the incubator, inspect them using an inverted microscope. Only use plates in which the cells in all wells giving a score of 1 according to **Table 11-1**.
2. Remove medium by inverting the plate onto blotter paper. Gently tap plate against the bench surface to remove residual liquid trapped in the wells.
3. Add 200 µL of reference standard, control, or test substance to each well (see **Sections 12.0** and **13.0** for specific plate layouts).
4. Return plates to incubator and incubate (see **Section 9.0** for details) for 19 to 24 hours to allow maximal induction of luciferase activity in the cells.

1.1.1 Preparation of Excel[®] Data Analysis Template For Range Finder Testing

1. In Excel[®], open a new “AgRFTemplate” and save it with the appropriate project name as indicated in the NICEATM Style Guide.
2. Add appropriate information regarding the assay to the “Compound Tracking” tab.

3. Enter substance testing information to the “List” page (i.e., Project /Sample ID, Concentration, and Comments[or compound name]). This should populate the “Template”, “Compound Mixing” and “Visual Inspection” tabs with the appropriate information for the experiment.
4. Save the newly named project file.
5. Print out either the “List” or “Template” page for help with dosing the 96-well plate. Sign and date the print out and store in study notebook.

11.1.2 Preparation of Excel® Data Analysis Template for Comprehensive Testing

1. In Excel®, open a new “AgCTTemplate” and save it with the appropriate project name as indicated in the NICEATM Style Guide.
2. Add appropriate information regarding the assay to the “Compound Tracking” tab.
3. Enter substance testing information to the “List” page (i.e., Project /Sample ID, Concentration, and Comments[or compound name]). This should populate the “Template”, “Compound Mixing” and “Visual Inspection” tabs with the appropriate information for the experiment.
4. Save the newly named project file.
5. Print out either the “List” or “Template” page for help with dosing the 96-well plate. Sign and date the print out and store in study notebook.

11.2 Visual Evaluation of Cell Viability

1. 19 to 24 hours after dosing the plate, remove the plate from the incubator and remove the media from the wells by inverting the plate onto blotter paper. Gently tap plate against the bench surface to remove residual liquid trapped in the wells.
2. Use a repeat pipetter to add 50 µL 1X PBS to all wells. Immediately remove PBS by inversion.
3. Using an inverted microscope, inspect all of the wells used in the 96-well plate and record the visual observations using the scores in **Table 11-1**.

Table 11-1 Visual Observation Scoring

Viability Score	Brief Description¹
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”

¹ Reference photomicrographs are provided in the BG1LUC4E2 ER TA Validation Study “Visual Observation Cell Viability Manual.”

11.3 Lysis of Cells for BG1LUC4E2 ER TA

1. Apply the reflective white backing tape to the bottom of the 96-well plate (this will increase the effectiveness of the luminometer).
2. Add 30 µL 1X lysis reagent to the assay wells and place the 96-well plate on an orbital shaker for one minute.
3. Remove plate from shaker and measure luminescence (as described in **Section 11.4**).

11.4 Measurement of Luminescence

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. The luminometer output is saved as raw data in an Excel[®] spread sheet. A hard copy of the luminometer raw data should be signed, dated and stored in the study notebook.

11.5 Data Analysis

BG1LUC4E2 ER TA uses an Excel[®] spreadsheet to collect and adjust the RLU values obtained from the luminometer and a GraphPad Prism[®] template to analyze and graph data. The Excel[®] spreadsheet subtracts background luminescence (average DMSO solvent control RLU value) from test substance, reference standard and control RLU values. Plate induction is calculated using these corrected RLU values. Test substance, reference standard, and control RLU values are then adjusted relative to the highest E2 reference standard RLU value, which is set to 10,000. After adjustment, values are transferred to GraphPad Prism[®] for data analysis and graphing.

11.5.1 Collection and Adjustment of Luminometer Data for Range Finder Testing

The following steps describe the procedures required to populate the Excel[®] spreadsheet that has been configured to collect and adjust the RLU values obtained from the luminometer.

