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Appendix B

BG1Luc ER TA – Agonist Protocol

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**BG1Luc ER TA TEST METHOD
AGONIST PROTOCOL**

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

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36	LIST OF ACRONYMS AND ABBREVIATIONS	
37	13 mm test tube	13 x 100 mm glass test tubes
38	DMEM	Dulbecco's Modification of Eagle's Medium
39	DMSO	Dimethyl Sulfoxide
40	DMSO control	1% v/v dilution of DMSO in tissue culture media used as a
41		vehicle control
42	E2	17 β -estradiol
43	E2 reference standard	11 Point Serial Dilution of 17 β -estradiol reference standard for
44		the LUMI-CELL [®] ER agonist assay
45	EC ₅₀ value	Concentration that produces a half-maximal response as
46		calculated using the four parameter Hill function.
47	ER	Estrogen Receptor
48	Estrogen-free DMEM	DMEM (phenol red free) supplemented with 1%
49		Penicillin/Streptomycin, 2% L-Glutamine, and 5% Charcoal-
50		dextran treated FBS
51	FBS	Fetal Bovine Serum
52	G418	Gentamycin
53	Methoxychlor	<i>p,p'</i> -Methoxychlor
54	Methoxychlor control	3.13 μ g/mL Methoxychlor Weak Positive Control for the
55		BG1Luc ER TA Agonist Assay
56	RPMI	RPMI 1640 growth medium
57	TA	Transcriptional Activation
58	T25	25 cm ² tissue culture flask
59	T75	75 cm ² tissue culture flask
60	T150	150 cm ² tissue culture flask
61		

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62 **1.0 PURPOSE**

63 This protocol is designed to evaluate coded test substances for potential estrogen receptor (ER) agonist
64 activity using the BG1Luc ER TA test method.

65 **2.0 SPONSOR**

66 (As Appropriate)

67 **3.0 DEFINITIONS**

- 68 • **Dosing Solution:** The test substance, control substance, or reference standard solution,
69 which is to be placed into the tissue culture wells for experimentation.
- 70 • **Raw Data:** Raw data includes information that has been collected but not formatted or
71 analyzed, and consists of the following:
- 72 ○ Data recorded in the Study Notebook
 - 73 ○ Computer printout of initial luminometer data
 - 74 ○ Other data collected as part of GLP compliance, e.g.:
 - 75 ▪ Equipment logs and calibration records
 - 76 ▪ Test substance and tissue culture media preparation logs
 - 77 ▪ Cryogenic freezer inventory logs
- 78 • **Soluble:** Test substance exists in a clear solution without visible cloudiness or
79 precipitate.
- 80 • **Study Notebook:** The study notebook contains recordings of all activities related to the
81 conduct of the BG1Luc ER TA agonist assay.
- 82 • **Test Substances:** Substances supplied to the testing laboratories that are coded and
83 distributed such that only the Project Officer, Study Management Team (SMT), and the
84 Substance Inventory and Distribution Management have knowledge of their true identity.
85 The test substances will be purchased, aliquoted, coded, and distributed by the Supplier
86 under the guidance of the Project Officer and the SMT.

87 **4.0 TESTING FACILITY AND KEY PERSONNEL**

88 **4.1 Testing Facility**

89 (As Appropriate)

90 **4.2 Key Personnel**

- 91 • Study Director: (As Appropriate)
- 92 • Quality Assurance Director: (As Appropriate)

93

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93 **5.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES**

94

95 **5.1 Test Substances**

96 Test substances are coded and will be provided to participating laboratories by the Substance Inventory
97 and Distribution Management team.

98 **5.2 Controls**

99 Controls for the ER agonist protocol are as follows:

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

102 *Reference standard (17 β -estradiol [E2]):* Three concentrations of E2 (CASRN 50-28-2) in duplicate for
103 range finder testing and a serial dilution consisting of 11 concentrations of E2 in duplicate for
104 comprehensive testing

105 *Positive control (p,p'-Methoxychlor [methoxychlor]):* Methoxychlor (CASRN 72-43-5), 3.13 $\mu\text{g/mL}$ in
106 tissue culture media, used as a weak positive control.

107 **6.0 OVERVIEW OF GENERAL PROCEDURES FOR AGONIST TESTING**

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

111 Agonist range finder testing is conducted on 96-well plates using four concentrations of E2
112 (5.00×10^{-5} , 1.25×10^{-5} , 3.13×10^{-6} and 7.83×10^{-7} $\mu\text{g/mL}$) in duplicate as the reference standard and four
113 replicate wells for the DMSO control. Range finder testing uses all wells of the 96-well plate to test six
114 substances as seven point 1:10 serial dilutions in duplicate.

115 Comprehensive testing is conducted on 96-well plates using 11 concentrations of E2 in duplicate as the
116 reference standard (**Table 6-1**). Four replicate wells for the DMSO control and four replicate wells for the
117 methoxychlor control are included on each plate. Comprehensive testing uses all wells of the 96-well
118 plate to test 2 substances as 11 point serial dilutions in triplicate.

119 **Table 6-1 Concentrations of E2 Reference Standard Used in Comprehensive Testing**

E2 Concentrations ¹		
1.00×10^{-4}	6.25×10^{-6}	3.92×10^{-7}
5.00×10^{-5}	3.13×10^{-6}	1.95×10^{-7}
2.50×10^{-5}	1.56×10^{-6}	9.78×10^{-8}
1.25×10^{-5}	7.83×10^{-7}	

120 ¹Concentrations are presented in $\mu\text{g/mL}$.

121 Visual observations for cell viability are conducted for all experimental plates just prior to luminescence
122 measurements, as outlined in **Section 11.2**.

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123 Luminescence data, measured in relative light units (RLUs), is corrected for background luminescence by
124 subtracting the mean RLU value of the vehicle control (DMSO) wells from the RLU measurements for
125 each of the other wells of the 96-well plate. Data is then transferred into Excel® data management
126 spreadsheets and GraphPad PRISM® 4.0 statistical software, graphed, and evaluated as follows:

- 127 • A response is considered positive for agonist activity when the average adjusted RLU for
128 a given concentration is greater than the mean RLU value plus three times the standard
129 deviation for the vehicle control.
- 130 • Any response below this threshold is considered negative for agonist activity.

131 For substances that are positive at one or more concentrations, the concentration that causes a half-
132 maximal response (EC₅₀) is calculated using a Hill function analysis. The Hill function is a four-
133 parameter logistic mathematical model relating the substance concentration to the response (typically
134 following a sigmoidal curve) using the equation below:

$$135 \quad Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log \text{EC}_{50} - X)\text{HillSlope}}}$$

136 where Y = response (i.e., relative light units); X = the logarithm of concentration; Bottom = the minimum
137 response; Top = the maximum response; log EC₅₀ = the logarithm of X as the response midway between
138 Top and Bottom; and HillSlope describes the steepness of the curve. The model calculates the best fit for
139 the Top, Bottom, HillSlope, and EC₅₀ parameters. See **Section 11.6.5** for more details.

140 Acceptance or rejection of a test is based on evaluation of reference standard and control results from
141 each experiment conducted on a 96-well plate. Results for these controls are compared to historical results
142 compiled in the historical database, as seen in **Section 14.0**.

143 **6.1 Range Finder Testing**

144 Agonist range finding for coded substances consists of a seven point, 1:10 serial dilution using duplicate
145 wells per concentration. Concentrations for comprehensive testing are selected based on the response
146 observed in range finder testing. If necessary, a second range finder test can be conducted to clarify the
147 optimal concentration range to test (see **Section 12.0**).

148 **6.2 Comprehensive Testing**

149 Comprehensive agonist testing for coded substances consists of 11 point, serial dilutions, with each
150 concentration tested in triplicate wells of the 96-well plate. Three separate experiments are conducted for
151 comprehensive testing on three separate days, except during Phases III and IV of the validation effort, in
152 which comprehensive testing experiments are conducted once (see **Section 13.0**).

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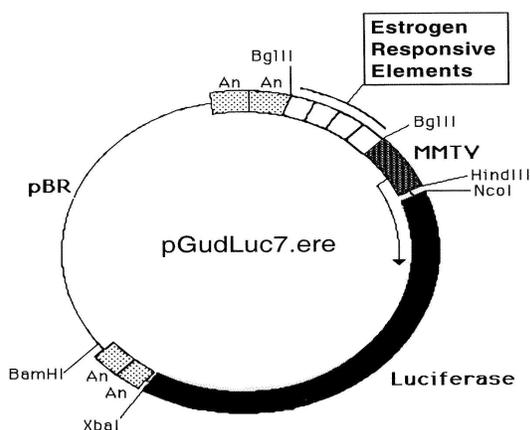
153 **7.0 MATERIALS FOR BG1LUC ER TA AGONIST TESTING**

154 This section provides the materials needed to conduct BG1Luc ER TA testing, with associated brand
 155 names/vendors¹ in brackets.

156 **7.1 BG1Luc4E2 Cells:**

157 Human ovarian cancer cell line stably transfected with a plasmid containing an estrogen response element
 158 pGudLuc7.0 (**Figure 7-1**) [XDS].

159 **Figure 7-1 pGudLuc7.ERE Plasmid.**



160

161 **7.2 Technical Equipment:**

162 All technical equipment may be obtained from Fisher Scientific International, Inc. (Liberty Lane
 163 Hampton, NH, USA 03842). Equivalent technical equipment from another commercial source
 164 can be used.

