- If there are no points on the test substance concentration curve that are above the line representing the mean plus three times the standard deviation of the DMSO control, the highest concentration used in comprehensive testing is the limit dose or the maximum soluble dose.

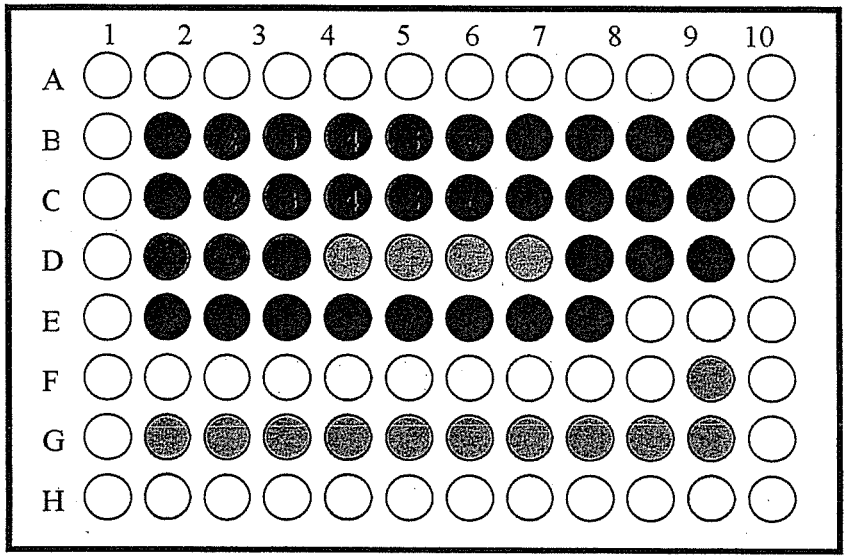
- If there are points on the test substance concentration curve that are above the line representing the mean plus three times the standard deviation of the DMSO control, select a concentration that is a single log dilution higher than the concentration giving the highest adjusted RLU value in the range finder, and use that as the highest concentration for comprehensive testing.

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

13.0 COMPREHENSIVE TESTING

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

Figure 13-1 Agonist Comprehensive Test Plate Layout



- E2 Reference Standard Dose Response Curve
Note: #9 in dilution series has been removed.

- Methoxychlor Control (3.13 µg/mL)

- DMSO Control (1% v/v)

- Comprehensive Dose Response Sample #1, Replicate #1

- Comprehensive Dose Response Sample #1, Replicate #2

- Comprehensive Dose Response Sample #1, Replicate #3

- Media only wells, not used for assay

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

- If the substance has been tested up to the limit dose or the maximum soluble dose, without causing a significant decrease in cell viability, and there are no points on the concentration curve that are above the line indicating the mean plus three times the standard deviation of the DMSO control, the substance is considered negative for agonism
- If the substance has a positive response (See **Section 6.0**) at any concentration, the substance is considered positive for agonism.

- Calculate an absolute EC_{50} value for all substances that have a positive response that reaches 50% of the E2 reference standard response.
- If a substance has a positive response that does not reach 50% of the E2 reference standard response, report the response as positive.
- If a substance has positive responses that are concentration-dependant, and that plateau at the highest and lowest concentrations tested, calculate a relative EC_{50} value.

14.0 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 LUMI-CELL® ER 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.

14.1 LUMI-CELL® ER Agonist QC Charts

1. Open the Excel® spreadsheet labeled LUMIAgonistQC.
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 ALUMIAgonistQC).

14.1.1 Methoxychlor Control

1. Open the Excel® spreadsheet from **Section 14.1 step 2**.

2. Click on the methoxychlor tab and enter the date, plate number (name), and average 2.5 x 10^{-5} µg RLU value for E2 (data located in column F on the "List" tab of the agonist report file).

