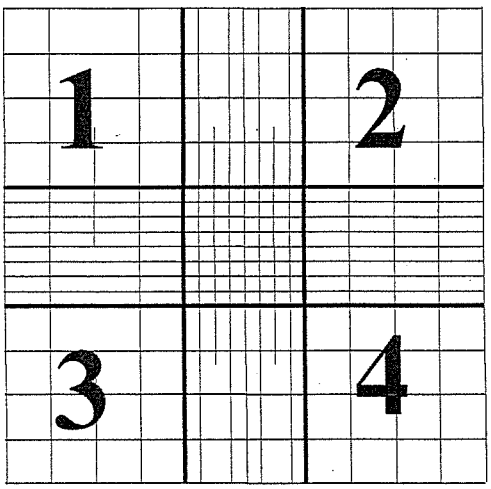
12. Using 100x magnification, view the counting grid.

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 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 200 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 200 mg/mL solution has visible precipitate or is cloudy, add an additional 9 mL of DMSO to the conical tube (20 mg/mL), then vortex and/or sonicate for 10 min. as before.
5. If the 20 mg/mL solution has visible precipitate or is cloudy, prepare a 2 mg/mL solution in a 15 mL conical tube. Vortex and/or sonicate for 10 min. as before.
6. If the 2 mg/mL solution has visible precipitate or is cloudy, add an additional 9 mL of DMSO to the conical tube (200 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 the 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 LUMI-CELL® ER testing.

8. Add 2 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 **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 5.0×10^{-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.	1.0×10^{-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.	5.0×10^{-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.	-	1.0×10^{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 **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

Step #	Action	DMSO	Discard	Raloxifene Concentration
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 9 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 10 mg/mL flavone
2. Add 1 mL of the $5x10^{-3}$ µg/mL E2 (prepared as in **Section 11.1.1**) to the 10 mg/mL flavone. This will make a working solution of 5 mg/mL flavone with $2.5x10^{-3}$ 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 tube.

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**.

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

Tube Number	Test Substance	E2	Remove	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.	Remove and discard 4 µL from Tube #1	400 µL	404 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.	Remove and discard 4 µL from Tube #2	400 µL	404 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.	Remove and discard 4 µL from Tube #3	400 µL	404 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.	Remove 4 µL from Tube #4	400 µL	404 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.	Remove 4 µL from Tube #5	400 µL	404 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.	Remove 4 µL from Tube #6	400 µL	404 µL

848

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

851

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	4 µL of Tube #1 from **Section 11.3.2**	400 µL	404 µL
2	4 µL of Tube #2 from **Section 11.3.2**	400 µL	404 µL
3	4 µL of Tube #3 from **Section 11.3.2**	400 µL	404 µL
4	4 µL of Tube #4 from **Section 11.3.2**	400 µL	404 µL
5	4 µL of Tube #5 from **Section 11.3.2**	400 µL	404 µL
6	4 µL of Tube #6 from **Section 11.3.2**	400 µL	404 µL
7	4 µL of Tube #7 from **Section 11.3.2**	400 µL	404 µL
8	4 µL of Tube #8 from **Section 11.3.2**	400 µL	404 µL
9	4 µL of Tube #9 from **Section 11.3.2**	400 µL	404 µL

12.3.2 Preparation of DMSO Control Comprehensive Testing 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.

12.3.3 Preparation of E2 Control Comprehensive Testing Dosing Solution

1. Add 2 µL of the E2 stock from **Section 11.1.1** to three 13 mm glass test tube.
2. Add 400 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 4 µL of flavone/E2 from **Section 11.4** to three 13 mm glass test tubes.
2. Add 400 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 LUMI-CELL® ER 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**.