- 165
- 166 • Analytical balance (Cat. No. 01-910-320)
 - 167 • Berthold Orion 1 Microplate Luminometer [Berthold CatNo.: Orion 1 MPL3] or
 168 equivalent and dedicated computer
 - 169 • Biological safety hood, class II, and stand (Cat. No. 16-108-99)
 - 170 • Centrifuge (low speed, tabletop with swinging bucket rotor) (Cat. No. 04-978-50
 171 centrifuge, and 05-103B rotor)
 - 172 • Combustion test kit (CO₂ monitoring) (Cat. No. 10-884-1)
 - 173 • Drummond diaphragm pipetter (Cat. No. 13-681-15)
 - 174 • Freezers, -20°C (Cat. No. 13-986-150), and -70°C (Cat. No. 13-990-86)
 - 175 • Hand tally counter (Cat. No. 07905-6)
 - Hemocytometer, cell counter (Cat. No. 02-671-5)

¹Brand names and vendors should not be considered an endorsement by the U.S. Government or any member of the U.S. Government; such information is provided as examples.

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- 176 • Light microscope, inverted (Cat. No. 12-561-INV)
- 177 • Light microscope, upright (Cat. No. 12-561-3M)
- 178 • Liquid nitrogen flask (Cat. No. 11-675-92)
- 179 • Micropipetter, repeating (Cat. No. 21-380-9)
- 180 • Pipetters, air displacement, single channel (0.5 –10µl (Cat. No. 21-377-191), 2 –
- 181 20 µl (Cat. No. 21-377-287), 20 – 200 µl (Cat. No. 21-377-298), 200 - 1000 µl
- 182 (Cat. No. 21-377-195))
- 183 • Refrigerator/freezer (Cat. No. 13-986-106A)
- 184 • Shaker for 96-well plates (Cat. No. 14-271-9)
- 185 • Sodium hydroxide (Cat. No. 5318-500)
- 186 • Sonicating water bath (Cat. No. 15-335-30)
- 187 • Tissue culture incubator with CO₂ and temperature control (Cat. No. 11-689-4)
- 188 • Vacuum pump with liquid trap (side arm Erlenmeyer) (Cat. No. 01-092-29)
- 189 • Vortex mixer (Cat. No. 12-814)

190 Equipment should be maintained and calibrated as per GLP guidelines and individual laboratory
191 SOPs.

192 **7.3 Reference Standard, Controls, and Tissue Culture Supplies**

193 All tissue culture reagents must be labeled to indicate source, identity, storage conditions and
194 expiration dates. Tissue culture solutions must be labeled to indicate concentration, stability
195 (where known), and preparation and expiration dates.

196 Equivalent tissue culture media and sera from another commercial source can be used, but must
197 first be tested as described in **Section 15.0** to determine suitability for use in this test method.

198 The following are the necessary tissue culture reagents and possible commercial sources (in
199 brackets) based on their use in the pre-validation studies:

- 200 • BackSeal-96/384, white adhesive bottom seal for 96-well and 384-well microplate
- 201 [Perkin-Elmer, Cat. No. 6005199]
- 202 • 17 β-estradiol (CAS RN: 50-28-2) [Sigma-Aldrich, Cat. No. E8875]
- 203 • Cryovial, 2 mL (Corning Costar) [Fisher Scientific Cat. No. 03-374-21]
- 204 • Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 10009186R38]²
- 205 • Culture tube, 50 mL conical (Corning Costar) [Fisher Scientific Cat. No. 05-
- 206 526C]
- 207 • DMSO, U.S.P. analytical grade. [Sigma-Aldrich, Cat. No. 34869-100ML]

² If glass tubes can not be obtained from Thomas Scientific, the preference is for flint glass, then lime glass, then borosilicate glass.

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- 208 • Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L
- 209 glucose, with sodium pyruvate, without phenol red or L-glutamine
- 210 [Mediatech/Cellgro, Cat. No. 17-205-CV]
- 211 • Fetal Bovine Serum [Mediatech/Cellgro Cat. No. MT 35-010-CV]
- 212 • Fetal Bovine Serum, charcoal/dextran treated, triple 0.1 µm sterile filtered
- 213 [Hyclone, Cat. No. SH30068.03]
- 214 • Gentamycin Sulfate (G418), 50 mg/mL [Mediatech/Cellgro Cat. No. 30-234-CR]
- 215 • L-glutamine, 29.2 mg/mL [Cellgro, Cat. No. 25005-CI]
- 216 • Luciferase Assay System (10-Pack) [Promega Cat. No. E1501]
- 217 • Lysis Solution 5X [Promega, Cat. No. E1531]
- 218 • Methoxychlor (CAS RN: 72-43-5) [Sigma-Aldrich, Cat. No. 49054]
- 219 • Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 µg/mL streptomycin
- 220 [Cellgro, Cat. No. 30-001-CI].
- 221 • Phosphate buffered saline (PBS, 1X) without calcium and magnesium [Cellgro,
- 222 Cat. No. 21-040-CV]
- 223 • Pipettes, serological: 2.0 mL [Sigma-Aldrich, Cat. No. P1736], 5.0 mL [Sigma-
- 224 Aldrich, Cat. No. P1986], 25 mL [Sigma-Aldrich, Cat. No. P2486]
- 225 • RPMI 1640 medium, containing L-glutamine [Mediatech, Cat. No. 10-040-CV]
- 226 • Tissue culture flasks (Corning-Costar): 25 cm² (T25) [Fisher Cat. No. 10-126-28];
- 227 75 cm² (T75) [Fisher Cat. No. 10-126-37]; and 150 cm² (T150) [Fisher Cat. No.
- 228 10-126-34]
- 229 • Tissue culture plates (Corning-Costar): 96-well [Thomas Scientific Cat. No.
- 230 6916A05]
- 231 • Trypsin (10X), 2.5% in Hank's balanced salt solution (HBSS), without calcium
- 232 and magnesium, without phenol red [Cellgro, Cat. No. 25-054-CI].

233 All reagent lot numbers and expiration dates must be recorded in the study notebook.

234 **8.0 PREPARATION OF TISSUE CULTURE MEDIA AND SOLUTIONS**

235 All tissue culture media and media supplements must be quality tested before use in experiment
236 (see Section 15.0).

237 **8.1 RPMI 1640 Growth Medium (RPMI)**

238 RPMI 1640 is supplemented with 0.9% Pen-Strep and 8.0% FBS to make RPMI growth medium
239 (RPMI).

240 Procedure for one 549 mL bottle:

- 241 1. Remove FBS from -70°C freezer, and Pen-Strep from -20°C freezer and allow to
- 242 equilibrate to room temperature.

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243 2. Add 44 mL of FBS and 5 mL Pen-Strep to the bottle of RPMI 1640.

244 3. Label RPMI bottle as indicated in **Section 7.3**

245 *Store at 2-8°C for no longer than six months or until the shortest expiration date of any media*
246 *component.*

247 **8.2 Estrogen-Free DMEM Medium**

248 DMEM is supplemented to contain 4.5% charcoal/dextran treated FBS, 1.9% L-glutamine, 0.9%
249 Pen-Strep.

250 Procedure for one 539 mL bottle:

251 1. Remove charcoal/dextran treated FBS from -70°C freezer, and L-glutamine and
252 Pen-Strep from -20°C freezer and allow to equilibrate to room temperature.

253 2. Add 24 mL of charcoal/dextran treated FBS, 10 mL L-glutamine, and 5 mL Pen-
254 Strep to one 500 mL bottle of DMEM.

255 3. Label estrogen-free DMEM bottle as indicated in **Section 7.3**

256 *Store at 2-8°C for no longer than six months or until the shortest expiration date of any media*
257 *component..*

258 **8.3 1X Trypsin Solution**

259 1X Trypsin solution is prepared by dilution from a 10X premixed stock solution. The 10X stock
260 solution should be stored in 10 mL aliquots in a -20°C freezer.

261 Procedure for making 100 mL of 1X trypsin:

262 1. Remove a 10 mL aliquot of 10X trypsin from -20°C freezer and allow to
263 equilibrate to room temperature.

264 2. Aliquot 1 mL Trypsin (10X) along with 9 mL of 1X PBS into ten 15 mL sterile
265 centrifuge tubes.

266 3. Label 1X trypsin aliquots as indicated in **Section 7.3**

267 *1X Trypsin should be stored at -20°C.*

268 **8.4 1X Lysis Solution**

269 Lysis solution is prepared by dilution from a 5X premixed stock solution. Both the 5X and 1X
270 solutions can be repeatedly freeze-thawed.

271 The procedure for making 10 mL of 1X lysis solution:

272 1. Thaw the 5X Promega Lysis solution and allow it to reach room temperature.

273 2. Remove 2 mL of 5X solution and place it in a 15 mL conical centrifuge tube.

274 3. Add 8 mL of distilled, de-ionized water to the conical tube.

275 4. Cap and shake gently until solutions are mixed.

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276 Store at -20°C for no longer than 1 year from receipt.

277 **8.5 Reconstituted Luciferase Reagent**

278 Luciferase reagent consists of two components, luciferase buffer and lyophilized luciferase
279 substrate.

280 For long term storage, unopened containers of the luciferase buffer and lyophilized luciferase
281 substrate can be stored at -70°C for up to one year.

282 To reconstitute luciferase reagent:

- 283 1. Remove luciferase buffer and luciferase substrate from -70°C freezer and allow
284 them to equilibrate to room temperature.
- 285 2. Add 10 mL of luciferase buffer solution to luciferase substrate container and swirl
286 or vortex gently to mix; the Luciferase substrate should readily go into solution.
- 287 3. After solutions are mixed, aliquot to a 15mL centrifuge tube.
- 288 4. Store complete solution at -20°C .

