Appendix F

BG1LUC4E2 ER TA Antagonist Protocol 11 June 2007

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BG1LUC4E2 ER TA ASSAY

ANTAGONIST PROTOCOL

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

Developed by:
Xenobiotic Detection Systems, Inc.
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11 June 2007

LIST OF ACRONYMS AND ABBREVIATIONS

13 mm test tube	13 x 100 mm glass test tubes
Absolute IC_{50} Value	Concentration of a substance that decreases the measured activity in an antagonist assay to 50% of the maximum activity induced by the reference substance
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	17β-estradiol
E2 Control	2.5×10^{-5} μg/mL E2 used as a control.
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
Flavone/E2 Control	25 μg/mL flavone + 2.5×10^{-5} μg/mL E2, used as a positive control.
G418	Gentamycin
Ral/E2 Reference Standard	Nine point dilution of raloxifene HCl + 2.5×10^{-5} 17β-estradiol reference standard for the LUMI-CELL® ER antagonist assay
Relative IC_{50} Value	Concentration that produces a half-maximal response as calculated using the four parameter Hill function.
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) antagonist 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 BG1LUC4E2 ER TA TA antagonist 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

Xenobiotic Detection Systems, Inc. (XDS), 1601 E. Geer St., Durham, NC 27704

4.2 Key Personnel

- Study Director: John Gordon, Ph.D.
- Laboratory Technician(s): Cynthia Matherly
- Scientific Advisor: Mike Denison, Ph.D.
- Quality Assurance Director: Andrew Chu
- Safety Manager: George Clark
- Facility Management: George Clark, Ph.D., Robert Clark, Tina Ginter
- Laboratory Director: Andrew Chu

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 antagonist protocol are as follows:

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

Ral/E2 reference standard for range finder testing: Three concentrations (1.25×10^{-1}, 1.25×10^{3}, and 5.00×10^{-5} μg/mL) of raloxifene HCl (raloxifene, CASRN 84449-90-1) plus a fixed concentration (2.5×10^{-5} μg/mL) of 17β-estradiol (E2, CASRN: 50-28-2).

Ral/E2 reference standard for comprehensive testing: A serial dilution of raloxifene HCl (raloxifene, CASRN 84449-90-1) plus a fixed concentration (2.5×10^{-5} μg/mL) of 17β-estradiol (E2, CASRN: 50-28-2), consisting of nine concentrations of Ral/E2 in duplicate wells.

E2 control: *17β-estradiol*, CASRN: 50-28-2, 2.5×10^{-5} μg/mL in tissue culture media used as a base line negative control.

Flavone/E2 Control: Flavone, CASRN 525-82-6, 25 μg/mL, with E2 2.5×10^{-5} μg/mL in tissue culture media used as a weak positive control.

6.0 Overview of General Procedures for Antagonist Testing

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

Antagonist range finder testing is conducted on 96-well plates using three concentrations of Ral/E2 (1.25×10^{-1}, 1.25×10^{-3}, and 5.00×10^{-5} μg/mL raloxifene with 2.50×10^{-5} μg/mL E2) in duplicate as the reference standard, with three replicate wells for the E2 and DMSO controls. Range finder testing uses all wells of the 96-well plate.

Comprehensive testing is conducted on 96-well plates using nine concentrations of Ral/E2 in duplicate as the reference standard (**Table 6-1**). Three replicate wells for the DMSO control, Flavone/E2 and E2 controls are included on each plate. In order to avoid edging effects[1], wells on the perimeter of the plate are not used for experiments. These wells should contain tissue culture media only.

[1] Edging effects are variations in response seen in the outermost wells in a cell culture plate. These variations are believed to be due to variations in temperature, evaporation, etc., that may occur in these wells that would ultimately affect cellular growth and health (ICCVAM 2001).

Table 6-1 Concentrations of Ral/E2 Reference Standard Used in Range Finder and Comprehensive Testing

Raloxifene Concentrations[1]	E2 Concentrations
1.25×10^{-2}	2.5×10^{-5}
6.25×10^{-3}	2.5×10^{-5}
3.13×10^{-3}	2.5×10^{-5}
1.56×10^{-3}	2.5×10^{-5}
7.81×10^{-4}	2.5×10^{-5}
3.91×10^{-4}	2.5×10^{-5}
1.95×10^{-4}	2.5×10^{-5}
9.77×10^{-5}	2.5×10^{-5}
4.88×10^{-5}	2.5×10^{-5}

[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. CellTiter-Glo® based cell viability testing (when used) is conducted concurrently in parallel plates on the same day, as outlined in **Section 11.4**.

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 for a positive or negative response as follows:

- A response is considered positive for antagonist activity when the average adjusted RLU for a given concentration is less than the mean RLU value minus three times the standard deviation for the E2 control.
- Any luminescence at or above this threshold is considered a negative response.

For substances that are positive at one or more concentrations, the concentration of test substance that causes a half-maximal response (the relative IC_{50}) 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

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log IC50 - X)\text{HillSlope}}}$$

where Y = response (i.e., relative light units); X = the logarithm of concentration; Bottom = the minimum response; Top = the maximum response; log IC_{50} = the logarithm of X as the response midway between Top and Bottom; and HillSlope describes the steepness of the curve. The model calculates the best fit for the Top, Bottom, HillSlope, and IC_{50} parameters. See **Section 13.6.5** 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 16.0**.

6.1 Range Finder Testing

Antagonist range finding for coded substances consists of a six-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 14.0)**.

6.2 Comprehensive Testing

Comprehensive antagonist 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 15.0)**.

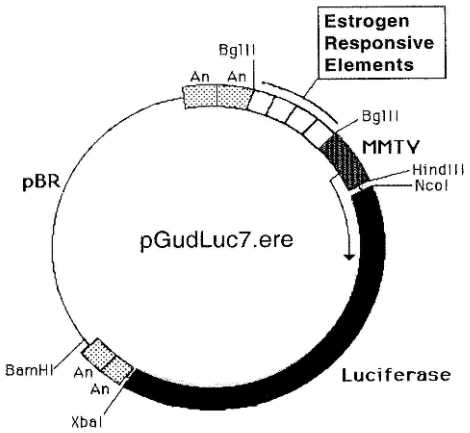
7.0 Materials for BG1LUC4E2 ER TA Antagonist Testing

This section provides the materials needed to conduct BG1LUC4E2 ER TA testing, with associated brand names/vendors[2] 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 (**Figure 7-1**) [XDS].

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 (CO_2 monitoring) (Cat. No. 10-884-1)

[2] 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.

- 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)
- Pipetters, 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 CO_2 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 17.0** to determine suitability for use in this test method.

