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 re-suspend it in 4 mL estrogen-free DMEM, drawing the pellet repeatedly through a 25 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.

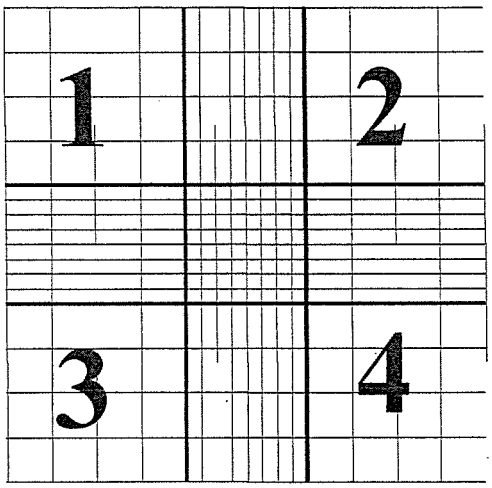
4. Tissue culture medium may need to be changed 24 hours after addition of G418 to remove cells that have died because they do not express reporter plasmid.
5. G418 does not need to be added to the flasks a second time.
6. Place the T150 flasks in the incubator (see conditions in **Section 9.0**) and 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 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).

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 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 DMEM Growth media 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 except the outside ring of wells.

16. Use a repeater pipetter to pipette 200 µL of estrogen-free DMEM to the outer wells of the 96-well plate.
17. 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 15 mL conical tube.
2. Vortex to mix.
3. If the substance does not go into solution after vigorous vortexing, sonicate for 10 min.
4. If the test substance does not dissolve at 100 mg/mL, add an additional 9 mL of DMSO to the conical tube (10 mg/mL), then vortex and/or sonicate for 10 min. as before.
5. If the test substance does not dissolve at 10 mg/mL solution, prepare a 1 mg/mL solution in a 15 mL conical tube. Vortex and/or sonicate for 10 min. as before.

6. If the test substance does not dissolve at 1 mg/mL, add an additional 9 mL of DMSO to the conical tube (100 µg/mL), then vortex and/or sonicate for 10 min. as before.

7. 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 LUMI-CELL® ER testing.

8. Add 4 µL of the highest concentration of the test substance/DMSO solution to a 13 mm test tube.

9. Add 400 µL estrogen-free DMEM to the test tube and vortex gently,

10. If cloudiness or precipitate develop, vortex for up to 10 minutes.

11. If vortexing does not dissolve test substance, sonicate test substance for up to 10 minutes.

12. 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.

735 10.2.1.1 *E2 Stock Solution*

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

738 **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.	0.1 µg/mL

739 10.2.1.2 *Methoxychlor Stock Solution*

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

741 To prepare the methoxychlor stock solution, proceed as follows:

742 1. Make a 10 mg/mL stock solution of Methoxychlor in 100% DMSO in a 4 mL
743 vial.

744 2. Remove 94 µL of the methoxychlor solution and place it in a new 4 mL vial.

745 3. Add 2.906 mL of 100% DMSO to the 4mL vial and gently vortex to mix.

746 10.2.2 Preparation of Reference Standard and Positive Control Dosing Solutions for Range
747 Finder Testing

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

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

752

10.2.2.1 *Preparation of E2 Reference Standard Dosing Solutions for Range Finder Testing*

In preparation for making E2 dosing solutions, label two sets of three glass 13 mm test tubes with the numbers one through three and place them in a test tube rack. Tube number 1 will contain the highest concentration of E2 (**Table 10-2**).

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

Tube Number	E2	Estrogen-free DMEM[1]	Final Volume	Final E2 Concentration
1	4 µl of 1.0 x 10^{-2} µg/mL working solution	400 µL	404 µL	1.00 x 10^{-3}µg/mL
2	8 µL of 1.0 x 10^{-3} µg/mL from Tube #1	400 µL	408 µL	2.00 x 10^{-5}µg/mL
3	1 µL of 1.0 x 10^{-3} µg/mL from Tube#1	4000 µL	4001 µL	1.00 x 10^{-6}µg/mL

[1]Vortex all tubes to mix media and E2.

10.2.2.2 *Preparation of DMSO Control Dosing Solution for Range Finder Testing*

1. Add 4 µL of 100% DMSO to six 13 mm tubes (solvent/negative controls).
2. Add 400 µL of estrogen-free DMEM to each tube and vortex vigorously.

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 six point, logarithmic serial dilutions run in duplicate.

Label two sets of six glass 13 mm test tubes with the numbers 1 through 6 and place them in a test tube rack. Tube number 1 will contain the highest concentration of test substance (**Table 10-3**).