1. Open the raw data file and the corresponding experimental Excel[®] spreadsheet from **Section 11.1.1**.
2. Copy the raw data using the Excel[®] copy function, then paste the copied data into cell B19 of the “RAW DATA” tab in the experimental Excel[®] spreadsheet using the Paste Special – Values command. This position corresponds to position A1 in the table labeled Table 1 in this tab.
3. Examine the DMSO data in Table 1 of the Excel[®] spreadsheet to determine whether there are any potential outliers. See **Section 11.6.2** for further explanation of outlier determinations.
4. If an outlier is identified, perform the following steps to remove the outlier from calculations:
 - Correct the equation used to calculate DMSO background in Table 1 [e.g., if outlier is located in cell F26, adjust the calculation in cell H40 to read =AVERAGE(G26:I26)]
 - Then correct the equation used to calculate the average DMSO value in Table 2 [e.g., following the above example, adjust cell M42 to read =AVERAGE(G26:I26)]
 - Then correct the equation used to calculate the standard deviation of the DMSO value in Table 2 [e.g., following the above example, adjust cell M43 to read =STDEV(G36:I36)]
5. Excel[®] will automatically subtract the background (the average DMSO control value) from all of the RLU values in Table 1 and populate Table 2 with these adjusted values.
6. To calculate plate induction, identify the cell containing the E2a replicate in Table 1, plate row H that has the highest RLU value (i.e., cell B28, C28, D38, or E28).
7. Click into cell D14 and enter the cell number from the previous step into the numerator.
8. Identify the cell containing the E2b replicate in Table 1, plate row H that has the highest RLU value (i.e., cell J26, K26, L26, or M26).
9. Click into cell E14 and enter the cell number from the previous step into the numerator.
10. Click on the “ER Agonist Report” tab.
11. The data for the E2 reference standard, methoxychlor, and DMSO replicates populate the left portion (columns A – F) of the spreadsheet. The data is automatically placed in an Excel[®] graph.
12. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell D2 of “ER Agonist Report” tab and check the formula contained within that cell. The divisor should be the cell number of the cell containing the highest averaged E2 RLU value (column D).
13. Copy the data into GraphPad Prism[®] for graphing and analysis as indicated in the NICEATM Prism[®] users guide.

11.5.2 Collection and Adjustment of Luminometer Data for Comprehensive Testing

The following steps describe the procedures required to populate the Excel[®] spreadsheet that has been configured to collect and adjust the RLU values obtained from the luminometer.

1. Open the raw data file and the corresponding experimental Excel[®] spreadsheet from **Section 11.1.2**.
2. Copy the raw data using the Excel[®] copy function, then paste the copied data into cell B19 of the “RAW DATA” tab in the experimental Excel[®] spreadsheet using the Paste Special – Values command. This position corresponds to position A1 in the table labeled Table 1 in this tab.
3. Examine the DMSO data in Table 1 of the Excel[®] spreadsheet to determine whether there are any potential outliers. See **Section 11.6.2** for further explanation of outlier determinations.
4. If an outlier is identified, perform the following steps to remove the outlier from calculations:
 - Correct the equation used to calculate DMSO background in Table 1 [e.g., if outlier is located in cell M20, adjust the calculation in cell H40 to read =AVERAGE(M19,M21:M22)]
 - Then correct the equation used to calculate the average DMSO value in Table 2 [e.g., following the above example, adjust cell M42 to read =AVERAGE(M31,M33:M34)]
 - Then correct the equation used to calculate the standard deviation of the DMSO value in Table 2 [e.g., following the above example, adjust cell M43 to read =STDEV(M31,M33:M34)]
5. Excel[®] will automatically subtract the background (the average DMSO control value) from all of the RLU values in Table 1 and populate Table 2 with these adjusted values.
6. To calculate plate induction, identify the cell containing the E2 replicate in plate row G that has the highest RLU value.
7. Click into cell D14 and enter the cell number from the previous step into the numerator.
8. Identify the cell containing the E2 replicate in plate row H that has the highest RLU value.
9. Click into cell E14 and enter the cell number from the previous step into the numerator.
10. Click on the “ER Agonist Report” tab.
11. The data for the E2 reference standard, methoxychlor, and DMSO replicates populate the left portion (columns A – F) of the spreadsheet. The data is automatically placed in an Excel[®] graph.
12. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell D2 of “ER Agonist Report” tab and check the formula contained within that cell. The divisor should be the cell number of the cell containing the highest averaged E2 RLU value (column D).
13. Copy the data into GraphPad Prism[®] for the calculation of EC₅₀ values and to graph experimental results as indicated in the NICEATM Prism[®] Users Guide.

11.5.3 Determination of Outliers

The Study Director will use good statistical judgment for determining “unusable” wells that will be excluded from the data analysis and will provide an explanation in the study notebook for any excluded data. This judgment for data acceptance will include Q-test analysis.