The following are the necessary tissue culture reagents and possible sources 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]
- CellTiter-Glo® Luminescent Cell Viability Assay [Promega Cat. No. G7572]
- Cryovial, 2 mL (Corning Costar) [Fisher Scientific Cat. No. 03-374-21]
- Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 1000 9186R38][3]
- 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 µm sterile filtered [Hyclone, Cat. No. SH30068.03]
- Flavone (CASRN: 525-82-6) [Sigma-Aldrich, Cat. No. F2003]
- 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]

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

- Lysis Solution 5X [Promega, Cat. No. E1531]
- Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 µg/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]
- Raloxifene (CASRN 84449-90-1) [Sigma-Aldrich Cat. No. R1402]
- RPMI 1640 medium, containing L-glutamine [Mediatech, Cat. No. 10-040-CV]
- Tissue culture flasks (Corning-Costar): 25 cm^2 (T25) [Fisher Cat. No. 10-126-28]; 75 cm^2 (T75) [Fisher Cat. No. 10-126-37]; and 150 cm^2 (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 experiments (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).

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℃ 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℃ 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 10mL 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 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, de-ionized water to the conical tube.
4. Cap and shake gently until solutions are mixed.

Store at -20℃ 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 six months.

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 to mix.
3. Luciferase substrate should readily go into solution.
4. After solutions are mixed aliquot to a 15mL centrifuge tube.
5. Store complete solution at –20°C.

Reconstituted luciferase reagent is stable for 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 37ºC ± 1ºC, 90% ± 5% humidity, and 5.0% ± 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 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 four 96-well plates for use in experiments.

9.1 Procedures for Thawing Cells and Establishing Tissue Cultures

Warm all 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 17.0**).

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 x *g* for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
7. Aspirate media from pellet and re-suspend 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 re-suspend 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,** re-suspending 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 5mL 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, then transfer 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 minutes. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
25. Aspirate the media from the pellet and re-suspend 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 minutes. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
11. Aspirate media from pellet and re-suspend 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 **Section 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.

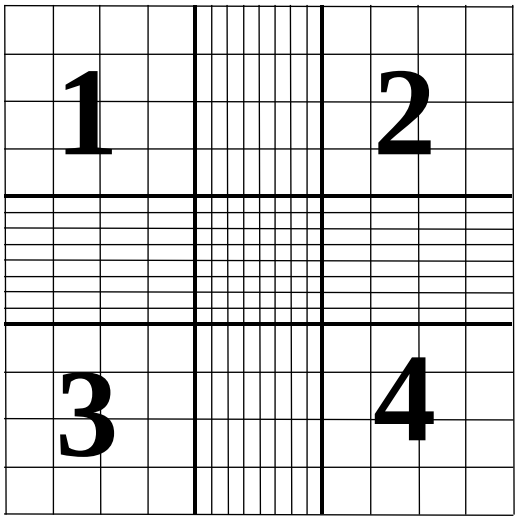
9.2.2 Conditioning in Estrogen-free Medium

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, 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 minutes. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
10. Aspirate off the media from the pellet and resuspend 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 µL 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:

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

<u>Starting dilution: 20mL (for T150 flasks)</u>

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 X 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 200 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 200 mg/mL, prepare a 20 mg/mL solution and vortex as above.
4. If the test substance does not dissolve at 20 mg/mL solution, prepare a 2 mg/mL solution in a 4 mL conical tube and vortex as above.
5. If the test substance does not dissolve at 2 mg/mL, prepare a 0.2 mg/mL solution in a 4 mL conical tube and vortex as above.
6. Continue testing, using 1/10 less substance in each subsequent attempt until test substance is solubilized in DMSO.

Once a solution of test substance has been obtained that does not have any visible precipitate or cloudiness 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.

7. Add 2 µL of the highest concentration of the test substance/DMSO solution to a 13 mm test tube.
8. Add 400 µL estrogen-free DMEM to the test tube and vortex gently,
9. If cloudiness or precipitate develop, vortex for up to 10 minutes.
10. If vortexing does not dissolve test substance, sonicate test substance for up to 10 minutes.
11. If test substance has visible precipitate or is cloudy return to **Section 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.

11.0 Preparation of Reference Standard, Control and Test Substance Stock Solutions for Range Finder and Comprehensive Testing

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

11.1 Preparation of Ral/E2 Stock Solutions

E2 and raloxifene stocks are prepared separately and then combined into Ral/E2 stocks, which are then used to prepare dosing solutions in **Section 12**.

11.1.1 E2 Stock Solution

The final concentration of the E2 stock solution is 5.0 x 10^{-3} µg/mL. Prepare the E2 stock as shown in **Table 11-1**.

Table 11-1 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 100 µL E2 solution from Step #3 to a new glass container large enough to hold 15 mL.	Add 9.90 mL of 100% DMSO. Vortex to mix.	1.0 x 10^{-2} µg/mL
5	Transfer 5 mL E2 solution from Step #4 to a new glass container large enough to hold 15 mL	Add 5 mL of 100% DMSO. Vortex to mix.	5.0 x 10^{-3} µg/mL

11.1.2 Raloxifene Working Stock Solution

Prepare a 2.5 µg/mL raloxifene working stock solution as shown in **Table 11-2**.

Table 11-2 Preparation of Raloxifene Working Stock Solution

Step #	Action	DMSO	Raloxifene Concentration
1	Make a 10 mg/mL solution of raloxifene in a 4 mL glass vial.	-	1.0 x 10^{4} µg/mL
2	Transfer 10 µL raloxifene solution from Step #1 to a new 4 mL vial.	Add 990 µL of 100% DMSO. Vortex to mix.	100 µg/mL
3	Transfer 150 µL raloxifene solution from Step #2 to a new 4 mL vial.	Add 2.850 mL of 100% DMSO. Vortex to mix.	5 µg/mL
4	Transfer 1.5 mL raloxifene solution from Step #3 to a new 13 mm test tube.	Add 1.5 mL of 100% DMSO. Vortex to mix.	2.5 µg/mL

11.2 Ral/E2 Range Finder Stock

11.2.1 Raloxifene Dilutions

Number three 4 mL vials with the numbers 1 to 3 and use the raloxifene solution prepared in **Section 11.1.2** to make raloxifene dilutions as shown **Table 11-3**.

Table 11-3 Preparation of Raloxifene Reference Standard for Range Finder Testing

Step #	Action	DMSO	Raloxifene Concentration
1	Transfer 500 µL of the raloxifene working stock solution to a new 4 mL vial.	-	2.5 µg/mL
2	Transfer 50 µL of the raloxifene working stock solution to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	2.50×10^{-1} µg/mL
3	Transfer 2 µL of the raloxifene working stock solution to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	1.00×10^{-2} µg/mL

11.2.2 Ral/E2 Dilutions for Range Finder Stock

Add 500 µL of the 5×10^{-3} µg/mL E2 solution prepared in **Section 11.1.1** to each of the raloxifene dilution vials prepared in **Section 11.2.1**. Vortex each tube to mix. The final concentrations for raloxifene and E2 are listed in **Table 11-4**.

Table 11-4 Concentrations of Raloxifene and E2 in the Ral/E2 Range Finder Stock Solution

Tube #	Raloxifene (µg/ml)	E2 (µg/ml)
1	1.25	2.5×10^{-3}
2	1.25×10^{-1}	2.5×10^{-3}
3	5.00×10^{-3}	2.5×10^{-3}

11.3 Ral/E2 Comprehensive Testing Stock

11.3.1 Raloxifene Dilutions for Comprehensive Testing Stock

Use the raloxifene solution prepared in **Section 11.1.2** to make a 9-point serial dilution of raloxifene as shown in **Table 11-5**.