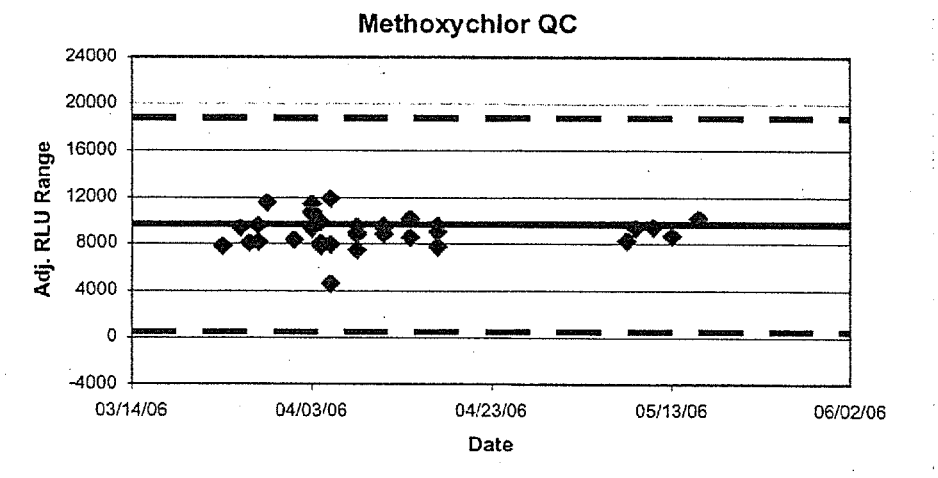
3. Enter the three values for methoxychlor into column D.

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 methoxychlor falls within the 2.5 times the standard deviation (**Figure 14-1**). If the average value falls within the 2.5 times the standard deviation area, methoxychlor passes QC. If the average value falls outside of the 2.5 times the standard deviation area, methoxychlor fails QC and the experiment must be repeated.

Acceptance or rejection of the methoxychlor 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 14-1 Example Scatter Chart of the Methoxychlor Control QC[1,2,3]

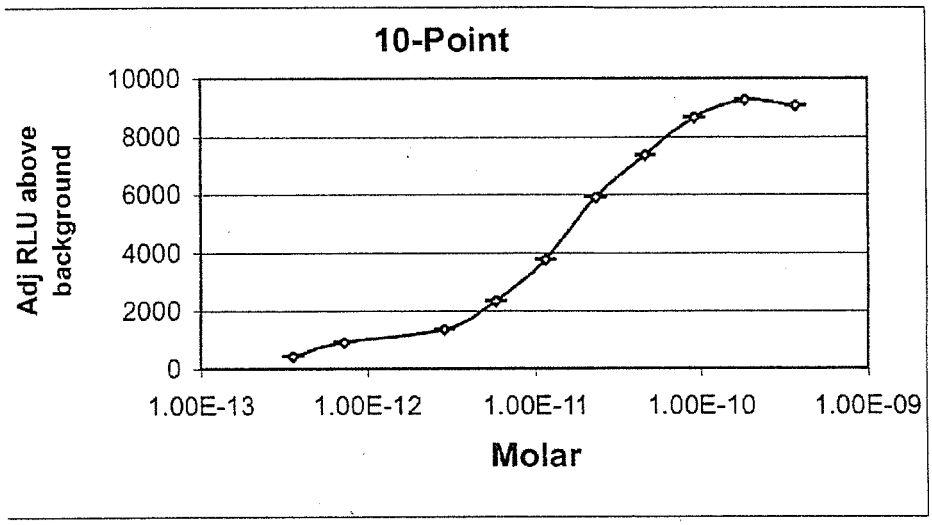


[1]Each point represents a single experiment.
[2]The solid line represents the historical mean RLU value for the methoxychlor control.
[3]The two dashed lines represent the historical mean RLU value for the methoxychlor control plus and minus 2.5 times the standard deviation from the historical mean.

14.1.2 10-Point E2 Reference Standard QC

1. Enter the experiment date and name, and copy and paste the adjusted RLU values for E2 into the appropriate slots in the tab labeled E2 Standard Curve.
2. The E2 standard curve is automatically graphed to ensure a normal sigmoidal shape (see **Figure 14-2** for an example curve).