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

Tube Number	100% DMSO	Test Substance[1]	Discard	E2 Testing Stock	Discard	Estrogen-free DMEM[2]	Final Volume
1	-	4 µL of test substance solution from **Section 10.2.4 step 1**	-	4 µL	4 µL	400 µL	404 µL
2	4 µL	4 µL of test substance solution from **Section 10.2.4 step 1**	-	4 µL	4 µL	400 µL	404 µL
3	4 µL	4 µL from Tube #2	-	4 µL	4 µL	400 µL	404 µL
4	4 µL	4 µL from Tube #3	-	4 µL	4 µL	400 µL	404 µL
5	4 µL	4 µL from Tube #4	-	4 µL	4 µL	400 µL	404 µL
6	4 µL	4 µL from Tube #5	-	4 µL	4 µL	400 µL	404 µL
7	4 µL	4 µL from Tube #6	-	4 µL	4 µL	400 µL	404 µL
8	4 µL	4 µL from Tube #7	-	4 µL	4 µL	400 µL	404 µL
9	4 µL	4 µL from Tube #8	-	4 µL	4 µL	400 µL	404 µL
10	4 µL	4 µL from Tube #9	-	4 µL	4 µL	400 µL	404 µL
11	4 µL	4 µL from Tube #10	4 µL	4 µL	4 µL	400 µL	404 µL

893 [1]Vortex tubes #2 through 10 before removing test substance/DMSO solution to place in the next tube in
894 the series.
895 [2]Vortex all tubes to mix media, test substance, and E2.
896

897 ## 13.0 GENERAL PROCEDURES FOR THE TESTING OF CODED SUBSTANCES

898 Range finder experiments are used to determine the concentrations of test substance to be used
899 during comprehensive testing. Comprehensive testing experiments are used to determine whether
900 a substance possesses ER antagonist activity in the LUMI-CELL® ER test method.

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

904

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
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.

13.3 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 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 11.5**).

13.4 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 pipetter 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 13-1**.
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.

13.5 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 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.

13.6 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. 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.

13.6.1 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 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 13.6.2** 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 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 13.6.1 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. Copy the data into GraphPad Prism® for graphing and analysis as indicated in the NICEATM Prism® users guide.

13.6.2 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:

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

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.

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

13.6.3 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.

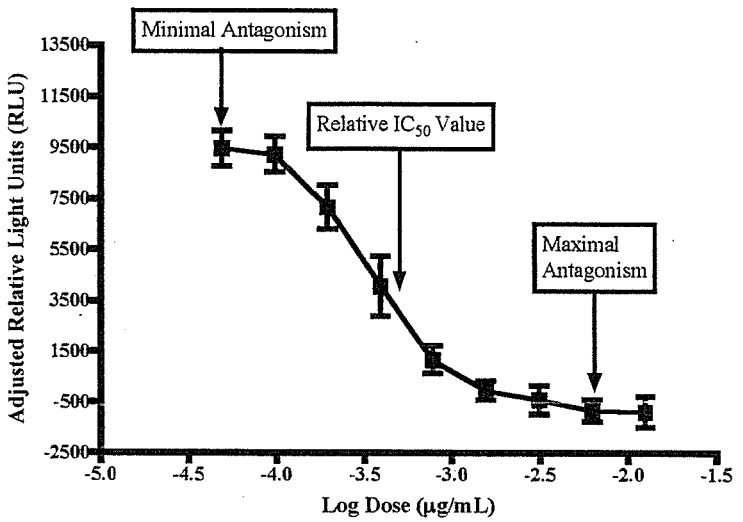
- Reduction: Plate reduction, as measured by dividing the averaged highest Ral/E2 reference standard RLU value by the averaged lowest Ral/E2 reference standard RLU value, must be greater than three fold.
- Reference standard results: Calculated Ral/E2 reference standard IC_{50} values must be within 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.

13.6.4 Calculation of Relative IC_{50} Values

Relative IC_{50} values are calculated as directed in the NICEATM Prism® users guide. The concentration curve must have a sigmoidal shape and reach saturation at the highest and lowest concentrations tested (**Figure 13-1)** for Prism® to calculate a valid relative IC_{50} value.

1059 **Figure 13-1 Example Concentration Curve for Calculation of Relative IC_{50} Value**



1060

1061 The mathematical model used by Prism® to calculate an IC_{50} value uses the Hill function as
1062 described in **Section 6.0**.

1063 13.6.5 Calculation of Absolute IC_{50} Values

1064 Calculate an absolute IC_{50} (Eli Lilly 2005) for all substances that are positive for antagonism at
1065 one or more concentrations that cause a 50% reduction in response compared to the E2 control
1066 (**Figure 13-2**).