Table 11-5 Preparation of Raloxifene 9-Point Serial Dilution

Step #	Action	DMSO	Discard	Raloxifene Concentration
1	Transfer 500 µL of the raloxifene working stock solution to a new 4 mL vial.	-	-	2.5 µg/mL
2	Transfer 500 µL of the raloxifene working stock solution to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	1.25 µg/mL
3	Transfer 500 µL raloxifene solution from Step #2 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	6.25×10^{-1} µg/mL
4	Transfer 500 µL raloxifene solution from Step #3 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	3.13×10^{-1} µg/mL
5	Transfer 500 µL raloxifene solution from Step #4 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	1.56×10^{-1} µg/mL
6	Transfer 500 µL raloxifene solution from Step #5 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	7.81×10^{-2} µg/mL
7	Transfer 500 µL raloxifene solution from Step #6 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	3.91×10^{-2} µg/mL
8	Transfer 500 µL raloxifene solution from Step #7 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.		1.95×10^{-2} µg/mL
9	Transfer 500 µL raloxifene solution from Step #8 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	Discard 500 µL from Tube #9	9.77×10^{-3} µg/mL

11.3.2 Ral/E2 Dilutions for Comprehensive Testing Stock

Add 500 µL of the 5×10^{-3} µg/mL E2 solution prepared in **Section 11.1.1** to each of the nine raloxifene dilution vials (including the working stock solution in Tube #1). Vortex each tube to mix. The final concentrations for raloxifene and E2 are listed in **Table 11-6**.

Table 11-6 Concentrations of Raloxifene and E2 in the Ral/E2 Stock Solution

Tube #	Raloxifene (μg/mL)	E2 (μg/mL)
1	1.25	2.5×10^{-3}
2	6.25×10^{-1}	2.5×10^{-3}
3	3.13×10^{1}	2.5×10^{-3}
4	1.56×10^{-1}	2.5×10^{-3}
5	7.81×10^{2}	2.5×10^{-3}
6	3.91×10^{-2}	2.5×10^{-3}
7	1.95×10^{-2}	2.5×10^{-3}
8	9.77×10^{-3}	2.5×10^{-3}
9	4.88×10^{-3}	2.5×10^{-3}

11.4 Flavone/E2 Stock Solution

To prepare the flavone/E2 stock solution, proceed as follows:

1. Prepare 1 mL of 5 mg/mL flavone
2. Add 1 mL of the 5×10^{-3} μg/mL E2 (prepared as in **Section 11.1.1**) to the 10 mg/mL flavone. This will make a working solution of 2.5 mg/mL flavone with 2.5×10^{-3} μg/mL E2.

12.0 Preparation of Reference Standard, Control and Test Substance Dosing Solutions for Range Finder and Comprehensive Testing

12.1 Preparation of Reference Standard and Control Dosing Solutions for Range Finder Testing

Range finder testing is conducted on 96-well plates using three concentrations of Ral/E2 in duplicate as the reference standard. Three replicate wells for the DMSO, and E2 controls are included on each plate.

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

Dosing solutions are to be used within 24 hours of preparation.

12.1.1 Preparation of Ral/E2 Reference Standard Range Finder Dosing Solutions

1. Label two sets of 13 mm glass tubes with the numbers 1 to 3.
2. Add 4 μL of Ral/E2 stock from tube #1 from **Section 11.2.2** to the two 13 mm glass test tubes labeled #1.
3. Add 4 μL of Ral/E2 stock from tube #2 from **Section 11.2.2** to the two 13 mm glass test tubes labeled #2. Repeat for tubes #3.
4. Add 400 μL of estrogen-free DMEM to each tube and vortex to mix.

12.1.2 Preparation of DMSO Control Range Finder Dosing Solution

1. Add 4 μL of 100% DMSO to three 13 mm glass test tubes.
2. Add 400 μL of estrogen-free DMEM to each tube and vortex to mix.

12.1.3 Preparation of E2 Control Range Finder Dosing Solution

1. Add 2 μL of the E2 stock from **Section 11.1.1** to three 13 mm glass test tubes.
2. Add 400 μL of estrogen-free DMEM to each tube and vortex to mix.

12.2 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. Antagonist range finding for coded substances consists of six-point logarithmic serial dilutions.

To prepare test substance dosing solutions:

1. Label two sets of six glass 13 mm test tubes with the numbers 1 through 6 and place them in a test tube rack. Perform a serial dilution of test substance as shown in **Table 12-1**.

Table 12-1 Preparation of Test Substance Serial Dilution for Range Finder Testing

Tube #	100% DMSO	Test Substance[1]	Final Volume
1	-	10 µL of test substance solution from **Section 10.1**	100 µL
2	90 µL	10 µL of test substance solution from **Section 10.1**	100 µL
3	90 µL	10 µL from Tube #2	100 µL
4	90 µL	10 µL from Tube #3	100 µL
5	90 µL	10 µL from Tube #4	100 µL
6	90 µL	10 µL from Tube #5	100 µL

[1] Vortex tubes #2 through 5 before removing test substance/DMSO solution to place in the next tube in the series.
[2] Transfer test substance/DMSO solutions to a new set of 13 mm test tubes.
[2] Vortex all tubes to mix media, test substance, and E2.

2. Transfer test substance/DMSO solutions to new tubes and add E2 as shown in **Table 12-2**.

Table 12-2 Addition of E2 to Test Substance Serial Dilution for Range Finder Testing

Tube Number	Test Substance	E2	Estrogen-free DMEM[3]	Final Volume
1	Transfer 4 µL of test substance from Tube #1 in **Section 12.2 step 1** to a new tube	Add 4 µL of the 5 x 10^{-3} µg/mL E2 solution prepared in **Section 11.1.1**. Vortex to mix.	800 µL	808 µL
2	Transfer 4 µL of test substance from Tube #2 to a new tube	Add 4 µL of the 5 x 10^{-3} µg/mL E2 solution prepared in **Section 11.1.1** Vortex to mix.	800 µL	808 µL
3	Transfer 4 µL of test substance from Tube #3 to a new tube	Add 4 µL of the 5 x 10^{-3} µg/mL E2 solution prepared in **Section 11.1.1**. Vortex to mix.	800 µL	808 µL
4	Transfer 4 µL of test substance from Tube #4 to a new tube	Add 4 µL of the 5 x 10^{-3} µg/mL E2 solution prepared in **Section 11.1.1**. Vortex to mix.	800 µL	808 µL
5	Transfer 4 µL of test substance from Tube #5 to a new tube	Add 4 µL of the 5 x 10^{-3} µg/mL E2 solution prepared in **Section 11.1.1**. Vortex to mix.	800 µL	808 µL
6	Transfer 4 µL of test substance from Tube #6 to a new tube	Add 4 µL of the 5 x 10^{-3} µg/mL E2 solution prepared in **Section 11.1.1**. Vortex to mix.	800 µL	808 µL

Determination of whether a substance is positive in range finder testing and selection of starting concentrations for comprehensive testing will be discussed in **Section 14.0**.