289 Reconstituted luciferase reagent is stable for up to 1 month at -20°C .

290 **9.0 OVERVIEW OF PROPAGATION AND EXPERIMENTAL PLATING OF** 291 **BG1LUC4E2 CELLS**

292 BG1Luc4E2 cells are based on a continuous ovarian carcinoma cell line (BG-1 cells) that
293 endogenously express $\text{ER}\alpha$ and $\text{ER}\beta$ and have been stably transfected with an ER responsive
294 reporter gene (*luc*). Although the cell line has proved to be stable over long-term passage *in*
295 *vitro*, careful handling and the use of quality cell culture materials is required to maintain the
296 stability and integrity of the cell line. Procedures specified in the Guidance on Good Cell Culture
297 Practice (Coecke, 2005) should be followed to assure the quality of all materials and methods in
298 order to maintain the integrity, validity, and reproducibility of any work conducted.

299 The BG1Luc4E2 cells are stored in liquid nitrogen in 2 mL cryovials. BG-1 cells are grown as a
300 monolayer in tissue culture flasks in a dedicated tissue culture incubator at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90\% \pm$
301 5% humidity, and $5.0\% \pm 1\%$ CO_2/air . The cells should be examined, on a daily basis during
302 working days, under an inverted phase contrast microscope and any changes in morphology
303 and/or adhesive properties must be noted in the study notebook.

304 Two T150 flasks containing cells at 80 to 90% confluence will usually yield a sufficient number
305 of cells to fill three 96-well plates for use in experiments.

306 **9.1 Procedures for Thawing Cells and Establishing Tissue Cultures**

307 Warm all of the tissue culture media and solutions to room temperature by placing them under
308 the tissue culture hood several hours before use.

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309 All tissue culture media, media supplements, and tissue culture plasticware must be quality
310 tested before use in experiments (**Section 15.0**).

311 9.1.1 Thawing Cells

- 312 1. Remove a cryovial of frozen BG-1 cells from the liquid nitrogen flask.
- 313 2. Facilitate rapid thawing by loosening the top slightly (do not remove top) to
314 release trapped gasses and retightening it. Roll vial between palms.
- 315 3. Use a micropipette to transfer cells to a 50 mL conical centrifuge tube.
- 316 4. Rinse cryovial twice with 1X PBS and add PBS rinse material to the conical tube.
- 317 5. Add 20 mL of RPMI to the conical tube.
- 318 6. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge
319 for an additional 5 minutes.
- 320 7. Aspirate media from pellet and re-suspend it in 5 mL RPMI, drawing the pellet
321 repeatedly through a 1.0 mL serological pipette to break up any clumps of cells.
- 322 8. Transfer cells to a T25 flask, place them in an incubator (see conditions in
323 **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

324 9.1.2 Establishing Tissue Cultures

325 Once cells have reached 80 to 90% confluence, transfer the cells to a T75 flask by performing,
326 for example, the following steps:

- 327 1. Remove the T25 flask from the incubator.
- 328 2. Aspirate the RPMI, then add 5 mL 1X PBS, making sure that the cells are coated
329 with PBS.
- 330 3. Aspirate 1X PBS, then add 1 to 2 mL 1X trypsin to the T25 flask, gently swirling
331 the flask to coat all cells with the trypsin.
- 332 4. Place the flask in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
- 333 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the
334 hand.
- 335 6. Confirm cell detachment by examination under an inverted microscope. If cells
336 have not detached, return the flask to the incubator for an additional 2 minutes,
337 then hit the flask again.
- 338 7. After cells have detached, add 5 mL PBS, and transfer the suspended cells to a 50
339 mL centrifuge tube. Wash the flask one additional time with 5 mL PBS.
- 340 8. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular
341 digestion by residual trypsin.
- 342 9. Pellet the cells by centrifugation, as described in **Section 9.1.1**, and re-suspend the
343 cells in 10 mL RPMI medium.

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- 344 10. Draw the pellet repeatedly through a 25 mL serological pipette to break up
345 clumps of cells
- 346 11. Transfer cells to a T75 flask, then place the flask in an incubator (see conditions
347 in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
348 When cells have reached 80% to 90% confluency, transfer them into a T150 flask by performing,
349 for example, the following steps:
- 350 12. Remove the T75 flask from the incubator, aspirate the old media and add 5 mL
351 1X PBS.
- 352 13. Aspirate 1X PBS, add 2 mL of 1X trypsin to the flask, and place it in an incubator
353 (see conditions in **Section 9.0**) for 5 to 10 min.
- 354 14. Repeat steps **5** through **11** in **Section 9.1.2**, re-suspending the pellet in 20 mL of
355 RPMI.
- 356 15. Transfer cells to a T150 flask and place it in the incubator (see conditions in
357 **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
- 358 16. Remove the T150 flask from the incubator.
- 359 17. Aspirate the RPMI and add 5 mL 1X PBS.
- 360 18. Aspirate 1X PBS and add 3 mL 1X trypsin to the T150 flask, making sure that the
361 cells are coated with the trypsin.
- 362 19. Incubate cells in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
- 363 20. Detach cells by hitting the side of the flask sharply against the palm or heel of the
364 hand.
- 365 21. Confirm cell detachment by examination under an inverted microscope. If cells
366 have not detached, return the flask to the incubator for an additional 2 minutes,
367 then hit the flask again.
- 368 22. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells
369 from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the
370 flask, swirl around the flask, and then transfer the PBS to the 50 mL conical tube.
- 371 23. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular
372 digestion by residual trypsin.
- 373 24. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge
374 for an additional 5 minutes.
- 375 25. Aspirate the media from the pellet and re-suspend it in 40 mL RPMI, drawing the
376 pellet repeatedly through a 25 mL serological pipette to break up any clumps of
377 cells.
- 378 26. Transfer 20 mL of cell suspension to each of two T150 flasks, place them in an
379 incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence
380 (approximately 48 to 72 hrs).

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381 **9.2 Ongoing Tissue Culture Maintenance, Conditioning in Estrogen-free Medium,**
382 **and Plating Cells for Experimentation**

383 The following procedure is used to condition the BG1Luc4E2 cells to an estrogen-free
384 environment prior to plating the cells in 96-well plates for analysis of estrogen dependent
385 induction of luciferase activity.

386 To start the tissue culture maintenance and estrogen-free conditioning, split the two T150 culture
387 flasks into four T150 flasks. Two of these flasks will be used for continuing tissue culture and
388 will use the RPMI media mentioned above. The other two flasks will be cultured in estrogen-free
389 DMEM for experimental use. Extra care must be taken to avoid contaminating the estrogen-free
390 cells with RPMI.

- 391 1. Remove both T150 flasks from the incubator.
- 392 2. Aspirate the medium and rinse the cells with 5 mL 1X PBS.
- 393 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask
394 to coat all cells with the trypsin.
- 395 4. Incubate cells in the incubator (see conditions in **Section 9.0**) for 5 to 10 min.
- 396 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the
397 hand.
- 398 6. Confirm cell detachment by examination under an inverted microscope. If cells
399 have not detached, return the flask to the incubator for an additional 2 minutes,
400 then hit the flask again.
- 401 7. After cells have detached, add 5 mL 1X PBS to the first T150 flask and transfer
402 the suspended cells to the second T150 flask.
- 403 8. Transfer the contents of both flasks to a 50 mL conical tube. Repeat step 7 with an
404 additional 5 mL 1X PBS and transfer to the 50 mL conical tube.
- 405 9. Immediately add 20 mL estrogen-free DMEM to the 50 mL conical tube to inhibit
406 further cellular digestion by residual trypsin.
- 407 10. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge
408 for an additional 5 minutes.
- 409 11. Aspirate media from pellet and re-suspend it in 4 mL estrogen-free DMEM,
410 drawing the pellet repeatedly through a 1 mL serological pipette to break up
411 clumps of cells.

412 At this point, cells are ready to be divided into the ongoing tissue culture and estrogen-free
413 conditioning groups.

414 **9.2.1 Ongoing Tissue Culture Maintenance**

- 415 1. Add 20 mL RPMI to two T150 flasks.

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- 416 2. Add 220 μ l G418 to the RPMI in the T150 flasks
- 417 3. Add 1 mL of cell suspension from **9.2 step 11** to each flask.
- 418 4. Place T150 flasks in tissue culture incubator (see conditions in **Section 9.0**) and
- 419 grow to 80% to 90% confluence (approximately 48 to 72 hrs).
- 420 5. Tissue culture medium may need to be changed 24 hours after addition of G418 to
- 421 remove cells that have died because they do not express reporter plasmid.
- 422 6. G418 does not need to be added to the flasks a second time.
- 423 7. Repeat **Section 9.2 steps 1-11** for ongoing tissue culture maintenance.