The formula for the Q test is:

Outlier – Nearest Neighbor

Range (Highest – Lowest)

where the outlier is the value proposed for exclusion, the nearest neighbor is the value closest to the outlier, and the range is the range of the three values (Q values for samples sizes from 3 to 10 are provided in **Table 11-2**). For example, if the value of this ratio is greater than 0.94 (the Q value for the 90% confidence interval for a sample size of three) or 0.76 (the Q value for the 90% confidence interval for a sample size of four), the outlier may be excluded from data analysis.

Table 11-2 Q Test Values

Number Of Observations	Q Value
2	-
3	0.94
4	0.76
5	0.64
6	0.56
7	0.51
8	0.47
9	0.44
10	0.41

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

11.5.4 Acceptance Criteria

11.5.3.1 Range Finder Testing

Acceptance or rejection of a test is based on 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 derived from the historical database, which are summarized below.

- Induction: Plate induction, as measured by dividing the averaged highest E2 reference standard RLU value by the averaged DMSO control RLU value, must be greater than three-fold.
- Solvent 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.

11.5.3.2 Comprehensive testing

Acceptance or rejection of a test is based on 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 derived from the historical database, which are summarized below.

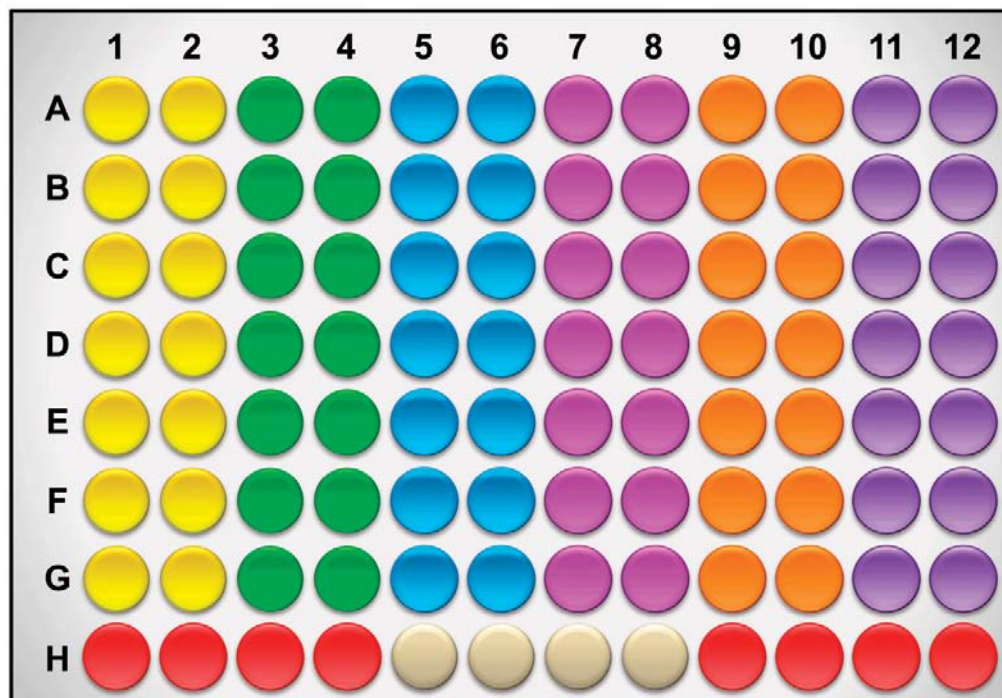
- Induction: Plate induction, as measured by dividing the averaged highest E2 reference standard RLU value by the averaged DMSO control RLU value, must be greater than three-fold.
- Reference standard results: Calculated E2 reference standard EC₅₀ values must be within 2.5 times the standard deviation of the historical database EC₅₀ mean value.
- Solvent control results: Solvent control RLU values must be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- Positive control results: Methoxychlor control RLU values must be within 2.5 times the standard deviation of the historical methoxychlor control mean RLU value.

An experiment that fails any single acceptance criterion will be discarded and repeated.

12.0 Range Finder Testing

Agonist range finding for coded substances consists of seven point, logarithmic serial dilutions, with each concentration tested in duplicate wells of the 96-well plate. **Figure 12-1** contains a template for the plate layout to be used in agonist range finder testing.

