

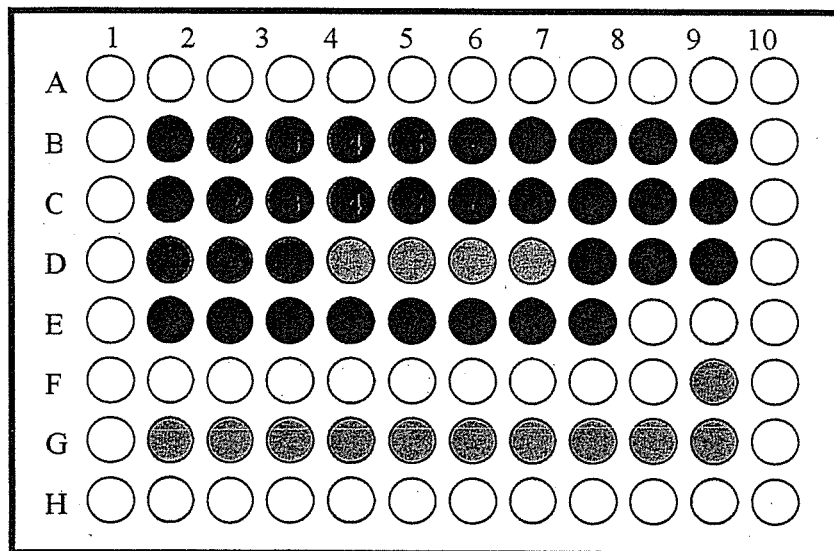
- 994 • If there are no points on the test substance concentration curve that are above the
995 line representing the mean plus three times the standard deviation of the DMSO
996 control, the highest concentration used in comprehensive testing is the limit dose
997 or the maximum soluble dose.
- 998 • If there are points on the test substance concentration curve that are above the line
999 representing the mean plus three times the standard deviation of the DMSO
1000 control, select a concentration that is a single log dilution higher than the
1001 concentration giving the highest adjusted RLU value in the range finder, and use
1002 that as the highest concentration for comprehensive testing.
- 1003 • If a substance exhibits a biphasic concentration curve, the range finder experiment
1004 should be repeated unless the proposed concentration range for the comprehensive
1005 studies will include all concentrations of the biphasic region in the range finding
1006 study. If the range finder experiment is repeated and the substance still exhibits a
1007 biphasic concentration curve, comprehensive testing must be conducted on the
1008 peak of the biphasic curve at the lowest test substance concentration. If the
1009 substance is negative at this lowest concentration, then test at the higher
1010 concentration. For either peak of the concentration curve, select a concentration
1011 that is a single log dilution higher than the concentration giving the highest
1012 adjusted RLU value in the range finder and use that as the highest concentration
1013 for comprehensive testing.

1014 **13.0 COMPREHENSIVE TESTING**

1015 Agonist comprehensive testing for coded substances consists of 11 point, double serial dilutions,
1016 with each concentration tested in triplicate wells of the 96-well plate. **Figure 13-1** contains a
1017 template for the plate layout to be used in agonist comprehensive testing.

1018

1018 **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**

1019

1020 Evaluate whether comprehensive experiments have met acceptance criteria (see **Section 11.6.4**)
1021 and graph the data as described in the NICEATM Prism® users guide.

- 1022 • If the substance has been tested up to the limit dose or the maximum soluble dose,
1023 without causing a significant decrease in cell viability, and there are no points on
1024 the concentration curve that are above the line indicating the mean plus three
1025 times the standard deviation of the DMSO control, the substance is considered
1026 negative for agonism
- 1027 • If the substance has a positive response (See **Section 6.0**) at any concentration,
1028 the substance is considered positive for agonism.

- 1029 ○ Calculate an absolute EC₅₀ value for all substances that have a positive
1030 response that reaches 50% of the E2 reference standard response.
- 1031 ○ If a substance has a positive response that does not reach 50% of the E2
1032 reference standard response, report the response as positive.
- 1033 ○ If a substance has positive responses that are concentration-dependant, and
1034 that plateau at the highest and lowest concentrations tested, calculate a
1035 relative EC₅₀ value.

1036 **14.0 USE OF THE HISTORICAL DATABASE TO GENERATE QC CHARTS**

1037 The historical database is maintained in order to ensure that the test method is functioning
1038 properly. The historical database is maintained as an Excel® spreadsheet that is separate from the
1039 spreadsheets used to report the data for individual experiments. The controls used to develop the
1040 historical database are used as one of the criteria for determining a valid test.