424 9.2.2 Conditioning in Estrogen-free Medium

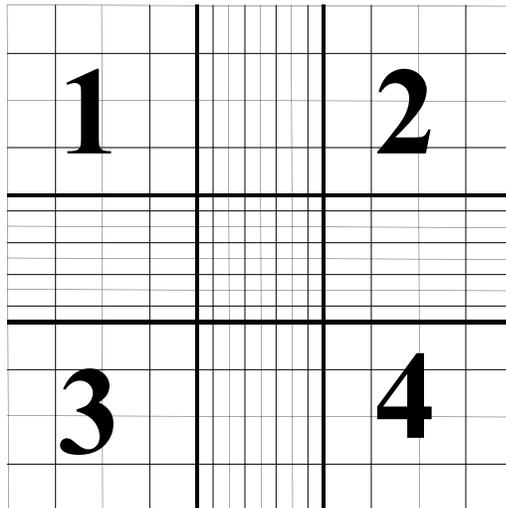
- 425 1. Add 20 mL estrogen-free DMEM to two T150 flasks.
- 426 2. Add 150 μ L G418 to the estrogen-free DMEM in the T150 flasks.
- 427 3. Add 1 mL of cell suspension from **Section 9.2 step 11** to each flask.
- 428 4. Tissue culture medium may need to be changed 24 hours after addition of G418 to
- 429 remove cells that have died because they do not express reporter plasmid.
- 430 5. G418 does not need to be added to the flasks a second time.
- 431 6. Place the T150 flasks in the incubator (see conditions in **Section 9.0**) and grow to
- 432 80% to 90% confluence (approximately 48 to 72 hrs).

433 9.2.3 Plating Cells Grown in Estrogen-free DMEM for Experimentation

- 434 1. Remove the T150 flasks that have been conditioned in estrogen-free DMEM for
- 435 48 to 72 hours from the incubator.
- 436 2. Aspirate the medium, then rinse the cells with 5 mL 1X PBS.
- 437 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask
- 438 to coat all cells with the trypsin.
- 439 4. Place the flasks in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
- 440 5. Detach cells by hitting the side of the flask sharply against the palm or the heel of
- 441 the hand.
- 442 6. Confirm cell detachment by examination under an inverted microscope. If cells
- 443 have not detached, return the flask to the incubator for 2 additional minutes, then
- 444 hit the flask again.
- 445 7. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells
- 446 from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the
- 447 flask, gently swirl around the flask, and then transfer to the 50 mL conical tube.
- 448 8. Immediately add 20 mL estrogen-free DMEM to each conical tube to inhibit
- 449 further cellular digestion by residual trypsin.
- 450 9. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge
- 451 for an additional 5 minutes.

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- 452 10. Aspirate the media from the pellet and re-suspend it in 20 mL DMEM, drawing
 453 the pellet repeatedly through a 25 mL serological pipette to break up any clumps
 454 of cells.
- 455 11. Pipette 15 μ L of the cell suspension into the “v” shaped slot on the
 456 hemocytometer. Ensure that the solution covers the entire surface area of the
 457 hemocytometer grid, and allow cells to settle before counting.
- 458 12. Using 100x magnification, view the counting grid.
- 459 13. The counting grid on the hemocytometer consists of nine sections, four of which
 460 are counted (upper left, upper right, lower left, and lower right, see **Figure 9-1**).
 461 Each section counted consists of four by four grids. Starting at the top left and
 462 moving clockwise, count all cells in each of the four by four grids. Some cells
 463 will be touching the outside borders of the square, but only count those that touch
 464 the top and right borders of the square. This value is then used in the calculation
 465 below to get to the desired concentration of 200,000 cells/mL.
 466

Figure 9-1 Hemocytometer Counting Grid.

467

468

The volume of each square is 10^{-4} mL, therefore:

469

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

470

Starting dilution: 20 mL (for T150 flasks)

471

472 Harvested cells for a T150 flask are suspended in 20 mL of estrogen-free DMEM and sampled
 473 for determination of concentration of cells/mL.

474

475 Example Calculation:

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476 • Grids 1, 2, 3, and 4 are counted and provide the following data:
477 ○ 50, 51, 49, and 50: average number of cells per grid is equal to 50.
478 Cells/mL = 50 cells per grid ÷ 10⁻⁴ mL volume of grid = 50 X 10⁴ cells/mL (or 500,000
479 cells/mL)

480 Total # of Cells Harvested = 500,000 cells/mL x 20 mL

481 Desired Concentration (or Concentration_{Final})= 200,000 cells/mL

482 Formula: (Concentration_{Final} x Volume_{Final} = Concentration_{Initial} x Volume_{Initial})

483 Concentration_{Final} = 200,000 cells/mL

484 Concentration_{Initial} = 500,000 cells/mL

485 Volume_{Initial} = 20 mL

486 Volume_{Final} – to be solved for.

487 Therefore: 200,000 cells/mL x Volume_{Final} = 500,000 cells/mL x 20 mL

488 Solving for Volume_{Final} we find = 50 mL

489 Therefore, add 30 mL of estrogen-free DMEM to the cell suspension for a total volume of 50
490 mL, which will yield the desired concentration of 200,000 cells/mL for plating.

491 14. This dilution scheme will give a concentration of 200,000 cells/mL. 200 µL of
492 this cell suspension is used for each well of a 96-well plate (i.e., 40,000 cells per
493 well).

494 15. Remove a 96-well plate from its sterile packaging. Use a repeater pipetter to
495 pipette 200 µL of cell suspension into each well for to be used for the testing of
496 coded substances, reference standard and controls (**note:** add 200 µL of estrogen-
497 free DMEM only to any wells not being used for testing).

498 16. Incubate plate(s) in an incubator (see conditions in **Section 9.0**) for a minimum of
499 24 hours, but no longer than 48 hours before dosing.

500 Two T150 flasks containing cells at 80% to 90% confluence will typically yield sufficient cells
501 to fill four 96-well plates.

502 **10.0 PREPARATION OF TEST SUBSTANCES**

503 The solvent used for dissolution of test substances is 100% DMSO. All test substances should be
504 allowed to equilibrate to room temperature before being dissolved and diluted. Test substance
505 solutions (except for reference standards and controls) should not be prepared in bulk for use in
506 subsequent tests. Test substances are to be used within 24 hours of preparation. Solutions should
507 not have noticeable precipitate or cloudiness.

508 All information on weighing, solubility testing, and calculation of final concentrations for test
509 substances, reference standards and controls is to be recorded in the study notebook.

510 **10.1 Determination of Test Substance Solubility**

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- 511 1. Prepare a 100 mg/mL solution of the test substance in 100% DMSO in a 4 mL
512 conical tube.
- 513 2. Vortex to mix.
- 514 3. If the test substance does not dissolve at 100 mg/mL, prepare a 10 mg/mL
515 solution and vortex as above.
- 516 4. If the test substance does not dissolve at 10 mg/mL solution, prepare a 1 mg/mL
517 solution in a 4 mL conical tube and vortex as above.
- 518 5. If the test substance does not dissolve at 1 mg/mL, prepare a 0.1 mg/mL solution
519 in a 4 mL conical tube and vortex as above.
- 520 6. Continue testing, using 1/10 less substance in each subsequent attempt until test
521 substance is solubilized in DMSO.

522 Once the test substance has fully dissolved in 100% DMSO, the test substance is ready to be
523 used for BG1Luc ER TA testing.

524 The Testing Facility shall forward the results from the solubility tests assay to the SMT through
525 the designated contacts in electronic format and hard copy upon completion of testing.

526 10.2 Preparation of Reference Standards, Control and Test Substances

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

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

531 10.2.1 Preparation of Reference Standard and Positive Control Stock Solutions

532 Stock solutions of E2 and methoxychlor are prepared in 100% DMSO and stored at room
533 temperature for up to three years or until the expiration date listed in the certificate of analysis
534 for that substance.

535 10.2.1.1 *E2 Stock Solution*

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

538 **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

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Step #	Action	DMSO	E2 Concentration
4	Transfer 10 μ L E2 solution from Step #3 to a 13 mm test tube to create the working solution.	Add 990 μ L of 100% DMSO. Vortex to mix.	1.0×10^{-2} μ g/mL

539

540 10.2.1.2 *Methoxychlor Stock Solution*541 The final concentration of the methoxychlor stock solution is 313 μ g/mL.

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

- 543 1. Make a 10 mg/mL stock solution of Methoxychlor in 100% DMSO in a 4 mL
544 vial.
- 545 2. Remove 94 μ L of the methoxychlor solution and place it in a new 4 mL vial.
- 546 3. Add 2.906 mL of 100% DMSO to the 4mL vial and gently vortex to mix.

547 10.2.2 Preparation of Reference Standard and DMSO Control for Range Finder Testing

548 Range finder testing is conducted on 96-well plates using four concentrations of E2 in duplicate
549 as the reference standard. Four replicate wells are used for the DMSO control. All wells on the
550 96 well plate are used during range finder testing.

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

552 10.2.2.1 *Preparation of E2 Reference Standard for Range Finder Testing*

553 To make E2 dosing solutions:

- 554 1. label four 4 mL conical tubes with numbers 1 through 4 and place them in a tube
555 rack
- 556 2. label four 13 mm glass test tubes with numbers 1 through 4, place them in a tube
557 rack and add 600 μ L of estrogen-free DMEM to each tube

558 Prepare dilutions to give final concentrations of the E2 as shown in **Table 10-2**.

559

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

Tube Number	100% DMSO	E2 ¹	Estrogen-free DMEM ²	Final Volume	E2 Concentration
1	6 µL	6 µl of 1.0 x 10 ⁻² µg/mL working solution	600 µL	606 µL	5.00 x 10 ⁻⁵ µL
2	18 µL	6 µL of 1.0 x 10 ⁻² µg/mL working solution	600 µL	606 µL	1.25 x 10 ⁻⁵ µL
3	18 µL	6 µL from conical tube #2	600 µL	606 µL	3.13 x 10 ⁻⁶ µL
4	18 µL	6 µL from conical tube #3	600 µL	606 µL	7.83 x 10 ⁻⁷ µL

561 ¹Add specified volume of 100% DMSO and 6 µl of the specified E2 solution to labeled 4 mL conical
 562 tubes, and vortex.