Figure 12-1 Agonist Range Finder Test Plate Layout



- 4 Point E2 Reference Standard
- DMSO (Solvent Control)
- Range Finder for Sample #1
- Range Finder for Sample #2
- Range Finder for Sample #3
- Range Finder for Sample #4
- Range Finder for Sample #5
- Range Finder for Sample #6

Evaluate whether range finder experiments have met the acceptance criteria (see **Section 11.6.3**) and graph the data as described in the NICEATM Prism[®] users guide.

To determine starting concentrations for comprehensive testing use the following criteria:

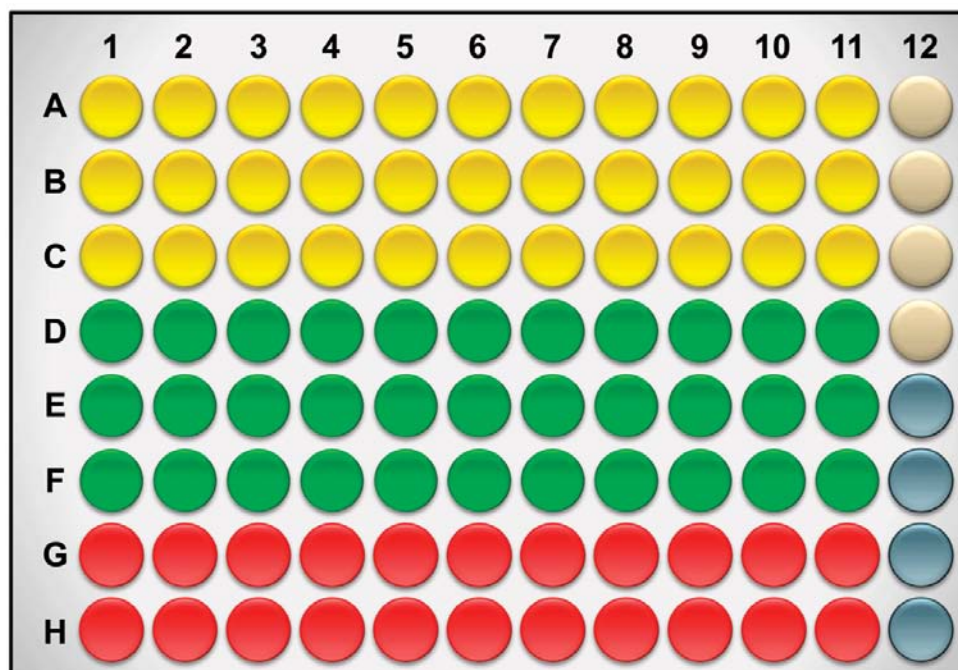
- If there are no points on the test substance concentration curve that are above the line representing the mean plus three times the standard deviation of the DMSO control, the highest concentration used in comprehensive testing is the limit dose or the maximum soluble dose.
- If there are points on the test substance concentration curve that are above the line representing the mean plus three times the standard deviation of the DMSO control, select a concentration that is a single log dilution higher than the concentration giving the highest adjusted RLU value in the range finder, and use that as the highest concentration for comprehensive testing.

- If a substance exhibits a biphasic concentration curve, the range finder experiment should be repeated unless the proposed concentration range for the comprehensive studies will include all concentrations of the biphasic region in the range finding study. If the range finder experiment is repeated and the substance still exhibits a biphasic concentration curve, comprehensive testing must be conducted on the peak of the biphasic curve at the lowest test substance concentration. If the substance is negative at this lowest concentration, then test at the higher concentration. For either peak of the concentration curve, select a concentration that is a single log dilution higher than the concentration giving the highest adjusted RLU value in the range finder and use that as the highest concentration for comprehensive testing.

13.0 Comprehensive Testing

Agonist comprehensive testing for coded substances consists of 11-point double serial dilutions, with each concentration tested in triplicate wells of the 96-well plate. **Figure 13-1** contains a template for the plate layout to be used in agonist comprehensive testing.

Figure 13-1 Agonist Comprehensive Test Plate Layout



- 11 Point Duplicate E2 Reference Standard
- DMSO (Solvent Control)
- Test Substance #1
- Test Substance #2
- Methoxychlor Control

Evaluate whether comprehensive experiments have met acceptance criteria (see **Section 11.6.4**) and graph the data as described in the NICEATM Prism[®] users guide.

- If the substance has been tested up to the limit dose or the maximum soluble dose, without causing a significant decrease in cell viability, and there are no points on the concentration curve that are above

the line indicating the mean plus three times the standard deviation of the DMSO control, the substance is considered negative for agonism.