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

Tube Number	100% DMSO	Test Substance[1]	Transfer[2]	Estrogen-free DMEM[3]	Final Volume
1	-	4 µL of test substance solution from **Section 10.1** step **14**	4 µL	400 µL	404 µL
2	90 µL	10 µL of test substance solution from **Section 10.1** step **14**	4 µL	400 µL	404 µL
3	90 µL	10 µL from Tube #2	4 µL	400 µL	404 µL
4	90 µL	10 µL from Tube #3	4 µL	400 µL	404 µL
5	90 µL	10 µL from Tube #4	4 µL	400 µL	404 µL
6	90 µL	10 µL from Tube #5	4 µL	400 µL	404 µL

[1]Vortex tubes #2 through 5 before removing test substance 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.
[3]Vortex all tubes to mix media and test substance solution.

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 10 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 Dosing Solutions for Comprehensive Testing*

In preparation for making E2 double serial dilutions, label two sets of 11 glass 13 mm test tubes with the numbers 1 through 11 and place them in a test tube rack. Tube number 1 will contain the highest concentration of E2 (**Table 10-4**).

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

Tube Number	100% DMSO	E2[1]	Discard	Estrogen-free DMEM[2]	Final Volume
1	-	4 µL of 1.0 x 10^{-2} µg/mL working solution	-	400 µL	404 µL
2	4 µL	4 µL of 1.0 x 10^{-2} µg/mL stock solution	-	400 µL	404 µL
3	4 µL	4 µL from Tube #2	-	400 µL	404 µL
4	4 µL	4 µL from Tube #3	-	400 µL	404 µL
5	4 µL	4 µL from Tube #4	-	400 µL	404 µL
6	4 µL	4 µL from Tube #5	-	400 µL	404 µL
7	4 µL	4 µL from Tube #6	-	400 µL	404 µL
8	4 µL	4 µL from Tube #7	-	400 µL	404 µL
9	4 µL	4 µL from Tube #8	Discard Tube #9	-	-
10	4 µL	4 µL from Tube #9	-	400 µL	404 µL
11	4 µL	4 µL from Tube #10	Remove and discard 4 µL from Tube #11	400 µL	404 µL

[1]Vortex tubes #2 through 11 before removing E2 solution to place in the next tube in the series.

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

10.2.4.2 *Preparation of Methoxychlor Control Dosing Solution for Comprehensive Testing*

1. Add 4 µL of the 313 µg/mL methoxychlor to three separate 13 mm tubes.
2. Add 400 µL of estrogen-free DMEM to each tube and vortex vigorously.

10.2.4.3 *Preparation of DMSO Control Dosing Solution for Comprehensive Testing*

1. Add 4 µL of 100% DMSO to four 13 mm tubes (solvent/negative controls).
2. Add 400 µL of estrogen-free DMEM to each tube and vortex vigorously.

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 LUMI-CELL® ER 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).

Label three sets of 11 glass 13 mm test tubes with the numbers 1 through 11 and place them in a test tube rack. Tube number 1 will contain the highest concentration of test substance (**Table 10-4**).

Table 10-4 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

Tube Number	100% DMSO	Test Substance[1]	Discard	Estrogen-free DMEM[2]	Final Volume
1	-	4 µL of highest concentration of test substance solution	-	400 µL	404 µL
2	4 µL	4 µL of highest concentration of test substance solution	-	400 µL	404 µL
3	4 µL	4 µL from Tube #2	-	400 µL	404 µL
4	4 µL	4 µL from Tube #3	-	400 µL	404 µL
5	4 µL	4 µL from Tube #4	-	400 µL	404 µL
6	4 µL	4 µL from Tube #5	-	400 µL	404 µL
7	4 µL	4 µL from Tube #6	-	400 µL	404 µL
8	4 µL	4 µL from Tube #7	-	400 µL	404 µL
9	4 µL	4 µL from Tube #8	-	400 µL	404 µL
10	4 µL	4 µL from Tube #9	-	400 µL	404 µL
11	4 µL	4 µL from Tube #10	4 µL	400 µL	404 µL

[1]Vortex tubes #2 through 11 before removing test substance solution to place in the next tube in the series.
[2]Vortex all tubes to mix media and test substance solution.

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 LUMI-CELL® ER 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 12.0**. For specific details of comprehensive testing, see **Section 13.0**.

11.1 Application of Reference Standard, Controls, and Test Substances

1. Remove the 96-well plates 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 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.

11.1.1 Preparation of Excel® Data Analysis Template

1. In Excel®, open a new "AgICCVAMTemplate" 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 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
1P	Score of 1 with Precipitate
2P	Score of 2 with Precipitate
3P	Score of 3 with Precipitate
4P	Score of 4 with Precipitate
5P	Unable to View Cells Due to Precipitate

[1]Reference micrographs will be provided by NICEATM.

11.3 Lysis of Cells for LUMI-CELL® ER

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 11.5**).