563 ²Transfer 6 µL of DMSO/E2 solution from 4 mL conical tube to labeled 13 mL glass tubes containing
 564 DMEM and vortex.

565 10.2.2.2 *Preparation of DMSO Control for Range Finder Testing*

- 566 1. Add 10 µL of 100% DMSO to a 13 mm glass tube.
 567 2. Add 1000 µL of estrogen-free DMEM to tube and vortex.

568 10.2.3 Preparation of Test Substance Dosing Solutions for Range Finder Testing

569 Range finder experiments are used to determine the concentrations of test substance to be used
 570 during comprehensive testing. Agonist range finding for coded substances consists of seven
 571 point, 1:10 serial dilutions run in duplicate.

572 To make dosing solutions for coded substances:

- 573 1. label seven 4 mL conical tubes with numbers 1 through 7 and place them in a tube
 574 rack
 575 2. label seven 13 mm glass test tubes with numbers 1 through 7, place them in a tube
 576 rack and add 600 µL of estrogen-free DMEM to each tube

577 Prepare dilutions as shown in **Table 10-3**.

578

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

Tube Number	100% DMSO	Test Substance ¹	Transfer	Estrogen-free DMEM	Final Volume
1	-	6 µL of test substance solution from Section 10.1 step 10	6 µL	600 µL	606 µL
2	90 µL	10 µL of test substance solution from Section 10.1 step 10	6 µL	600 µL	606 µL
3	90 µL	10 µL from conical tube #2	6 µL	600 µL	606 µL
4	90 µL	10 µL from conical tube #3	6 µL	600 µL	606 µL
5	90 µL	10 µL from conical tube #4	6 µL	600 µL	606 µL
6	90 µL	10 µL from conical tube #5	6 µL	600 µL	606 µL
7	90 µL	10 µL from conical tube #6	6 µL	600 µL	606 µL

579 ¹Add specified volume of 100% DMSO and test substance solution to labeled 4 mL conical tubes, and
580 vortex.

581 ²Transfer 6 µL of DMSO/E2 solution from 4 mL conical tube to labeled 13 mL glass tubes containing
582 DMEM and vortex.

583
584 Determination of whether a substance is positive in range finder testing and selection of starting
585 concentrations for comprehensive testing will be discussed in **Section 12.0**.

586 10.2.4 Preparation of Reference Standard and Positive Control Dosing Solutions for
587 Comprehensive Testing

588 Comprehensive testing is conducted on 96-well plates using 11 concentrations of E2 in duplicate
589 as the reference standard. Four replicate wells for the DMSO control and three replicate wells for
590 the methoxychlor control are included on each plate.

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

592 10.2.4.1 *Preparation of E2 Reference Standard for Comprehensive Testing*

593 To make E2 dosing solutions:

- 594 1. label 11 4 mL conical tubes with numbers 1 through 11 and place them in a tube
595 rack
- 596 2. label 11 13 mm glass test tubes with numbers 1 through 11, place them in a tube
597 rack and add 600 µL of DMEM to each tube

598 Prepare dilutions to give final concentrations of E2 as shown in **Table 10-4**.

599

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

Tube Number	100% DMSO	E2 ¹	Estrogen-free DMEM ²	Final Volume	E2 Concentration
1	-	6 µL of 1.0 x 10 ⁻² µg/mL working solution	600 µL	606 µL	1.00 x 10 ⁻⁴ µL
2	6 µL	6 µL of 1.0 x 10 ⁻² µg/mL working solution	600 µL	606 µL	5.00 x 10 ⁻⁵ µL
3	6 µL	6 µL from conical tube #2	600 µL	606 µL	2.50 x 10 ⁻⁵ µL
4	6 µL	6 µL from conical tube #3	600 µL	606 µL	1.25 x 10 ⁻⁵ µL
5	6 µL	6 µL from conical tube #4	600 µL	606 µL	6.25 x 10 ⁻⁶ µL
6	6 µL	6 µL from conical tube #5	600 µL	606 µL	3.13 x 10 ⁻⁶ µL
7	6 µL	6 µL from conical tube #6	600 µL	606 µL	1.56 x 10 ⁻⁶ µL
8	6 µL	6 µL from conical tube #7	600 µL	606 µL	7.83 x 10 ⁻⁷ µL
9	6 µL	6 µL from conical tube #8	600 µL	606 µL	3.92 x 10 ⁻⁷ µL
10	6 µL	6 µL from conical tube #9	600 µL	606 µL	1.95 x 10 ⁻⁷ µL
11	6 µL	6 µL from conical tube #10	600 µL	606 µL	9.78 x 10 ⁻⁸ µL

601 ¹Add specified volume of 100% DMSO and 6 µL of the specified E2 solution to labeled 4 mL conical
 602 tubes, and vortex.

603 ²Transfer 6 µL of DMSO/E2 solution from 4 mL conical tube to labeled 13 mL glass tubes containing
 604 DMEM and vortex.

605

606 10.2.4.2 Preparation of Methoxychlor Control Dosing Solution for Comprehensive Testing

607 1. Add 10 µL of the 313 µg/mL methoxychlor to a 13 mm glass tube.

608 2. Add 1000 µL of estrogen-free DMEM to the tube and vortex.

609 10.2.4.3 Preparation of DMSO Control Dosing Solution for Comprehensive Testing

610 1. Add 10 µL of 100% DMSO to four 13 mm tubes (solvent/negative controls).

611 2. Add 1000 µL of estrogen-free DMEM to the tube and vortex.

612 10.2.5 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

613 Comprehensive testing experiments are used to determine whether a substance possesses ER
 614 agonist activity in the BG1Luc ER TA test method. Agonist comprehensive testing for coded
 615 substances consists of either an 11 point 1:2 serial dilution or an 11 point 1:5 serial dilution,
 616 depending on the results from range finder testing (**see Section 12.0**) with each concentration
 617 tested in triplicate wells of the 96-well plate.

618 10.2.5.1 Preparation of Test Substance 1:2 Serial Dilutions for 619 Comprehensive Testing

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620 Start the 11-point serial dilution according to criteria in **Section 12.0**.

621 To make test substance 1:2 serial dilutions for comprehensive testing:

- 622 1. label eleven 4 mL conical tubes with numbers 1 through 11 and place them in a
623 tube rack
- 624 2. label eleven 13 mm glass test tubes with numbers 1 through 11, place them in a
625 tube rack and add 800 μ L of estrogen-free DMEM to each tube

626 Prepare dilution of test substance as shown in **Table 10-6**.

627 **Table 10-5 Preparation of Test Substance 1:2 Serial Dilutions for Comprehensive**
628 **Testing**

Tube Number	100% DMSO	Test Substance ¹	Transfer	Estrogen-free DMEM	Final Volume
1	-	8 μ L of highest concentration of test substance solution	8 μ L	800 μ L	808 μ L
2	8 μ L	8 μ L of highest concentration of test substance solution	8 μ L	800 μ L	808 μ L
3	8 μ L	8 μ L from conical tube #2	8 μ L	800 μ L	808 μ L
4	8 μ L	8 μ L from conical tube #3	8 μ L	800 μ L	808 μ L
5	8 μ L	8 μ L from conical tube #4	8 μ L	800 μ L	808 μ L
6	8 μ L	8 μ L from conical tube #5	8 μ L	800 μ L	808 μ L
7	8 μ L	8 μ L from conical tube #6	8 μ L	800 μ L	808 μ L
8	8 μ L	8 μ L from conical tube #7	8 μ L	800 μ L	808 μ L
9	8 μ L	8 μ L from conical tube #8	8 μ L	800 μ L	808 μ L
10	8 μ L	8 μ L from conical tube #9	8 μ L	800 μ L	808 μ L
11	8 μ L	8 μ L from conical tube #10	8 μ L	800 μ L	808 μ L

629 ¹Add specified volume of 100% DMSO and test substance solution to labeled 4 mL conical tubes, and
630 vortex.

631

632 10.2.5.2 *Preparation of Test Substance 1:5 Serial Dilutions for Comprehensive*
633 *Testing*

634 Start the 11-point serial dilution according to criteria in **Section 12.0**.

635 To make test substance 1:5 serial dilutions for comprehensive testing:

- 636 3. label eleven 4 mL conical tubes with numbers 1 through 11 and place them in a
637 tube rack
- 638 4. label eleven 13 mm glass test tubes with numbers 1 through 11, place them in a
639 tube rack and add 800 μ L of estrogen-free DMEM to each tube

640 Prepare dilution of test substance as shown in **Table 10-6**.

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641 **Table 10-6 Preparation of Test Substance 1:5 Serial Dilutions for Comprehensive**
 642 **Testing**

Tube Number	100% DMSO	Test Substance ¹	Transfer	Estrogen-free DMEM	Final Volume
1	-	8 μ L of highest concentration of test substance solution	8 μ L	800 μ L	808 μ L
2	16 μ L	4 μ L of highest concentration of test substance solution	8 μ L	800 μ L	808 μ L
3	16 μ L	4 μ L from conical tube #2	8 μ L	800 μ L	808 μ L
4	16 μ L	4 μ L from conical tube #3	8 μ L	800 μ L	808 μ L
5	16 μ L	4 μ L from conical tube #4	8 μ L	800 μ L	808 μ L
6	16 μ L	4 μ L from conical tube #5	8 μ L	800 μ L	808 μ L
7	16 μ L	4 μ L from conical tube #6	8 μ L	800 μ L	808 μ L
8	16 μ L	4 μ L from conical tube #7	8 μ L	800 μ L	808 μ L
9	16 μ L	4 μ L from conical tube #8	8 μ L	800 μ L	808 μ L
10	16 μ L	4 μ L from conical tube #9	8 μ L	800 μ L	808 μ L
11	16 μ L	4 μ L from conical tube #10	8 μ L	800 μ L	808 μ L

643 ¹Add specified volume of 100% DMSO and test substance solution to labeled 4 mL conical tubes, and
 644 vortex.