1041 Results collected during Phase I will be compared to historical control data established during
1042 the LUMI-CELL® ER Protocol Standardization Study. Reference standard and control data
1043 collected during Phase I will be used to compile the initial historical database. Reference
1044 standard and control data collected during Phase IIa will be added to the historical database
1045 compiled in Phase I and this combined historical database will be used to establish acceptance
1046 criteria for Phase II. Reference standard and control data collected during Phase IIb will be added
1047 to the historical database compiled in Phases I and IIa and this combined historical database will
1048 be used to establish acceptance criteria for Phases III and IV.

1049 **14.1 LUMI-CELL® ER Agonist QC Charts**

- 1050 1. Open the Excel® spreadsheet labeled LUMIAgonistQC.
- 1051 2. Save this sheet under a new name, adding the laboratory designator to the file
1052 name (e.g., for Laboratory A, the new name would be ALUMIAgonistQC).

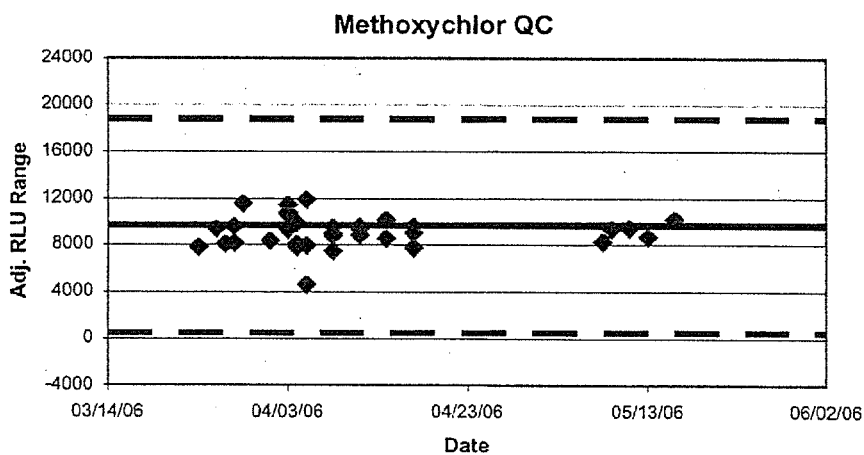
1053 14.1.1 Methoxychlor Control

- 1054 1. Open the Excel® spreadsheet from **Section 14.1 step 2**.

- 1055 2. Click on the methoxychlor tab and enter the date, plate number (name), and
1056 average 2.5×10^{-5} µg RLU value for E2 (data located in column F on the “List”
1057 tab of the agonist report file).
- 1058 3. Enter the three values for methoxychlor into column D.
- 1059 4. The mean and 2.5 times the standard deviation plus (and minus) the mean are
1060 calculated automatically.
- 1061 5. Check the scatter charts tab to see if the average value for methoxychlor falls
1062 within the 2.5 times the standard deviation (**Figure 14-1**). If the average value
1063 falls within the 2.5 times the standard deviation area, methoxychlor passes QC. If
1064 the average value falls outside of the 2.5 times the standard deviation area,
1065 methoxychlor fails QC and the experiment must be repeated.

1066 Acceptance or rejection of the methoxychlor control data is based on whether the data for a given
1067 experiment falls within 2.5 times the standard deviation from the historical mean RLU value.

1068 **Figure 14-1 Example Scatter Chart of the Methoxychlor Control QC^{1,2,3}**



1069

1070 ¹Each point represents a single experiment.

1071 ²The solid line represents the historical mean RLU value for the methoxychlor control.

1072 ³The two dashed lines represent the historical mean RLU value for the methoxychlor control

1073 plus and minus 2.5 times the standard deviation from the historical mean.