12.3 Preparation of Reference Standard and Control Dosing Solutions for Comprehensive Testing

Comprehensive testing is conducted on 96-well plates using nine concentrations of Ral/E2 in duplicate as the reference standard. Three replicate wells for the DMSO, E2 and flavone/E2 controls are included on each plate.

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

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

12.3.1 Preparation of Ral/E2 Reference Standard Dosing Solutions for Comprehensive Testing

In preparation for making Ral/E2 double serial dilutions, label two sets of nine glass 13 mm test tubes with the numbers 1 through 9 and place them in a test tube rack. Tube number 1 will contain the highest concentration of raloxifene (**Table 12-3**).

Table 12-3 Preparation of Ral/E2 Reference Standard Dosing Solution for Comprehensive Testing

Tube Number	Ral/E2 Stock	Estrogen-free DMEM	Final Volume
1	6 µL of Tube #1 from **Section 11.3.2**	600 µL	606 µL
2	6 µL of Tube #2 from **Section 11.3.2**	600 µL	606 µL
3	6 µL of Tube #3 from **Section 11.3.2**	600 µL	606 µL
4	6 µL of Tube #4 from **Section 11.3.2**	600 µL	606 µL
5	6 µL of Tube #5 from **Section 11.3.2**	600 µL	606 µL
6	6 µL of Tube #6 from **Section 11.3.2**	600 µL	606 µL
7	6 µL of Tube #7 from **Section 11.3.2**	600 µL	606 µL
8	6 µL of Tube #8 from **Section 11.3.2**	600 µL	606 µL
9	6 µL of Tube #9 from **Section 11.3.2**	600 µL	606 µL

12.3.2 Preparation of DMSO Control Comprehensive Testing Dosing Solution

1. Add 8 µL of 100% DMSO to three 13 mm glass test tubes.
2. Add 800 µL of estrogen-free DMEM to each tube and vortex to mix.

12.3.3 Preparation of E2 Control Comprehensive Testing Dosing Solution

1. Add 4 µL of the E2 stock from **Section 11.1.1** to three 13 mm glass test tube.
2. Add 800 µL of estrogen-free DMEM to each tube and vortex to mix.

12.3.4 Preparation of Flavone/E2 Control Comprehensive Dosing Solution

1. Add 8 µL of flavone/E2 from **Section 11.4** to three 13 mm glass test tubes.
2. Add 800 µL of estrogen-free DMEM to each tube and vortex to mix.

12.4 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

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

To prepare test substance dosing solutions for comprehensive testing:

1. Determine the concentration at which maximal antagonism occurs in the range finder experiment. Start an 11-point serial dilution curve at a 20-fold higher concentration than the concentration causing a maximal antagonist response (i.e., if the maximum antagonist response occurred at 0.01 mg/mL, start the serial dilution curve at 0.2 mg/mL).
2. If there is no observable antagonist response in the range finder experiment, start the serial dilution at the maximum soluble concentration.
3. Label three sets of 11 glass 13 mm test tubes with the numbers 1 through 11 and place them in a test tube rack. Prepare three serial dilutions as indicated in **Table 12-4**.

Table 12-4 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

Tube Number	100% DMSO	Test Substance[1]	Discard	E2 Testing Stock	Estrogen-free DMEM[2]	Final Volume
1	-	4 µL of test substance solution from **Section 10.2.4 step 1**	-	4 µL	800 µL	808 µL
2	4 µL	4 µL of test substance solution from **Section 10.2.4 step 1**	-	4 µL	800 µL	808 µL
3	4 µL	4 µL from Tube #2	-	4 µL	800 µL	808 µL
4	4 µL	4 µL from Tube #3	-	4 µL	800 µL	808 µL
5	4 µL	4 µL from Tube #4	-	4 µL	800 µL	808 µL
6	4 µL	4 µL from Tube #5	-	4 µL	800 µL	808 µL
7	4 µL	4 µL from Tube #6	-	4 µL	800 µL	808 µL
8	4 µL	4 µL from Tube #7	-	4 µL	800 µL	808 µL
9	4 µL	4 µL from Tube #8	-	4 µL	800 µL	808 µL
10	4 µL	4 µL from Tube #9	-	4 µL	800 µL	808 µL
11	4 µL	4 µL from Tube #10	4 µL	4 µL	800 µL	808 µL

[1] Vortex tubes #2 through 10 before removing test substance/DMSO solution to place in the next tube in the series.

[2] Vortex all tubes to mix media, test substance, and E2.

13.0 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 antagonist activity in the BG1LUC4E2 ER TA test method.

General procedures for range finder and comprehensive testing are nearly identical. For specific details (such as plate layout) of range finder testing see **Section 14.0**. For specific details of comprehensive testing, see **Section 15.0**.

13.1 Application of Reference Standard, Control and Test Substances

1. Remove the 96-well plates (from **Section 9.2.3 step 18)** from the incubator; inspect them using an inverted microscope. Only use plates in which the cells in all wells receive 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 medium, reference standard, control or test substance to each well (see **Sections 14.0** and **15.0** for specific plate layouts).
4. Return plates to incubator (see **Section 9.0** for details) for 19 to 24 hours to allow maximal induction of luciferase activity in the cells.

13.1.1 Preparation of Excel® Data Analysis Template

1. In Excel®, open a new "AntICCVAMTemplate" 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.

13.2 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 13-1**.

Table 13-1 Visual Observation Scoring

Viability Score	Brief Description[1]
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.”

13.3 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 30µL 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 13.4**).

13.4 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 relative light units (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.

13.5 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. Plate reduction is calculated using unadjusted RLU values.

The Excel® spreadsheet subtracts background luminescence (average DMSO solvent control RLU value) from test substance, reference standard and control RLU values. Test substance, reference standard, and control RLU values are then adjusted relative to the highest Ral/E2 reference standard RLU value, which

is set to 10,000. After adjustment, values are transferred to GraphPad Prism® for data analysis and graphing.