645

646 11.0 GENERAL PROCEDURES FOR THE TESTING OF CODED SUBSTANCES

647 Range finder experiments are used to determine the concentrations of test substance to be used
 648 during comprehensive testing. Comprehensive testing experiments are used to determine whether
 649 a substance possesses ER agonist activity in the BG1Luc ER TA assay.

650 General procedures for range finder and comprehensive are similar. For specific details (such as
 651 plate layout) of range finder testing see **Section 12.0**. For specific details of comprehensive
 652 testing, see **Section 13.0**.

653 11.1 Application of Reference Standard, Controls, and Test Substances

- 654 1. Remove seeded 96-well plates from the incubator, inspect them using an inverted
 655 microscope. Only use plates in which the cells in all wells giving a score of 1
 656 according to **Table 11-1**.
- 657 2. Remove medium by inverting the plate onto blotter paper. Gently tap plate against
 658 the bench surface to remove residual liquid trapped in the wells.
- 659 3. Add 200 μ L of reference standard, control, or test substance to each well (see
 660 **Sections 12.0** and **13.0** for specific plate layouts).
- 661 4. Return plates to incubator and incubate (see **Section 9.0** for details) for 19 to 24
 662 hours to allow maximal induction of luciferase activity in the cells.

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663 11.1.1 Preparation of Excel® Data Analysis Template For Range Finder Testing

- 664 1. In Excel®, open a new “AgRFTemplate” and save it with the appropriate project
665 name as indicated in the NICEATM Style Guide.
- 666 2. Fill out the table at the top of the “Raw Data” worksheet with information
667 regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot.
668 Meas. Time/Well (s), etc. (**note**: this information can be permanently added to the
669 default template “AgRFTemplate” on a laboratory specific basis).
- 670 3. Add the following information regarding the assay to the “Compound Tracking”
671 worksheet.
- 672 ▪ Plate # - Enter the experiment ID or plate number into cell E1
 - 673 ▪ Cell Lot # - Enter the passage or lot number of the cells used for this
674 experiment into cell B5
 - 675 ▪ DMSO and Media Lot #'s – Enter the lot numbers for the DMSO and
676 Media in cells B6 and B7
 - 677 ▪ Test Substance Code – Enter the test substance codes into cells C13 to
678 C18
 - 679 ▪ Name: Enter the experimenter name into cell G6
 - 680 ▪ Date: Enter the experiment date in the format day\month\year into cell
681 G10
 - 682 ▪ Comments: - Enter any comments about the experiment in this box (e.g.,
683 plate contaminated)
- 684 4. Enter the following substance testing information to the “List” page:
- 685 ▪ Concentration – Type in the test substance concentration in µg/ml in
686 descending order.
 - 687 ▪ Also add any replicate-specific comments on this page (e.g, spilled tube,
688 etc.), in the comments section
 - 689 ▪ All of the remaining cells on the List tab should populate automatically.
 - 690 ▪ The “Template”, “Compound Mixing” and “Visual Inspection” tabs
691 should automatically populate with the information entered into the
692 Compound Tracking and List tabs.
- 693 5. Save the newly named project file.
- 694 6. Print out either the “List” or “Template” page for help with dosing the 96-well
695 plate. Sign and date the print out and store in study notebook.

696 11.1.2 Preparation of Excel® Data Analysis Template for Comprehensive Testing

- 697 1. In Excel®, open a new “AgCTTemplate” and save it with the appropriate project
698 name as indicated in the NICEATM Style Guide.

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- 699 2. Fill out the table at the top of the “Raw Data” worksheet with information
700 regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot.
701 Meas. Time/Well (s), etc. (**note**: this information can be permanently added to the
702 default template “AgCTTemplate” on a laboratory specific basis).
703
- 704 3. On the “Compound Tracking” tab, enter the following information:
705 ▪ Plate # - Enter the experiment ID or plate number into cell E1
706 ▪ Cell Lot # - Enter the passage or lot number of the cells used for this
707 experiment into cell C5
708 ▪ DMSO and Media Lot #'s – Enter the lot numbers for the DMSO and
709 Media in cells C6 and C7
710 ▪ Test Substance Code – Enter the test substance codes into cells C15 and
711 C16. Enter the test substance dilution into cells E25 and E26.
712 ▪ Name: Enter the experimenter name into cell G6
713 ▪ Date: Enter the experiment date in the format day\month\year into cell
714 G10
715 ▪ Comments: - Enter any comments about the experiment in this box (e.g.,
716 plate contaminated)
- 717 4. Enter substance testing concentrations to the “List” page. Also add any replicate-
718 specific comments on this page (e.g, spilled tube, etc.).
719 5. Save the newly named project file.
720 6. Print out either the “List” or “Template” page for help with dosing the 96-well
721 plate. Sign and date the print out and store in study notebook.

722 **11.2 Visual Evaluation of Cell Viability**

- 723 1. 19 to 24 hours after dosing the plate, remove the plate from the incubator and
724 remove the media from the wells by inverting the plate onto blotter paper. Gently
725 tap plate against the bench surface to remove residual liquid trapped in the wells.
726 2. Use a repeat pipetter to add 50 μ L 1X PBS to all wells. Immediately remove PBS
727 by inversion.
728 3. Using an inverted microscope, inspect all of the wells used in the 96-well plate
729 and record the visual observations using the scores in **Table 11-1**.
730

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730 **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
P	Wells containing precipitation are to be noted with "P"

731 ¹Reference photomicrographs are provided in the BG1Luc ER TA Validation Study "Visual Observation Cell
 732 Viability Manual."
 733

734 **11.3 Lysis of Cells for BG1Luc ER TA**

- 735 1. Apply the reflective white backing tape to the bottom of the 96-well plate (this
 736 will increase the effectiveness of the luminometer).
 737 2. Add 30 μ L 1X lysis reagent to the assay wells and place the 96-well plate on an
 738 orbital shaker for one minute.
 739 3. Remove plate from shaker and measure luminescence (as described in **Section**
 740 **11.4**).

741 **11.4 Measurement of Luminescence**

742 Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and
 743 with software that controls the injection volume and measurement interval. Light emission from
 744 each well is expressed as RLU per well. The luminometer output is saved as raw data in an
 745 Excel[®] spread sheet. A hard copy of the luminometer raw data should be signed, dated and stored
 746 in the study notebook.

747 **11.5 Data Analysis**

748 BG1Luc ER TA uses an Excel[®] spreadsheet to collect and adjust the RLU values obtained from
 749 the luminometer and a GraphPad Prism[®] template to analyze and graph data. The Excel[®]
 750 spreadsheet subtracts background luminescence (average DMSO solvent control RLU value)
 751 from test substance, reference standard and control RLU values. Plate induction is calculated
 752 using these corrected RLU values. Test substance, reference standard, and control RLU values
 753 are then adjusted relative to the highest E2 reference standard RLU value, which is set to 10,000.
 754 After adjustment, values are transferred to GraphPad Prism[®] for data analysis and graphing.

755 **11.5.1 Collection and Adjustment of Luminometer Data for Range Finder Testing**

756 The following steps describe the procedures required to populate the Excel[®] spreadsheet that has
 757 been configured to collect and adjust the RLU values obtained from the luminometer.

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- 758 1. Open the raw data file and the corresponding experimental Excel[®] spreadsheet
759 from **Section 11.1.1**.
- 760 2. Copy the raw data using the Excel[®] copy function, then paste the copied data into
761 cell B19 of the “RAW DATA” tab in the experimental Excel[®] spreadsheet using
762 the **Paste Special – Values** command. This position corresponds to position A1 in
763 the table labeled Table 1 in this tab.
- 764 3. Examine the DMSO data in Table 1 of the Excel[®] spreadsheet to determine
765 whether there are any potential outliers. See **Section 11.6.2** for further explanation
766 of outlier determinations.
- 767 4. If an outlier is identified, perform the following steps to remove the outlier from
768 calculations:
- 769 ▪ correct the equation used to calculate DMSO background in Table 1 [e.g.,
770 if outlier is located in cell F26, adjust the calculation in cell H40 to read
771 =AVERAGE(G26:I26)]
 - 772 ▪ then correct the equation used to calculate the average DMSO value in
773 Table 2 [e.g., following the above example, adjust cell M42 to read
774 =AVERAGE(G26:I26)]
 - 775 ▪ then correct the equation used to calculate the standard deviation of the
776 DMSO value in Table 2 [e.g., following the above example, adjust cell
777 M43 to read =STDEV(G36:I36)]
- 778 5. Excel[®] will automatically subtract the background (the average DMSO control
779 value) from all of the RLU values in Table 1 and populate Table 2 with these
780 adjusted values.
- 781 6. To calculate plate induction, identify the cell containing the E2a replicate in Table
782 1, plate row H that has the highest RLU value (i.e., cell B26, C26, D26, or E26).
- 783 7. Click into cell D14 and enter the cell number from the previous step into the
784 numerator.
- 785 8. Identify the cell containing the E2b replicate in Table 1, plate row H that has the
786 highest RLU value (i.e., cell J26, K26, L26, or M26).
- 787 9. Click into cell E14 and enter the cell number from the previous step into the
788 numerator.
- 789 10. Click on the “ER Agonist Report” worksheet.
- 790 11. The data for the E2 reference standard, methoxychlor, and DMSO replicates
791 populate the left portion (columns A – F) of the spreadsheet. The data is
792 automatically placed in an Excel[®] graph.
- 793 12. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell
794 D2 of “ER Agonist Report” tab and check the formula contained within that cell.