- If the substance has a positive response (See **Section 6.0**) at any concentration, the substance is considered positive for agonism.

14.0 Compilation of the Historical Quality Control Database

Historical databases are maintained in order to ensure that the assay is functioning properly. Individual historical databases are maintained for agonist range finder and comprehensive testing as Excel[®] spreadsheets and are separate from the spreadsheets used to collect the data for individual test plates. Reference standard and control data is used to develop and maintain the historical database and are used as quality controls to determine acceptance of individual test plates.

Results collected during Phase I will be compared to historical control data established during the BG1LUC4E2 ER TA Protocol Standardization Study. Reference standard and control data collected during Phase I will be used to compile the initial historical database. Reference standard and control data collected during Phase IIa will be added to the historical database compiled in Phase I and this combined historical database will be used to establish acceptance criteria for Phase IIb. Reference standard and control data collected during Phase IIb will be added to the historical database compiled in Phases I and IIa and this combined historical database will be used to establish acceptance criteria for Phases III and IV.

14.1 Compilation of Historical Quality Control Database for Phase IIa Testing

Reference standard and control data from all plates that met Phase I acceptance criteria for induction (see **Section 11.5.3.2**) will be used to compile the historical database for Phase IIa agonist testing. The source of the reference standard and control data needed to compile the historical database for plate induction and methoxychlor and DMSO controls is the experiment specific Excel[®] data collection and analysis spreadsheet (see **Section 11.5.2**). The source of data needed to compile the historical database for EC₅₀ values is the experiment specific GraphPad Prism[®] file used to calculate and average the value (see **Section 11.5.2, Step 13**).

1. Open the standard BG1LUC4E2 ER TA Assay historical database Excel[®] spreadsheet (LUMIAgQC.xls).
2. Save this sheet under a new name, adding the laboratory designator to the file name (e.g., for Laboratory H, the new name would be HLUMIAgQC.xls).

14.1.1 Methoxychlor Control

1. Open Excel[®] spreadsheet HLUMIAgQC.xls
2. Open the Methoxychlor Control worksheet and enter the date and experiment name into worksheet columns A and B respectively. The experiment date and name entered into the Methoxychlor Control worksheet is used to automatically populate this information into the EC₅₀, DMSO and Reduction worksheets. Enter BG1Luc4E2 cell subculture information into column C. Enter the experimental mean adjusted methoxychlor value (from cell E30 in the ER Agonist Report worksheet of the Excel[®] data collection and analysis spreadsheet) into worksheet column D. Acceptance or rejection of plate methoxychlor control data for comprehensive testing is based on whether the mean plate methoxychlor RLU value falls within 2.5 times the standard deviation of the methoxychlor value in the historical database (columns G and H in the Methoxychlor worksheet).

14.1.2 DMSO Control

Open the DMSO worksheet and enter the experimental mean DMSO control value (from cell H40 in the RAW DATA worksheet of the Excel[®] data collection and analysis spreadsheet) into worksheet column C.

Acceptance or rejection of the plate DMSO control data for range finding and comprehensive testing is based on whether the mean plate DMSO RLU value falls within 2.5 times the standard deviation of the DMSO value in the historical database (columns F and G in the DMSO worksheet).

14.1.3 Induction

Open the Induction worksheet and enter the experimental mean induction value (from cell F14 in the RAW DATA worksheet of the Excel[®] data collection and analysis spreadsheet) into worksheet column C. Acceptance or rejection of range finding and comprehensive testing plates is based on whether the mean induction value is three or greater.

14.1.4 EC₅₀

Open the EC₅₀ worksheet and enter the GraphPad Prism[®] calculated mean EC₅₀ value (see Section 11.5.2, Step 13) into worksheet column C. Acceptance or rejection of plate EC₅₀ control data for comprehensive testing is based on whether the mean plate EC₅₀ value falls within 2.5 times the standard deviation of the EC₅₀ value in the historical database (columns F and G in the EC₅₀ worksheet).

14.2 Compilation of Historical Quality Control Database for Phase IIb Testing

Reference standard and control data from all plates that met all acceptance criteria for comprehensive testing (see Section 11.5.3.2) during Phase IIa will be added to the historical database compiled in Phase I (as per instructions in Sections 14.1.1 through 14.1.4) and this combined historical database will be used to establish acceptance criteria for Phase IIb.