11.4 CellTiter-Glo® Assessment of Cell Viability

When considered necessary, a quantitative evaluation of cell viability will be performed with the Promega CellTiter-Glo® assay system. CellTiter-Glo® uses luminescence as an indicator of the

number of cells per plate and therefore must be conducted in parallel with the LUMI-CELL® ER test method (i.e., both test methods cannot be conducted on the same plate).

1. Dose and incubate cells under the same conditions as for LUMI-CELL® ER.
2. Remove plates from incubator and discard the medium by inverting the plate onto blotter paper. Gently tap plate against the bench surface to remove residual liquid trapped in the wells.
3. Use a repeat pipettor to add 50 µL 1X PBS to all assay wells. Immediately remove PBS by inversion.
4. Examine all wells used under an inverted microscope. Make notes of any well with codes described in **Table 11-1**.
5. Place white backing tape on the bottom of the 96-well plate.
6. Add 100 µL estrogen-free DMEM to each well containing cells.
7. Add 100 µL CellTiter-Glo® reagent to each well containing cells.
8. Place plate on an orbital shaker for 1 minute to induce cell lysis.
9. Incubate (see **Section 9.0** for details) for 10 min.
10. Measure luminescence promptly. Do *not* add luciferase reagent to CellTiter-Glo® plates.

11.5 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.

11.6 Data Analysis

LUMI-CELL® ER 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.

11.6.1 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 11.1.1**.
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 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 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 induction, identify the cell containing the E2 replicate that has the highest RLU value and the cell containing the RLU values for the same concentration in the corresponding E2 replicate (e.g., if the highest RLU value for E2 is located in cell E23, the corresponding cell would be E22).

7. Click into cell D16 and enter the cell number from the previous step into the numerator.

8. Click on the "ER Agonist Report" tab.

9. 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.

10. 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 E).

11. Copy the data into GraphPad Prism® for determination of outliers, graphing, and analysis as indicated in the NICEATM Prism® user's guide.

11.6.2 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:

943 $$\frac{\textit{Outlier} - \textit{Nearest Neighbor}}{\textit{Range (Highest} - \textit{Lowest)}}$$

944 where the outlier is the value proposed for exclusion, the nearest neighbor is the value closest to
945 the outlier, and the range is the range of the three values.

946 If the value of this ratio is greater than 0.94 (the Q value for the 90% confidence interval for a
947 sample size of three) or 0.76 (the Q value for the 90% confidence interval for a sample size of
948 four), the outlier may be excluded from data analysis. For E2 reference standard replicates
949 (sample size of two), any adjusted RLU value for a replicate at a given concentration of E2 is
950 considered and outlier if its value is more than 20% above or below the adjusted RLU value for
951 that concentration in the historical database.

952 11.6.3 Acceptance Criteria

953 Acceptance or rejection of a test is based on evaluation of reference standard and control results
954 from each experiment conducted on a 96-well plate. Results are compared to quality controls
955 (QC) for these parameters derived from the historical database, which are summarized below.

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

959 • Reference standard results: Calculated E2 reference standard EC_{50} values must be
960 within 2.5 times the standard deviation of the historical database EC_{50} mean
961 value.

962 • Solvent control results: Solvent control RLU values must be within 2.5 times the
963 standard deviation of the historical solvent control mean RLU value.

964 • Positive control results: Methoxychlor control RLU values must be within 2.5
965 times the standard deviation of the historical methoxychlor control mean RLU
966 value.

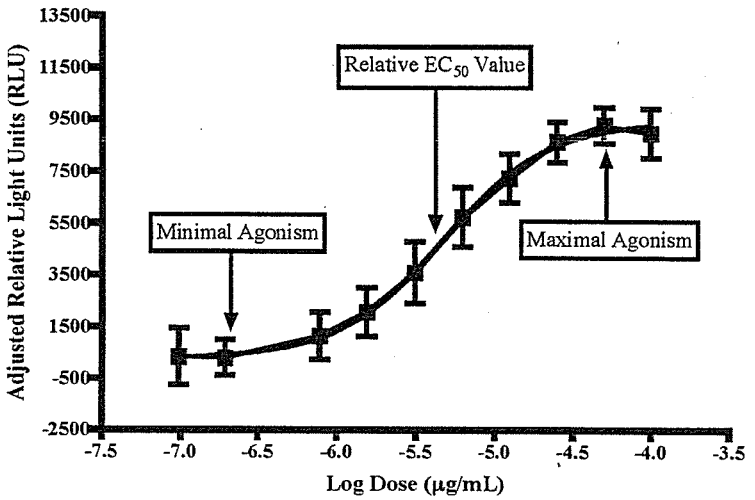
967 An experiment that fails any single acceptance criterion will be discarded and repeated.