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- 795 The divisor should be the cell number of the cell containing the highest Mean E2
796 RLU value ((i.e., cell A16, A17, A18, or A19).
- 797 13. Open the “Visual Observation Scoring” worksheet. Enter the visual observation
798 scores for each well on the 96-well plate. This data will be linked to the “ER
799 Agonist Report” worksheet.
- 800 14. After the testing results have been evaluated and reviewed for quality control,
801 enter the following information into the Compound Tracking worksheet:
- 802 ▪ Enter pass/fail results for plate reference standard and control parameters
803 into the Plate Pass/Fail Table
 - 804 ▪ Enter information from the testing of coded substances into the Testing
805 Results Table
 - 806 ▪ Reviewer Name – Enter the name of the person who Reviewed\QC’ed the
807 data into cell A34
 - 808 ▪ Date – Enter the date on which the data was reviewed into cell D34

809 11.5.2 Collection and Adjustment of Luminometer Data for Comprehensive Testing

810 The following steps describe the procedures required to populate the Excel[®] spreadsheet that has
811 been configured to collect and adjust the RLU values obtained from the luminometer.

- 812 1. Open the raw data file and the corresponding experimental Excel[®] spreadsheet
813 from **Section 11.1.2**.
- 814 2. Copy the raw data using the Excel[®] copy function, then paste the copied data into
815 cell B16 of the “RAW DATA” worksheet in the experimental Excel[®] spreadsheet
816 using the **Paste Special – Values** command. This position corresponds to position
817 A1 in the table labeled Table 1 in this worksheet.
- 818 3. Fill out the table at the top of the “Raw Data” worksheet with information
819 regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot.
820 Meas. Time/Well (s), etc. If desired, this information can be added to the
821 Laboratory Template File.
- 822 4. Examine the DMSO data in Table 1 of the Excel[®] spreadsheet to determine
823 whether there are any potential outliers. See **Section 11.6.2** for further explanation
824 of outlier determinations.
- 825 5. If an outlier is identified, perform the following steps to remove the outlier from
826 calculations:
 - 827 ▪ correct the equation used to calculate DMSO background in Table 1[e.g.,
828 if outlier is located in cell M17, adjust the calculation in cell H37 to read
829 =AVERAGE(M16,M18:M19)]

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- 830 ▪ then correct the equation used to calculate the DMSO mean and SD
831 values [e.g., following the above example, adjust cell M39 to read
832 = AVERAGE(M28,M30:M31), and adjust cell M40 to read
833 =STDEV(M28,M30:M31)]
- 834 6. Excel[®] will automatically subtract the background (the average DMSO control
835 value) from all of the RLU values in Table 1 and populate Table 2 with these
836 adjusted values.
- 837 7. To calculate plate induction, identify the cell in containing the E2 replicate in
838 Table 1, plate row G that has the highest RLU value.
- 839 8. Click into cell D11 and enter the cell number from the previous step into the
840 numerator.
- 841 9. Identify the cell containing the E2 replicate in plate row H that has the highest
842 RLU value.
- 843 10. Click into cell E11 and enter the cell number from the previous step into the
844 numerator.
- 845 11. Open the “ER Agonist Report” worksheet.
- 846 12. The data for the E2 reference standard, methoxychlor, and DMSO replicates
847 populate the left portion (columns A – E) of the spreadsheet. The data is
848 automatically placed in an Excel[®] graph.
- 849 13. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell
850 E2 of “ER Agonist Report” tab and check the formula contained within that cell.
851 The divisor should be the cell number of the cell containing the highest Avg E2
852 RLU value (cells A16 through A26).
- 853 14. Open the “Visual Observation Scoring” worksheet. Enter the visual observation
854 scores for each well on the 96-well plate. This data will be linked to the “ER
855 Agonist Report” worksheet.
- 856 15. Copy the data from the “ER Agonist Report” worksheet into GraphPad Prism[®] for
857 the calculation of EC₅₀ values and to graph experimental results as indicated in the
858 NICEATM Prism[®] Users Guide.
- 859 16. After the testing results have been evaluated and reviewed for quality control,
860 enter the following information into the Compound Tracking worksheet:
- 861 ▪ Enter pass/fail results for plate reference standard and control parameters
862 into the Plate Pass/Fail Table
- 863 ▪ Enter information from the testing of coded substances into the Testing
864 Results Table
- 865 ▪ Reviewer Name – Enter the name of the person who Reviewed\QC’ed the
866 data into cell A32

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867 ▪ Date – Enter the date on which the data was reviewed into cell D32

868 11.5.3 Determination of Outliers

869 The Study Director will use good statistical judgment for determining “unusable” wells that will
870 be excluded from the data analysis and will provide an explanation in the study notebook for any
871 excluded data. This judgment for data acceptance will include Q-test analysis.

872 The formula for the Q test is:

873 Outlier – Nearest Neighbor

Range (Highest – Lowest)

874 where the outlier is the value proposed for exclusion, the nearest neighbor is the value closest to
875 the outlier, and the range is the range of the three values (Q values for samples sizes from 3 to 10
876 are provided in **Table 11-2**). For example, if the value of this ratio is greater than 0.94 (the Q
877 value for the 90% confidence interval for a sample size of three) or 0.76 (the Q value for the 90%
878 confidence interval for a sample size of four), the outlier may be excluded from data analysis.

879 **Table 11-2 Q Test Values**

Number Of Observations	Q Value
2	-
3	0.94
4	0.76
5	0.64
6	0.56
7	0.51
8	0.47
9	0.44
10	0.41

880
881 For E2 reference standard replicates (sample size of two), any adjusted RLU value for a replicate
882 at a given concentration of E2 is considered and outlier if its value is more than 20% above or
883 below the adjusted RLU value for that concentration in the historical database.

884 11.5.4 Acceptance Criteria

885 11.5.4.1 *Range Finder Testing*

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

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

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

894 An experiment that fails either acceptance criterion will be discarded and repeated.

895 11.5.4.2 *Comprehensive testing*

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

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

902 • Reference standard results: The E2 reference standard concentration-response
903 curve should be sigmoidal in shape and have at least three values within the linear
904 portion of the concentration-response curve.

905 • DMSO control results: DMSO control RLU values must be within 2.5 times the
906 standard deviation of the historical solvent control mean RLU value.

907 • Positive control results: Methoxychlor control RLU values must be above the line
908 representing the DMSO mean plus three times the standard deviation from the
909 DMSO mean.

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

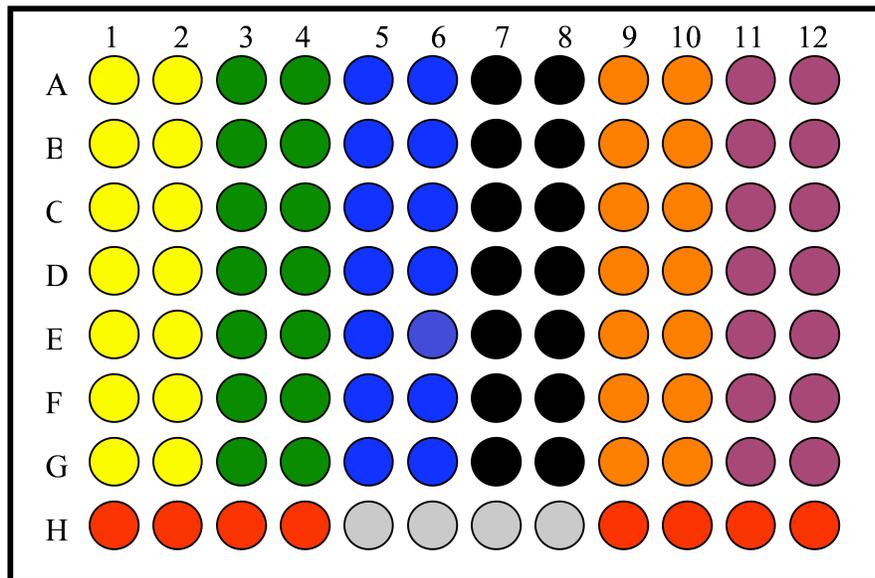
911

912 **12.0 RANGE FINDER TESTING**

913 Agonist range finding for coded substances consists of seven point, 1:10 serial dilutions, with
914 each concentration tested in duplicate wells of the 96-well plate. **Figure 12-1** contains a template
915 for the plate layout to be used in agonist range finder testing.

916

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

 **Four Point E2 Reference Standard**

 **DMSO (Solvent Control)**

 **Range Finder for Sample #1**

 **Range Finder for Sample #2**

 **Range Finder for Sample #3**

 **Range Finder for Sample #4**

 **Range Finder for Sample #5**

 **Range Finder for Sample #6**

917

918 Evaluate whether range finder experiments have met the acceptance criteria

919 (see **Section 11.5.4.1**).