13.5.1 Correction and Adjustment of Luminometer Data

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 13.1.1**.
2. Copy the raw data using the Excel® copy function, then paste the copied data into cell C22 of the "RAW DATA" tab in the experimental Excel® spreadsheet using the Paste Special – Values command. This position corresponds to position B2 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 13.5.2** 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 G24, adjust the calculation in cell H42 to read =AVERAGE(F24.H24,I24)
   - Then correct the equation used to calculate the average DMSO value in Table 2, e.g., following the above example, adjust cell M44 to read =AVERAGE(F36,H36,I36)
   - 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 M45 to read =STDEV(F36,H36,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 reduction, identify the cell containing the Ral/E2 replicate that has the lowest RLU value and the cell containing the RLU value for the same concentration in the corresponding Ral/E2 replicate (e.g., if the lowest RLU value for Ral/E2 is located in cell E23, the corresponding cell would be E22).
7. Identify the cell containing the Ral/E2 replicate that has the highest RLU value and the cell containing the RLU value for the same concentration in the corresponding Ral/E2 replicate.
8. Click into cell D16 and enter the cell numbers from **Section 13.5.1 step 6** into the numerator and the cell numbers from **step 7** into the denominator.
9. Click on the "ER Antagonist Report" tab.
10. The data for the Ral/E2 reference standard, DMSO, E2, and Flavone/E2 replicates populate the left portion (columns A-F) of the spreadsheet. The data is automatically placed into an Excel® graph.
11. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell D2 of "ER Antagonist Report" tab and check the formula contained within that cell. The divisor should be the cell number of the cell containing the highest averaged Ral/E2 RLU value (column E).
12. Forecast the EC50 value using the formula (FORECAST(x,known_y's,known_x's), where x = the data point for which you wish to predict a value [i.e., the median RLU], known y's = the three RLU values that constitute the middle of the linear portion of the concentration-response curve, and known x's = the concentrations corresponding to the known y's.
13. Copy the data into GraphPad Prism® for graphing and analysis as indicated in the NICEATM Prism® users guide.

13.5.2 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:

$$\frac{Outlier - Nearest\ Neighbor}{Range\ (Highest - Lowest)}$$

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 13-2). 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 13-2 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 an outlier if its value is more than 20% above or below the adjusted RLU value for that concentration in the historical database.

13.5.3 Acceptance Criteria

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.

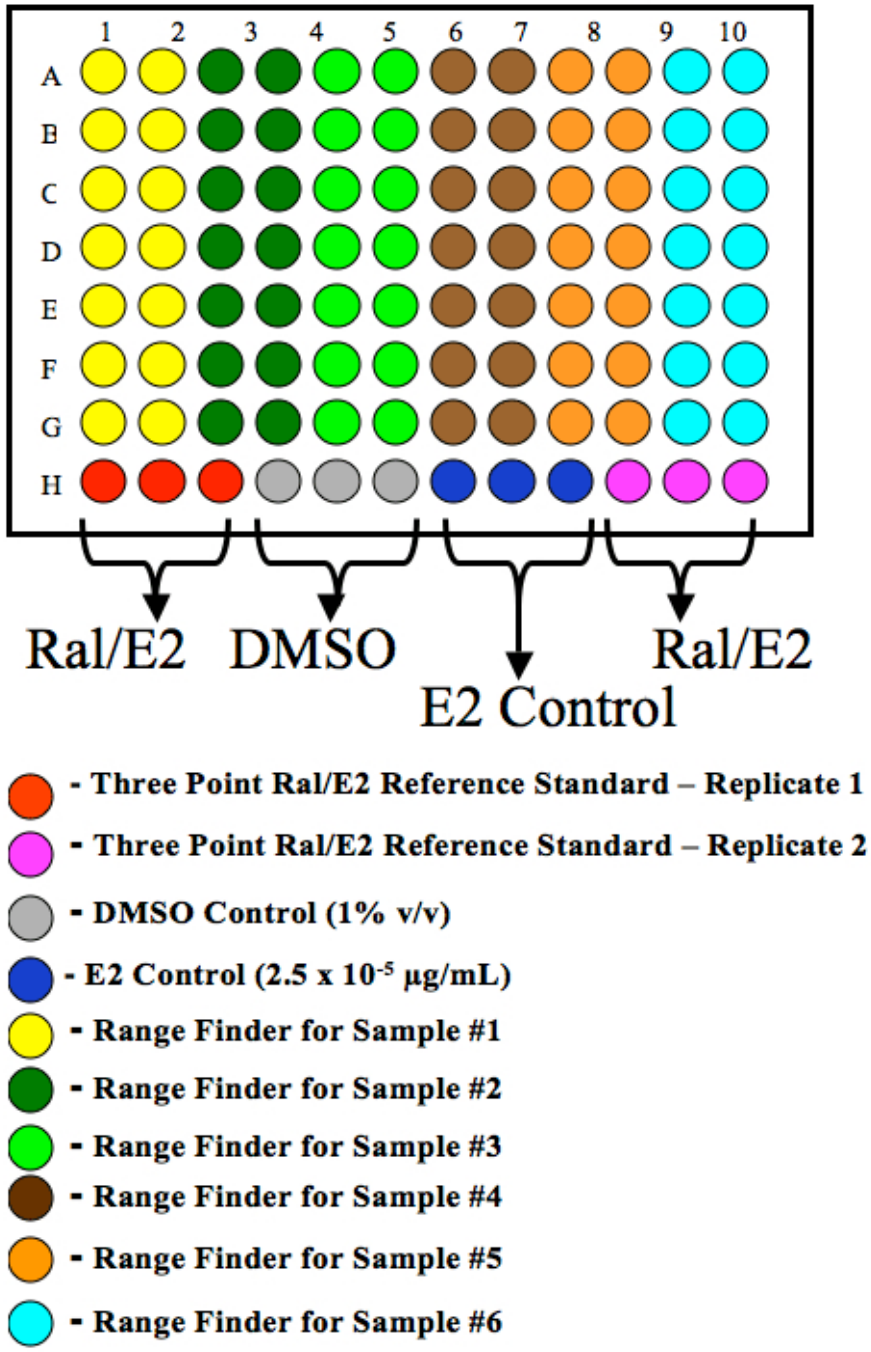
- Reduction: Plate reduction, as measured by dividing the averaged highest Ral/E2 reference standard RLU value by the averaged lowest Ral/E2 reference standard RLU value, must be greater than three fold.
- Reference standard results: Calculated Ral/E2 reference standard IC_{50} values must be within 2.5 times the standard deviation of the historical database IC_{50} mean value.
- Solvent control results: DMSO control RLU values must be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- E2 control results: E2 control RLU values must be within 2.5 times the standard deviation of the historical E2 control mean RLU value.
- Positive control results: Flavone/E2 control RLU values must be within 2.5 times the standard deviation of the historical database flavone/E2 control mean RLU value.

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

14.0 Range Finder Testing

Antagonist range finding for coded substances consists of six-point logarithmic serial dilutions tested in duplicate wells of the 96-well plate. **Figure 14-1** contains a template for the plate layout used in antagonist range finder testing.

Figure 14-1 Antagonist Range Finder Test Plate Layout



Evaluate whether range finder experiments have met the acceptance criteria (see **Section 13.6.3**) and graph the data as described in the NICEATM Prism® users guide.