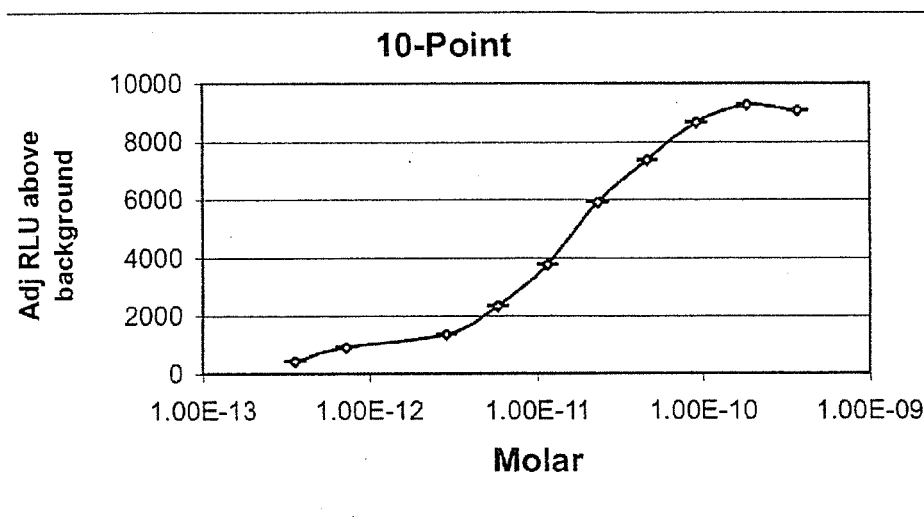
1074

1075

1075 14.1.2 10-Point E2 Reference Standard QC

- 1076 1. Enter the experiment date and name, and copy and paste the adjusted RLU values
1077 for E2 into the appropriate slots in the tab labeled E2 Standard Curve.
1078 2. The E2 standard curve is automatically graphed to ensure a normal sigmoidal
1079 shape (see **Figure 14-2** for an example curve).

1080 **Figure 14-2 Example Figure of a Sigmoidal E2 Concentration Response Curve¹**



1081

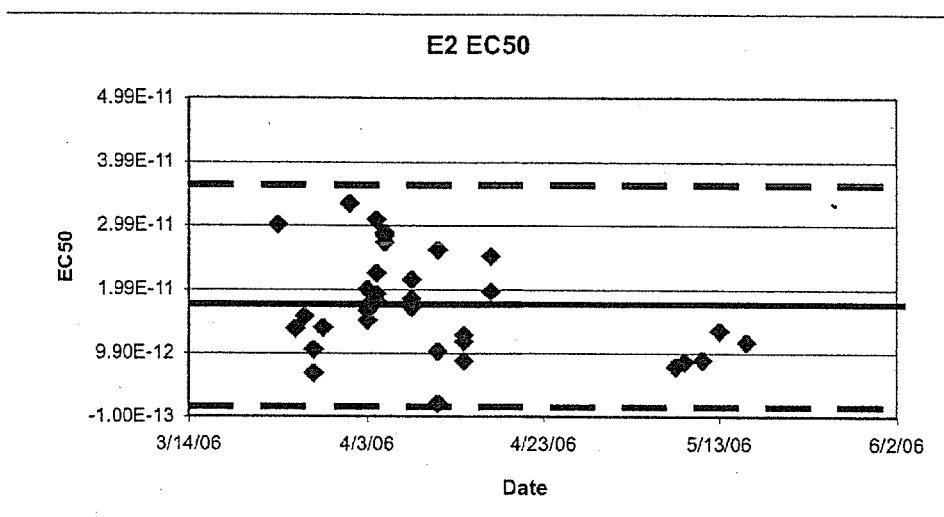
1082 ¹The line represents the averaged E2 values for a single experiment.

1083 14.1.3 EC₅₀ Tracking Data

- 1084 1. Enter the date and plate ID into the first two columns of the EC₅₀ Tracking Data
1085 tab.
1086 2. Link the EC₅₀ data from the 10-point E2 Curve QC tab to the column to the right
1087 of the plate information.
1088 3. Column E calculates the percent deviation from the historical database EC₅₀
1089 value.
1090 4. The mean and 2.5 times the standard deviation plus (and minus) the mean for the
1091 EC₅₀ deviation are calculated automatically.
1092 5. Check the Scatter Charts tab to see whether the experimental EC₅₀ value falls
1093 within the 2.5 times the standard deviation (**Figure 14-3**). If the value falls within

1094 the 2.5 times the standard deviation area, it passes QC. If the value does not fall
1095 within the 2.5 times the standard deviation, it fails QC and the experiment must be
1096 repeated.

1097 **Figure 14-3 Example Scatter Chart of the E2 EC₅₀ Control QC^{1,2,3}**



1098

1099 ¹Each point represents a single experiment.

1100 ²The solid line represents the historical mean RLU value for the E2 EC₅₀.

1101 ³The two dashed lines represent the historical mean RLU value for the E2 EC₅₀
1102 control plus and minus 2.5 times the standard deviation from the historical mean.

1103

1104 Acceptance or rejection of E2 EC₅₀ data is based on whether the data falls within 2.5 times the
1105 standard deviation from the mean for the historical E2 EC₅₀ RLU value.