968

968 11.6.4 Calculation of Relative EC_{50} Values

969 Relative EC_{50} values are calculated as directed in the NICEATM Prism® users guide. The
970 concentration curve must have a sigmoidal shape and reach saturation at the highest and lowest
971 concentrations tested (**Figure 11-1**) for Prism® to calculate a valid relative EC_{50} value.

972 **Figure 11-1 Example Concentration Curve for Calculation of Relative EC_{50} Value**



973

974 The mathematical model used by Prism® to calculate a relative EC_{50} value uses the Hill function
975 as described in **Section 6.0**.

976 11.6.5 Calculation of Absolute EC_{50} Values

977 Calculate an absolute EC_{50} value for all substances that have a positive response that reaches
978 50% of the E2 reference standard response (Eli Lilly 2005).

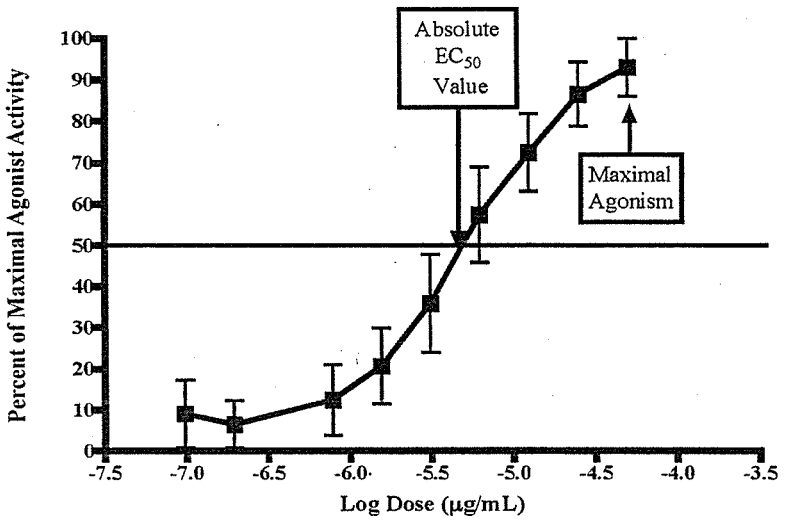
979 To calculate the absolute EC_{50}:

980 1. Convert the test substance data to the percentage of the maximum agonist
981 response for E2, the reference substance, excluding any negative values.

982 2. Find the concentration of the test substance that is 50% of the maximum E2
983 response.

984

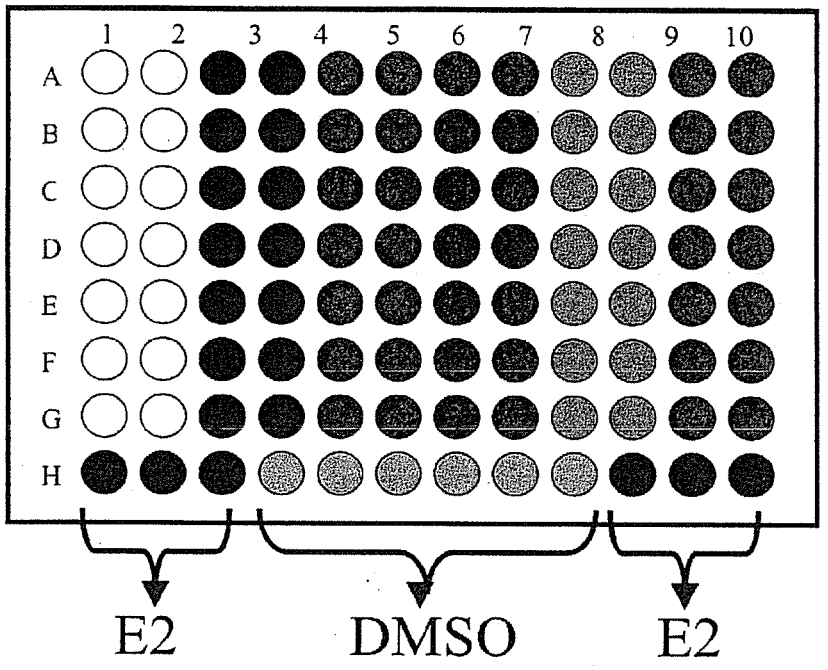
Figure 11-2 Example Concentration Curve for Calculation of Absolute EC_{50} Value



12.0 RANGE FINDER TESTING

Agonist range finding for coded substances consists of six 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.

990 **Figure 12-1 Agonist Range Finder Test Plate Layout**



- **Three Point E2 Reference Standard – Replicate 1**
- **Three Point E2 Reference Standard – Replicate 2**
- **DMSO Control (1% v/v)**
- **Range Finder for Sample #2**
- **Range Finder for Sample #1**
- **Range Finder for Sample #3**
- **Range Finder for Sample #4**
- **Range Finder for Sample #5**
- **Range Finder for Sample #6**

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

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