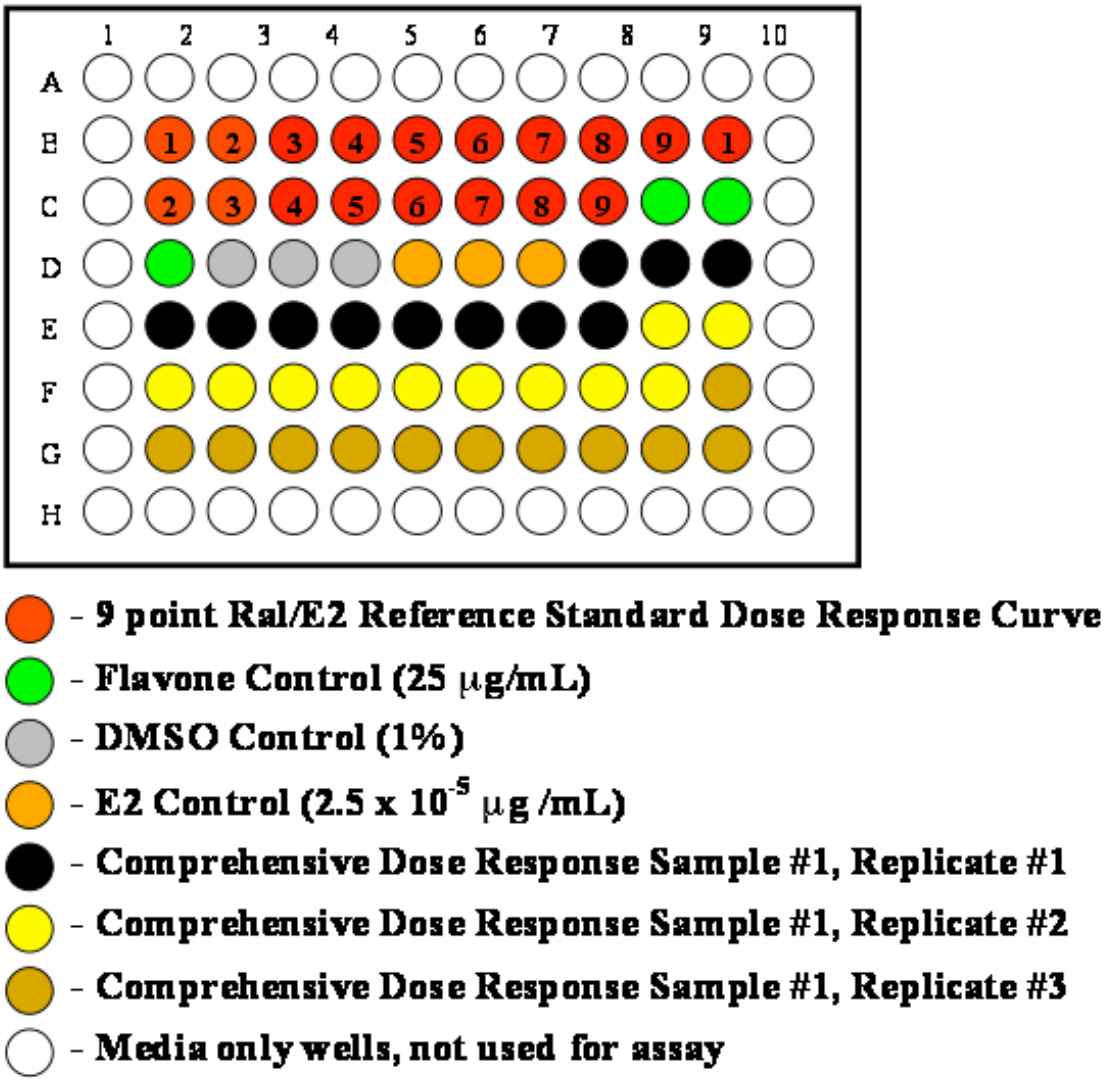
To determine starting concentrations for comprehensive testing use the following criteria:

- If there are no points on the test substance concentration curve that are less than the mean value of the E2 control minus three times the standard deviation from that mean, 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 below the mean value of the E2 standard control minus three times the standard deviation from that mean, select a concentration that is a single log dilution higher than the concentration giving the lowest 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 lowest adjusted RLU value in the range finder and use that as the highest concentration for comprehensive testing.

15.0 Comprehensive Testing

Antagonist 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 15-1** contains a template for the plate layout to be used in antagonist comprehensive testing.

Figure 15-1 Antagonist Comprehensive Test Plate Layout



Evaluate whether comprehensive experiments have met acceptance criteria (see **Section 13.6.3**) 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 below the line indicating the mean minus three times the standard deviation of the E2 control, the substance is considered negative for antagonism.
- If the substance has been tested up to the limit dose and there are points on the concentration curve that are below the line indicating the mean minus three times the standard deviation of the E2 control, but cell viability has a visual inspection score of 2 or greater, at all points falling below the E2 line, the substance is considered negative for antagonism.
- If there are points on the test substance concentration curve that are below the line indicating the mean minus three times the standard deviation of the E2 control that do not cause a visual inspection score of 2 or greater, the substance is positive for antagonism.

– Points in the test substance concentration curve that cause a visual inspection score of 2 or greater are not included in data analyses.

16.0 Use of the Historical Database to Generate QC Charts

The historical database is maintained in order to ensure that the test method is functioning properly. The historical database is maintained as an Excel® spreadsheet that is separate from the spreadsheets used to report the data for individual experiments. The controls used to develop the historical database are used as one of the criteria for determining a valid test.

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 II. 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.

16.1 LUMI-CELL® ER Antagonist QC Charts

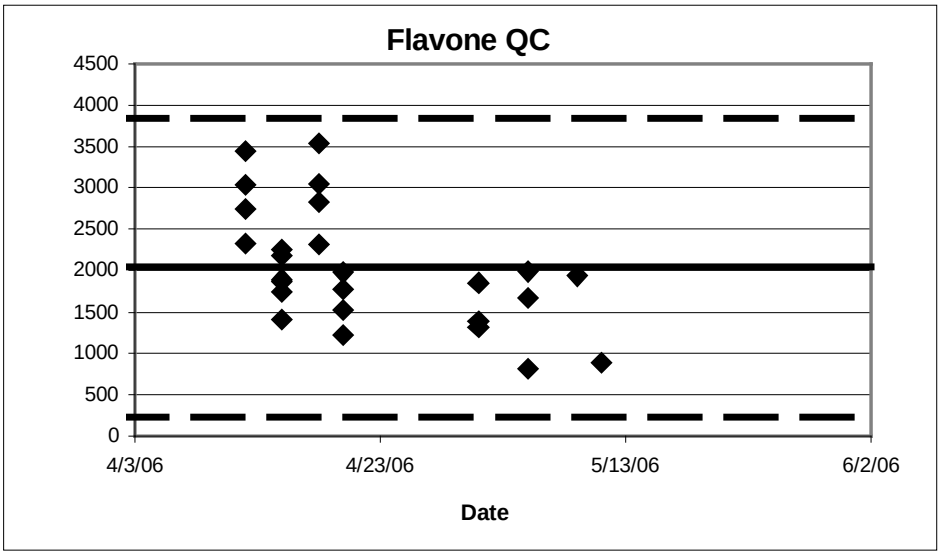
1. Open the Excel® spreadsheet labeled LUMIAntagonistQC.
2. Save this sheet under a new name, adding the laboratory designator to the file name (e.g., for Laboratory A, the new name would be ALUMIAntagonistQC).