Figure 14-2 Example Figure of a Sigmoidal E2 Concentration Response Curve[1]



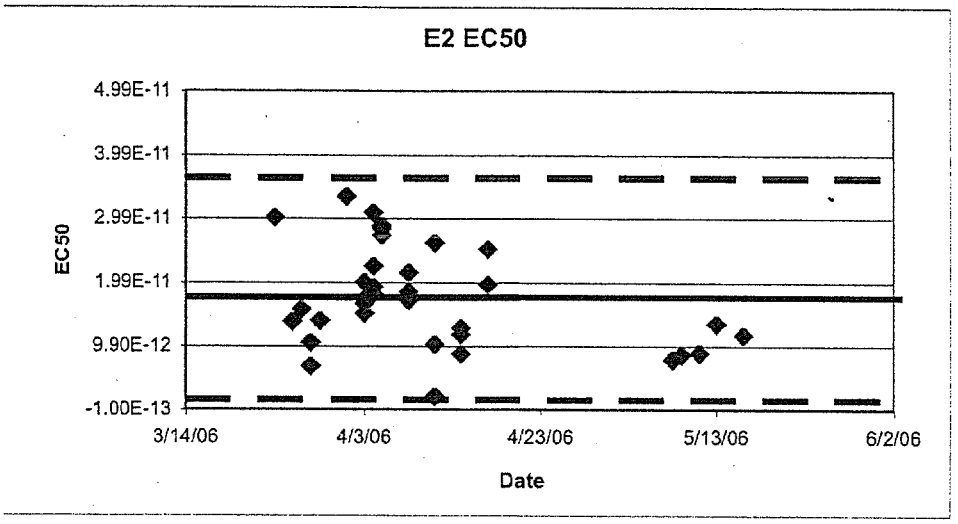
[1]The line represents the averaged E2 values for a single experiment.

14.1.3 EC_{50} Tracking Data

1. Enter the date and plate ID into the first two columns of the EC_{50} Tracking Data tab.
2. Link the EC_{50} data from the 10-point 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 EC_{50} value.
4. The mean and 2.5 times the standard deviation plus (and minus) the mean for the EC_{50} deviation are calculated automatically.
5. Check the Scatter Charts tab to see whether the experimental EC_{50} value falls within the 2.5 times the standard deviation (**Figure 14-3**). 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 14-3 Example Scatter Chart of the E2 EC_{50} Control QC[1,2,,3]



[1]Each point represents a single experiment.
[2]The solid line represents the historical mean RLU value for the E2 EC50.
[3]The two dashed lines represent the historical mean RLU value for the E2 EC50 control plus and minus 2.5 times the standard deviation from the historical mean.

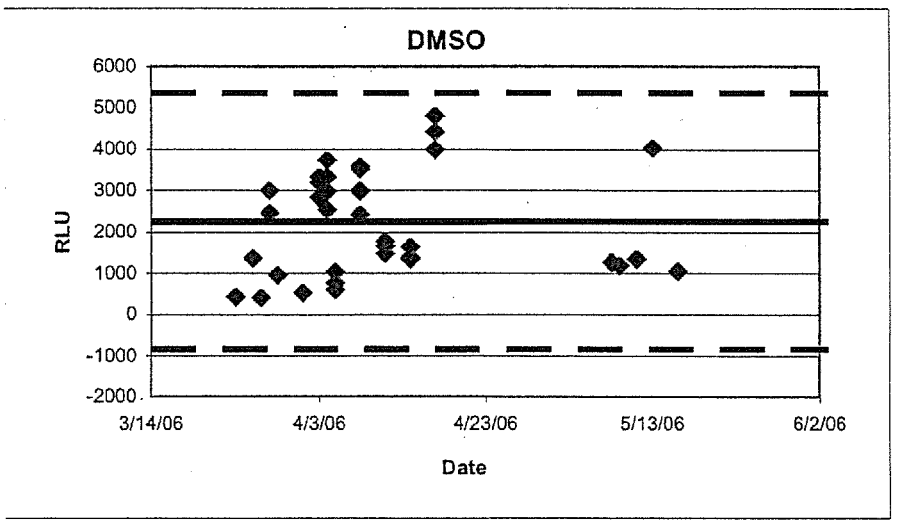
Acceptance or rejection of E2 EC_{50} data is based on whether the data falls within 2.5 times the standard deviation from the mean for the historical E2 EC_{50} RLU value.