1106 14.1.4 DMSO Control

1107 1. The date and experiment name should populate automatically

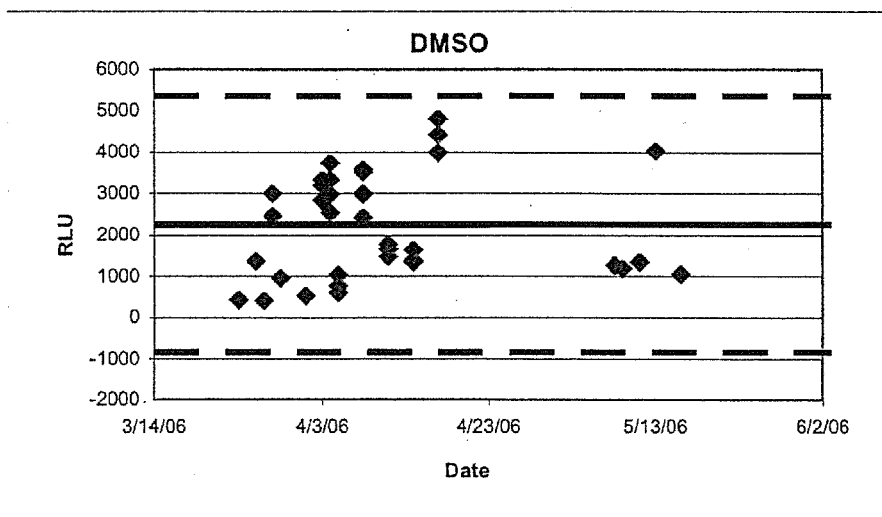
1108 2. Enter all of the DMSO values from Table 1 on the "Raw Data" tab on the Excel®
1109 spreadsheet which passed the outlier test, into the areas marked DMSO 1, DMSO
1110 2, DMSO 3, and DMSO 4.

1111 3. The average RLU value for DMSO is then calculated under the "mean" column.

1112 4. The mean and 2.5 times the standard deviation plus (and minus) the mean for the
1113 DMSO deviation are calculated automatically.

- 1114 5. Check the Scatter Charts tab to see whether the average value for DMSO falls
1115 within 2.5 times the standard deviation (**Figure 14-4**) from the mean. If the value
1116 falls within the 2.5 times the standard deviation area, the DMSO passes QC. If
1117 the value does not fall within the 2.5 times the standard deviation, it fails QC and
1118 the experiment must be repeated.

1119 **Figure 14-4 Example Scatter Chart of the DMSO Control QC^{1,2,3}**



1120

- 1121 ¹Each point represents a single experiment.
1122 ²The solid line represents the historical mean RLU value for the DMSO control.
1123 ³The two dashed lines represent the historical mean RLU value for the DMSO
1124 control plus and minus 2.5 times the standard deviation from the historical mean.
1125

1126 Acceptance or rejection of the DMSO control data is based on whether the data for a given
1127 experiment falls within 2.5 times the standard deviation from the historical mean RLU value.

1128 14.1.5 Induction

1129 Enter the induction value from the “Raw Data” tab on the Excel® spreadsheet. If the value is
1130 greater than or equal to 3, the experiment passed QC. An induction value of less than 3 fails
1131 induction QC and the experiment must be repeated.

1132 15.0 QUALITY TESTING OF MATERIALS

1133 All information pertaining to the preparation and testing of media, media supplements, and other
1134 materials should be recorded in the Study Notebook.

1135 **15.1 Tissue Culture Media**

1136 Each bottle of tissue culture medium must be tested in a single growth flask of cells before use in
1137 ongoing tissue culture or experimentation.

- 1138 1. Every new lot of media (RPMI and DMEM) and media components (FBS,
1139 Charcoal/Dextran treated FBS, and L-glutamine) must first be tested on the
1140 LUMI-CELL® ER assay prior to being used in any GLP acceptable assays.
- 1141 2. Add 4 µL of DMSO (previously tested) into four separate 13 mm tubes.
- 1142 3. Add 400 µL media (to be tested) to the same tubes.
- 1143 4. Dose an experimental plate as in **Section 12.0**, treating the media being tested as a
1144 test substance.
- 1145 5. Analyze 96-well plate as described in **Section 12.0**, comparing the data from the
1146 DMSO controls made using previously tested tissue culture media to the new
1147 media being tested.
- 1148 6. Use the QC charts to determine if the new media with DMSO lies within 2.5
1149 standard deviation of the mean for the media. If the RLU values for the new
1150 media with DMSO lie within 2.5 standard deviation of the mean for the historical
1151 data on DMSO, the new lot of media is acceptable. If the RLU values for the new
1152 media with DMSO do not lie within 2.5 standard deviation of the mean for the
1153 historical data the new lot may not be used in the assay.
- 1154 7. Note date and lot number in study notebook.
- 1155 8. If the new bottle passes the QC as described in **Section 15.1 step 6**, apply the
1156 media to a single flask cells and observe the cells growth and morphology over
1157 the following 2 – 3 days. If there is no change in growth or morphology, the new
1158 media is acceptable for use.