16.1.1 Flavone/E2

1. Open Excel® spreadsheet XDSLUMIAntQC.
2. Click on flavone tab and enter the date, plate number (name), and the average 1.56×10^{-3} µg/mL RLU value for ral/E2 (data located in column F on the “List” tab of the antagonist report file).
3. Enter the three values for flavone/E2 into column D.
4. The mean and 2.5 times the standard deviation plus (and minus) the mean are calculated automatically.
5. Check the Scatter Charts tab to see if the average value for flavone/E2 falls within 2.5 times the standard deviation (e.g., **Figure 16-1**) of the historical mean. If the mean flavone/E2 value falls within the 2.5 times the standard deviation area, it passes QC. Otherwise it fails QC and the experiment must be repeated.

Acceptance or rejection of the flavone/E2 control data is based on whether the data for a given experiment falls within 2.5 times the standard deviation from the historical mean RLU value.

Figure 16-1 Example Scatter Chart of the Flavone/E2 Control QC[1,2,3]



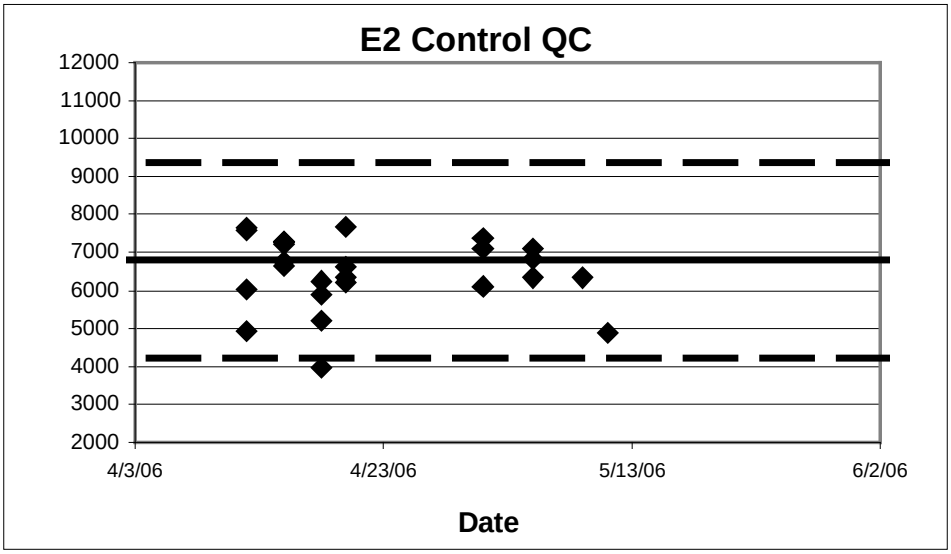
[1] Each point represents a single experiment.
[2] The solid line represents the historical mean RLU value for the flavone control.
[3] The two dashed lines represent the historical mean RLU value for the flavone control plus and minus 2.5 times the standard deviation from the historical mean.

16.1.2 E2 Control

1. Click on the E2 control tab. The date, study name, and 1.56 x 10^{-3} μg/mL ral/E2 data should be populated in the appropriate position.
2. Enter the values for the E2 control into column D.
3. The mean and 2.5 times the standard deviation plus (and minus) the mean are calculated automatically.
4. Check the scatter charts tab to see if the average value for the E2 control falls within the 2.5 times the standard deviation (**Figure 16-2**) from the historical mean. If the value falls within the 2.5 times the standard deviation area, the E2 control passes QC. Otherwise it fails QC and the study must be repeated.

Acceptance or rejection of the E2 control data is based on whether the data for a given experiment falls within 2.5 times the standard deviation from the historical mean RLU value.

Figure 16-2 Example Scatter Chart of the E2 Control QC



[1] Each point represents a single experiment.

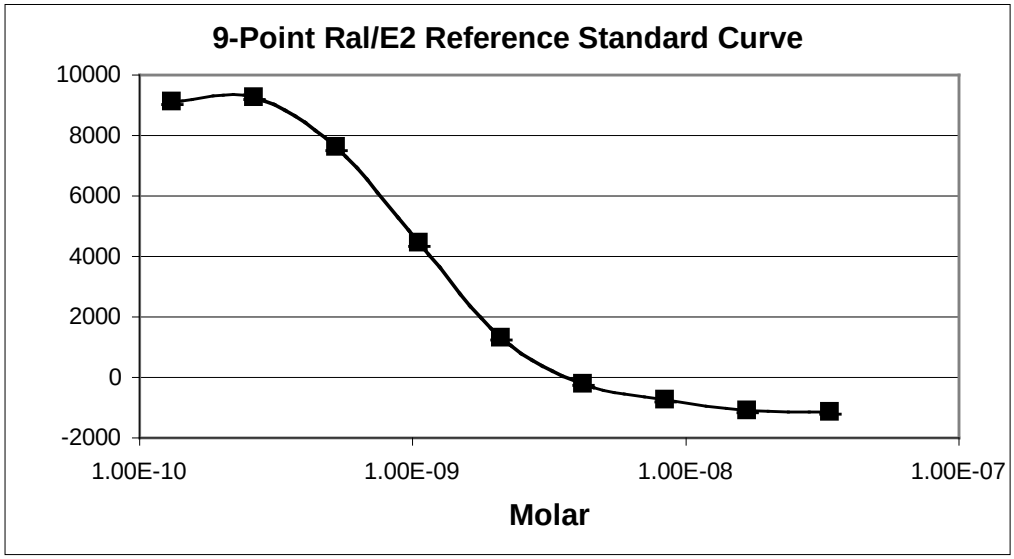
[2] The solid line represents the historical mean RLU value for the E2 control.

[3] The two dashed lines represent the historical mean RLU value for the E2 control plus and minus 2.5 times the standard deviation from the historical mean.

16.1.3 9-Point Ral/E2 Reference Standard QC

1. Enter the experiment date and name, and copy and paste the adjusted RLU values for Ral/E2 into the appropriate slots on the tab labeled Ral E2 Standard Curve.
2. The Ral/E2 standard curve is automatically graphed to ensure a normal sigmoidal shape (see **Figure 16-3** for an example curve).

Figure 16-3 Example Figure of a Sigmoidal Ral/E2 Concentration Response Curve[1]

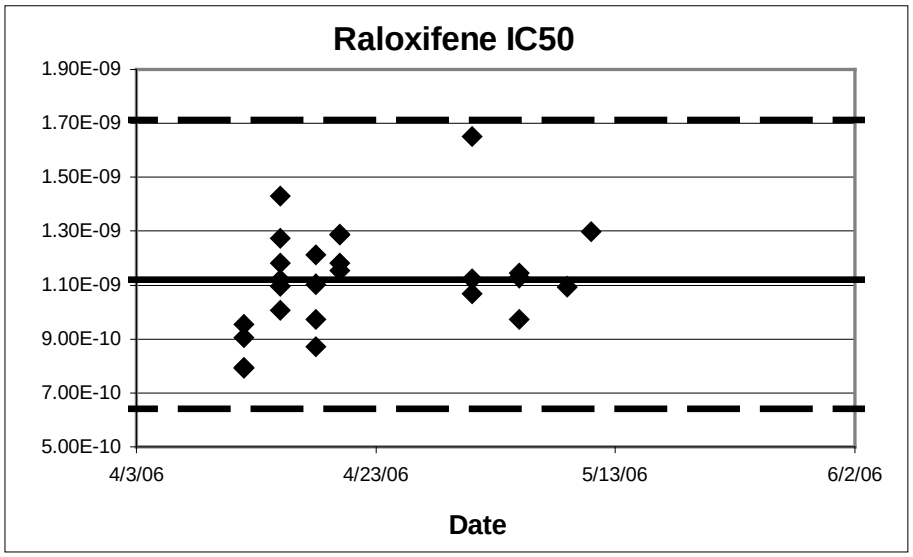


[1] The points on the line represent the averaged Ral/E2 values for a single experiment.