14.1.4 DMSO Control

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 14-4**) 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 14-4 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.

14.1.5 Induction

Enter the induction 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.

15.0 QUALITY TESTING OF MATERIALS

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

15.1 Tissue Culture Media

Each bottle of tissue culture medium must be tested in a single growth flask of cells before use in ongoing tissue culture or experimentation.

1. Every new lot of media (RPMI and DMEM) and media components (FBS, Charcoal/Dextran treated FBS, and L-glutamine) must first be tested on the LUMI-CELL® ER assay prior to being used in any GLP acceptable assays.
2. Add 4 µL of DMSO (previously tested) into four separate 13 mm tubes.
3. Add 400 µL media (to be tested) to the same tubes.
4. Dose an experimental plate as in **Section 12.0,** treating the media being tested as a test substance.
5. Analyze 96-well plate as described in **Section 12.0,** comparing the data from the DMSO controls made using previously tested tissue culture media to the new media being tested.
6. Use the 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 lie within 2.5 standard deviation of the mean for the historical data on DMSO, the new lot of media is acceptable. If the RLU values for the new media with DMSO do not lie within 2.5 standard 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 – 3 days. If there is no change in growth or morphology, the new media is acceptable for use.

15.2 G418:

1. New lots of G418 must first be tested on the LUMI-CELL® ER assay prior to being used in any GLP acceptable assays.

2. Add 220 µL of G418 (previously tested) to a single flask containing cells growing in RPMI.
3. Add 220 µL of G418 (to be tested) to a different flask containing cells growing in RPMI.
4. Observe cellular growth and morphology in both tissue culture flasks over a 48 to 72 hour period. If there are no differences in observed growth rate and morphology between the two flasks, the new G418 lot is acceptable.
5. If cellular growth is decreased, or the cells exhibit abnormal morphology, the new lot of G418 is not acceptable.
6. Note date and lot number in study book.

15.3 DMSO

1. Every new bottle of DMSO must be tested on the LUMI-CELL® ER assay prior to use in any GLP acceptable assays.
2. Add 4 µL of DMSO (to be tested) into four separate 13 mm tubes.
3. Add 400 µL media (previously tested) the same tubes.
4. Dose an experimental plate as in **Section 12.0,** treating the media being tested as a test substance.
5. Analyze 96-well plate as described in **Section 12.0,** comparing the data from the DMSO controls made using previously tested tissue culture media to the new media being tested.
6. Use the 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 lie within 2.5 standard deviation of the mean for the historical data on DMSO, the new lot of media is acceptable. If the RLU values for the new media with DMSO do not lie within 2.5 standard 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 lies within 2.5 standard deviation of the mean for DMSO background. If the RLU for the new DMSO does lie 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 lower average RLU than the other bottle(s) tested. Use the DMSO with the lowest average RLU for official experiments.

15.4 Plastic Tissue Culture Materials

1. Grow one set of cells, plate them for experiments on plastic ware from the new lot and one set of cells in the plastic ware from a previous lot, and dose them with E2 reference standard and controls.

2. Perform the LUMI-CELL® ER experiment with both sets of cells.

3. If all of the analysis falls within acceptable QC criteria, then the new manufacturer's products may be used.

16.0 REFERENCES

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]

ANTAGONIST PROTOCOL

LUMI-CELL® ESTROGEN RECEPTOR TRANSCRIPTIONAL ACTIVATION TEST METHOD FOR IDENTIFYING ESTROGEN AGONISTS AND ANTAGONISTS

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

Developed by:

Xenobiotic Detection Systems, Inc.

1601 E. Geer St., Suite S

Durham, NC 27704

07 November, 2006

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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

153	Relative IC_{50} Value	Concentration that produces a half-maximal response as
154		calculated using the four parameter Hill function.
155	RPMI	RPMI 1640 growth medium
156	TA	Transcriptional Activation
157	T25	25 cm^2 tissue culture flask
158	T75	75 cm^2 tissue culture flask
159	T150	150 cm^2 tissue culture flask
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