1159 **15.2 G418:**

- 1160 1. New lots of G418 must first be tested on the LUMI-CELL® ER assay prior to
1161 being used in any GLP acceptable assays.

- 1162 2. Add 220 µL of G418 (previously tested) to a single flask containing cells growing
1163 in RPMI.
- 1164 3. Add 220 µL of G418 (to be tested) to a different flask containing cells growing in
1165 RPMI.
- 1166 4. Observe cellular growth and morphology in both tissue culture flasks over a 48 to
1167 72 hour period. If there are no differences in observed growth rate and
1168 morphology between the two flasks, the new G418 lot is acceptable.
- 1169 5. If cellular growth is decreased, or the cells exhibit abnormal morphology, the new
1170 lot of G418 is not acceptable.
- 1171 6. Note date and lot number in study book.

1172 **15.3 DMSO**

- 1173 1. Every new bottle of DMSO must be tested on the LUMI-CELL® ER assay prior
1174 to use in any GLP acceptable assays.
- 1175 2. Add 4 µL of DMSO (to be tested) into four separate 13 mm tubes.
- 1176 3. Add 400 µL media (previously tested) the same tubes.
- 1177 4. Dose an experimental plate as in **Section 12.0**, treating the media being tested as a
1178 test substance.
- 1179 5. Analyze 96-well plate as described in **Section 12.0**, comparing the data from the
1180 DMSO controls made using previously tested tissue culture media to the new
1181 media being tested.
- 1182 6. Use the QC charts to determine if the new media with DMSO lies within 2.5
1183 standard deviation of the mean for the media. If the RLU values for the new
1184 media with DMSO lie within 2.5 standard deviation of the mean for the historical
1185 data on DMSO, the new lot of media is acceptable. If the RLU values for the new
1186 media with DMSO do not lie within 2.5 standard deviation of the mean for the
1187 historical data the new lot may not be used in the assay.

- 1188 7. Use the QC charts to determine if the new DMSO lies within 2.5 standard
1189 deviation of the mean for DMSO background. If the RLU for the new DMSO
1190 does lie within 2.5 standard deviation of the mean for the historical data on
1191 DMSO, then the new bottle of DMSO is acceptable; otherwise the new bottle may
1192 not be used in the assay.
- 1193 8. Note the date, lot number, and bottle number in study book.
- 1194 9. If no DMSO has been previously tested, test several bottles as described in
1195 **Section 15.3**, and determine whether any of the bottles of DMSO have a lower
1196 average RLU than the other bottle(s) tested. Use the DMSO with the lowest
1197 average RLU for official experiments.

1198 **15.4 Plastic Tissue Culture Materials**

- 1199 1. Grow one set of cells, plate them for experiments on plastic ware from the new lot
1200 and one set of cells in the plastic ware from a previous lot, and dose them with E2
1201 reference standard and controls.
- 1202 2. Perform the LUMI-CELL[®] ER experiment with both sets of cells.
- 1203 3. If all of the analysis falls within acceptable QC criteria, then the new
1204 manufacturer's products may be used.

1205 **16.0 REFERENCES**

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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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131

131 **LIST OF ACRONYMS AND ABBREVIATIONS**

132	13 mm test tube	13 x 100 mm glass test tubes
133	Absolute IC ₅₀ Value	Concentration of a substance that decreases the measured
134		activity in an antagonist assay to 50% of the maximum
135		activity induced by the reference substance
136	DMEM	Dulbecco's Modification of Eagle's Medium
137	DMSO	Dimethyl Sulfoxide
138	DMSO Control	1% v/v dilution of DMSO in tissue culture media
139		used as a vehicle control
140	E2	17β-estradiol
141	E2 Control	2.5 x 10 ⁻⁵ µg/mL E2 used as a control.
142	ER	Estrogen Receptor
143	Estrogen-free DMEM	DMEM (phenol red free), supplemented with 1 %
144		Penicillin/Streptomycin, 2 % L-Glutamine, and 5%
145		Charcoal-dextran treated FBS
146	FBS	Fetal Bovine Serum
147	Flavone/E2 Control	25 µg/mL flavone + 2.5 x 10 ⁻⁵ µg/mL E2,
148		used as a positive control.
149	G418	Gentamycin
150	Ral/E2 Reference Standard	Nine point dilution of raloxifene HCl + 2.5 x 10 ⁻⁵ 17β-
151		estradiol reference standard for the LUMI-CELL® ER
152		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
160		

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