14.3 Compilation of Historical Quality Control Database for Phase III and IV Testing

Reference standard and control data from all plates that met all acceptance criteria for comprehensive testing (see Section 13.5.3.2) during Phase IIb will be added to the historical database compiled in Phase I and IIa (as per instructions in Sections 14.1.1 through 14.1.4) and this combined historical database will be used to establish acceptance criteria for Phases III and IV.

15.0 Quality Testing of Materials

All information pertaining to the preparation and testing of media, media supplements, and other materials should be recorded in the Study Notebook.

15.1 Tissue Culture Media

Each lot of tissue culture medium must be tested in a single growth flask of cells before use in ongoing tissue culture or experimentation (**note:** each bottle within a given lot of charcoal/dextran-treated FBS must be tested separately).

1. Every new lot of media (RPMI and DMEM) and media components (FBS, charcoal/dextran-treated FBS, and L-glutamine) must first be tested on the LUMI-CELL[®] ER assay prior to being used in any GLP-acceptable assays.
2. Add 4 µL of DMSO (previously tested) into four separate 13 mm tubes.
3. Add 400 µL media (to be tested) to the same tubes.
4. Dose an experimental plate as in Section 12.0, treating the media being tested as a test substance.
5. Analyze 96-well plate as described in Section 12.0, comparing the data from the DMSO controls made using previously tested tissue culture media to the new media being tested.
6. Use the agonist historical database to determine if the new media with DMSO lies within 2.5 standard deviations of the mean for the media. If the RLU values for the new media with DMSO lie within 2.5 standard deviation of the mean for the historical data on DMSO, the new lot of media is acceptable. If the RLU values for the new media with DMSO do not lie within 2.5 standard deviations of the DMSO mean from historical database, the new lot may not be used in the assay.

7. Note date and lot number in study notebook.
8. If the new bottle passes quality testing as described in **Section 15.1 step 6**, apply the media to a single flask of cells and observe cell growth and morphology over the following 2–3 days. If there is no change in growth or morphology, the new media is acceptable for use.

15.2 G418

1. New lots of G418 must first be tested on the LUMI-CELL[®] ER assay prior to being used in any GLP-acceptable assays.
2. Add 220 µL of G418 (previously tested) to a single flask containing cells growing in RPMI.
3. Add 220 µL of G418 (to be tested) to a different flask containing cells growing in RPMI.
4. Observe cellular growth and morphology in both tissue culture flasks over a 48 to 72 hour period. If there are no differences in observed growth rate and morphology between the two flasks, the new G418 lot is acceptable.
5. If cellular growth is decreased, or the cells exhibit abnormal morphology, the new lot of G418 is not acceptable.
6. Note date and lot number in study book.

15.3 DMSO

1. Every new bottle of DMSO must be tested on the LUMI-CELL[®] ER assay prior to use in any GLP-acceptable assays.
2. Add 4 µL of DMSO (to be tested) into four separate 13 mm tubes.
3. Add 400 µL media (previously tested) to the same tubes.
4. Dose an experimental plate as in Section 12.0, treating the DMSO containing media being tested as a test substance.
5. Analyze 96-well plate as described in Section 12.0, comparing the data from the DMSO controls made using previously tested tissue culture media.
6. Use the agonist historical database to determine if media with new DMSO lies within 2.5 standard deviations of the DMSO mean from historical database. If the RLU values for the media with new DMSO lie within 2.5 standard deviations of the DMSO mean from the historical database, the new lot of DMSO is acceptable. If the RLU values for media with new DMSO do not lie within 2.5 standard deviations of the DMSO mean from historical database, the new lot may not be used in the assay.
7. Note the date, lot number, and bottle number in study book.
8. If no DMSO has been previously tested, test several bottles as described in Section 15.3, and determine whether any of the bottles of DMSO have a lower average RLU than the other bottle(s) tested. Use the DMSO with the lowest average RLU for official experiments.

15.4 Plastic Tissue Culture Materials

1. Grow one set of cells, plate them for experiments on plasticware from the new lot and one set of cells in the plasticware from a previous lot, and dose them with E2 reference standard and controls.
2. Perform the BG1LUC4E2 ER TA experiment with both sets of cells.
3. If all of the analysis falls within acceptable QC criteria, then the new manufacturer's products may be used.

16.0 References

ICCVAM. 2001. Guidance Document on Using *In Vitro* Data to Estimate *In Vivo* Starting Doses for Acute Toxicity. NIH Pub. No. 01-4500. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/invidocs/guidance/iv_guide.pdf [accessed 31 August 2006]

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