16.1.4 IC_{50} Tracking Data

1. Enter the date and plate ID into the first two columns of the IC_{50} Tracking Data tab.
2. Link the EC_{50} data from the 9-point Ral/E2 Curve QC tab to the column to the right of the plate information.
3. Column E calculates the percent deviation from the historical database IC_{50} value.
4. The mean and 2.5 times the standard deviation plus (and minus) the mean for the IC_{50} deviation are calculated automatically.
5. Check the Scatter Charts tab to see whether the experimental IC_{50} value falls within the 2.5 times the standard deviation (**Figure 16-4**). If the value falls within the 2.5 times the standard deviation area, it passes QC. If the value does not fall within the 2.5 times the standard deviation, it fails QC and the experiment must be repeated.

Figure 16-4 Example Scatter Chart of the Ral/E2 IC_{50} Control QC



[1] Each point represents a single experiment.

[2] The solid line represents the historical mean RLU value for the Ral/E2 EC_{50}.

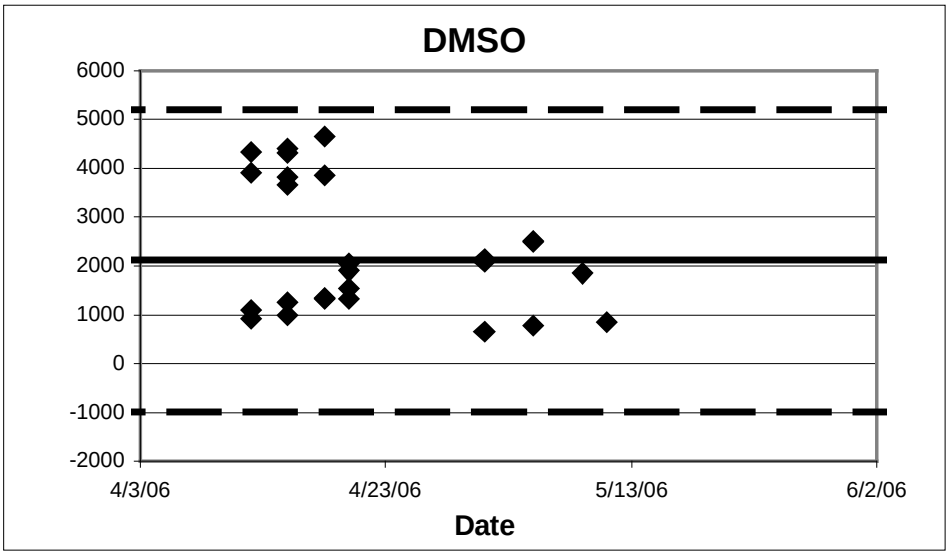
[3] The two dashed lines represent the historical mean RLU value for the Ral/E2 control plus and minus 2.5 times the standard deviation from the historical mean.

Acceptance or rejection of Ral/E2 IC_{50} data is based on whether the data falls within 2.5 times the standard deviation from the mean for the historical Ral/E2 IC_{50} RLU value.

16.1.5 DMSO

1. The date and experiment name should populate automatically.
2. Enter all of the DMSO values from Table 1 on the "Raw Data" tab on the Excel® spreadsheet which passed the outlier test, into the areas marked DMSO 1, DMSO 2, DMSO 3, and DMSO 4.
3. The average RLU value for DMSO is then calculated under the "mean" column.
4. The mean and 2.5 times the standard deviation plus (and minus) the mean for the DMSO deviation are calculated automatically.
5. Check the Scatter Charts tab to see whether the average value for DMSO falls within 2.5 times the standard deviation (**Figure 16-5**) from the mean. If the value falls within the 2.5 times the standard deviation area, the DMSO passes QC. If the value does not fall within the 2.5 times the standard deviation, it fails QC and the experiment must be repeated.

Figure 16-5 Example Scatter Chart of the DMSO Control QC[1,2,3]



[1] Each point represents a single experiment.
[2] The solid line represents the historical mean RLU value for the DMSO control.
[3] The two dashed lines represent the historical mean RLU value for the DMSO control plus and minus 2.5 times the standard deviation from the historical mean.

Acceptance or rejection of the DMSO control data is based on whether the data for a given experiment falls within 2.5 times the standard deviation from the historical mean RLU value.

16.1.6 Reduction

Reduction is calculated by dividing the highest RLU value for each of the Ral/E2 standards by the lowest Ral/E2 RLU value. To calculate reduction, follow the steps below:

Enter the reduction value from the “Raw Data” tab on the Excel® spreadsheet. If the value is greater than or equal to 3, the experiment passed QC. An induction value of less than 3 fails induction QC and the experiment must be repeated.

17.0 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.

17.1 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 13 mm tube.
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 QC charts to determine if the new media with DMSO lies within 2.5 standard deviation of the mean for the media. If the RLU values for the new media with DMSO are 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 are not within 2.5 standard deviation of the mean for the historical data, 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 the QC as described in **Section 15.1 step 6**, apply the media to a single flask cells and observe the cells growth and morphology over the following 2 to 3 days. If there is no change in growth or morphology, the new media is acceptable for use.

17.2 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.

17.3 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) the same tubes.
4. Dose an experimental plate as in **Section 15.0,** treating the media being tested as a test substance.
5. Analyze 96-well plate as described in **Section 15.0,** comparing the data from the DMSO controls made using previously tested tissue culture media to the new media being tested.
6. Use the QC charts to determine if the new media with DMSO lies within 2.5 standard deviation of the mean for the media. If the RLU values for the new media with DMSO are 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 are not within 2.5 standard deviation of the mean for the historical data the new lot may not be used in the assay.
7. Use the QC charts to determine if the new DMSO is within 2.5 standard deviation of the mean for DMSO background. If the RLU for the new DMSO is within 2.5 standard deviation of the mean for the historical data on DMSO, then the new bottle of DMSO is acceptable; otherwise the new bottle may not be used in the assay.
8. Note the date, lot number, and bottle number in study book.
9. 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 higher average RLU than the other bottle(s) tested. Use the DMSO with the lowest average RLU for official experiments.

17.4 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.

18.0 References

Eli Lilly and Company and National Institutes of Health Chemical Genomics Center. 2005. Assay Guidance Manual Version 4.1. Bethesda, MD: National Institutes of Health. Available: http://www.ncgc.nih.gov/guidance/manual_toc.html [accessed 05 September 2006]

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]