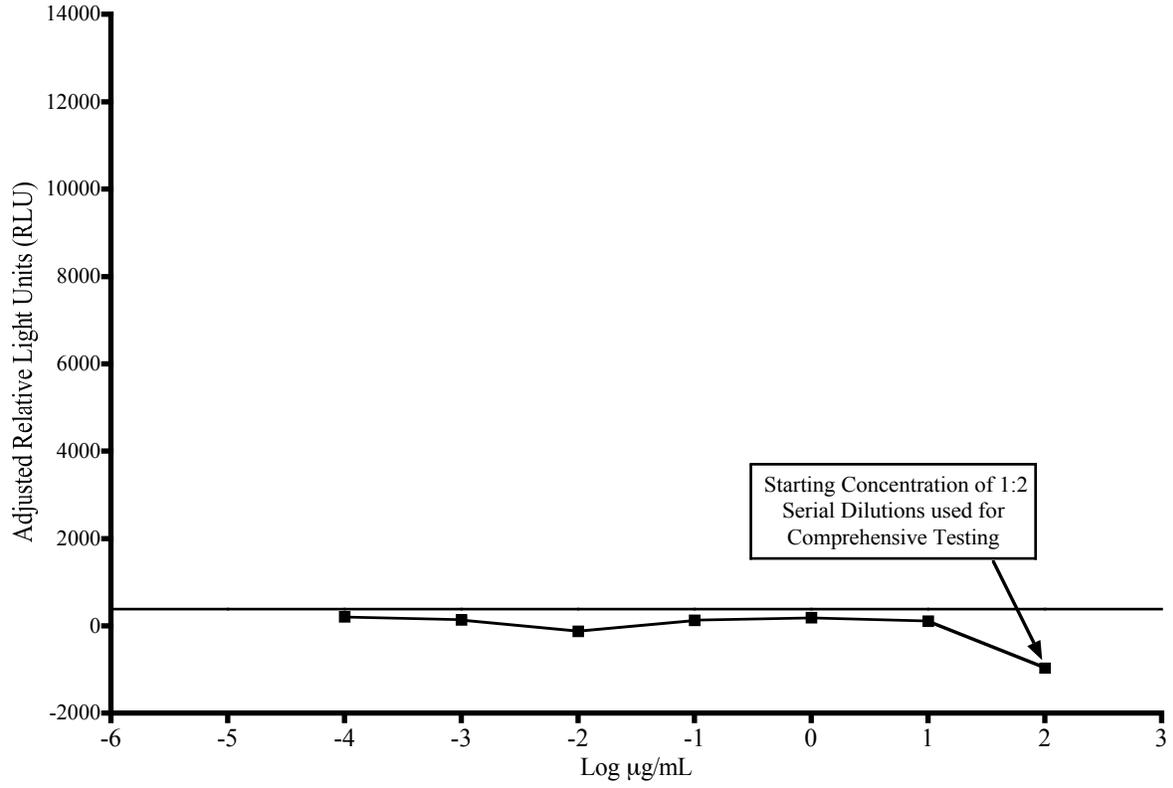
920 To determine starting concentrations for comprehensive testing use the following criteria:

- 921
- 922 • If results in the range finder test suggest that the test substance is negative for
923 agonist activity (i.e., if there are no points on the test substance concentration
924 curve that are greater than the mean plus three times the standard deviation of the
925 DMSO control, see **Figure 12-2**), comprehensive testing will be conducted using
an 11 point 1:2 serial dilution starting at the maximum soluble concentration.

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- If results in the range finder test suggest that the test substance is negative for agonist activity (i.e., if there are no points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control), and the higher concentrations in the range finder are cytotoxic, comprehensive testing will be conducted using an 11 point 1:2 serial dilution with the lowest cytotoxic concentration as the starting concentration (see **Figure 12-3**).
 - If results in the range finder test suggest that the test substance is positive for agonist activity (i.e., if there are points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control), the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one log higher than the concentration giving the highest adjusted RLU value in the range finder. The 11-point dilution scheme will be based on either 1:2 or 1:5 dilutions according to the following criteria:
 - An 11-point 1:2 serial dilution should be used if the resulting concentration range (note: an 11-point 1:2 serial dilution will cover a range of concentrations over approximately three orders of magnitude [three logs]) will encompass the full range of responses based on the concentration response curve generated in the range finder test (see **Figure 12-4**).
 - If the concentration range that would be generated with the 1:2 serial dilution will not encompass the full range of responses based on the concentration response curve in the range finder test (see **Figure 12-5** and **12-6**), an 11-point 1:5 serial dilution should be used instead.
 - If a substance exhibits a biphasic concentration response curve in the range finder test, both phases should also be resolved in comprehensive testing. In order to resolve both curves, the starting concentration should be based on the peak associated with the higher concentration and should be one log higher than the concentration giving the highest adjusted RLU value in the range finder. As an example, an 11-point 1:5 serial dilution should be used based on the range finder results presented in **Figure 12-7**.

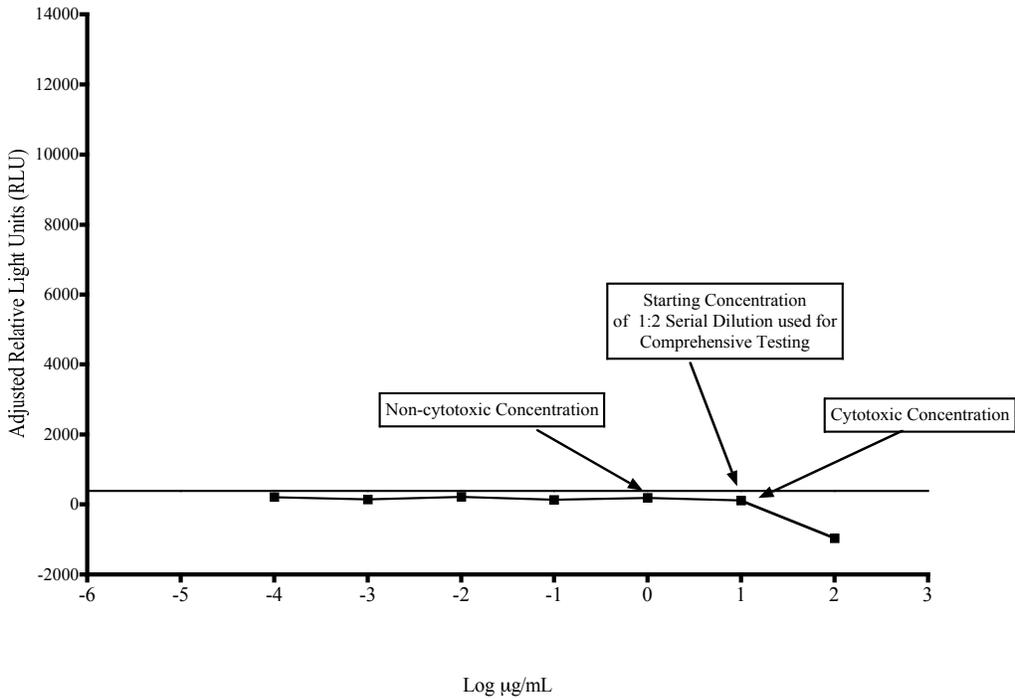
957 **Figure 12-2 Agonist Range Finder (example 1)**



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959
960
961

The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

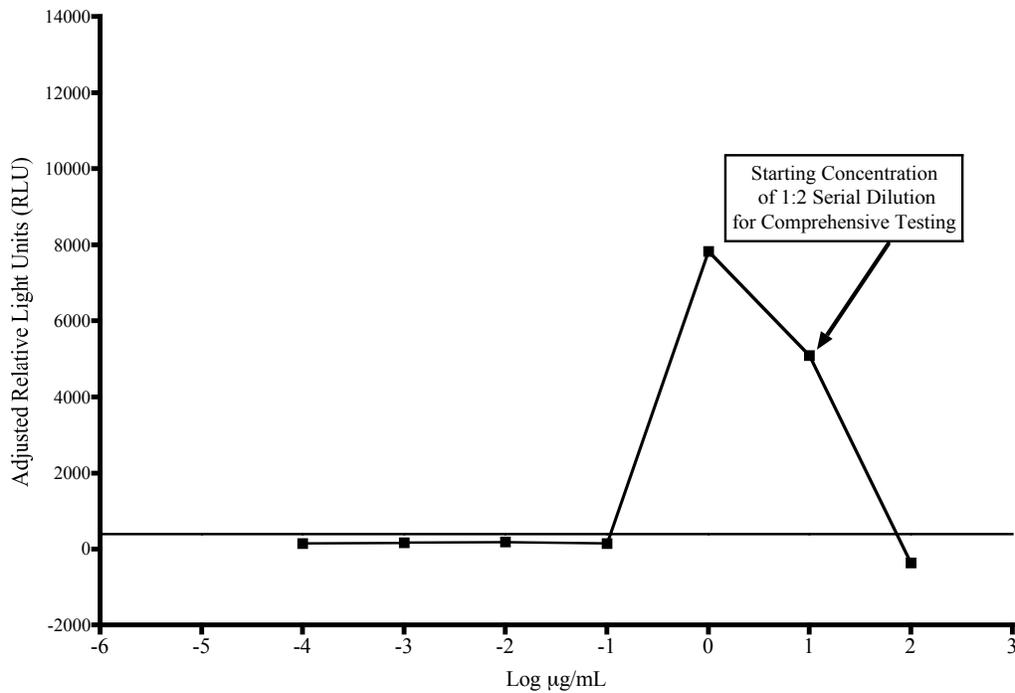
961 **Figure 12-3 Agonist Range Finder (example 2)**



962

963 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

964 **Figure 12-4 Agonist Range Finder (example 3)**

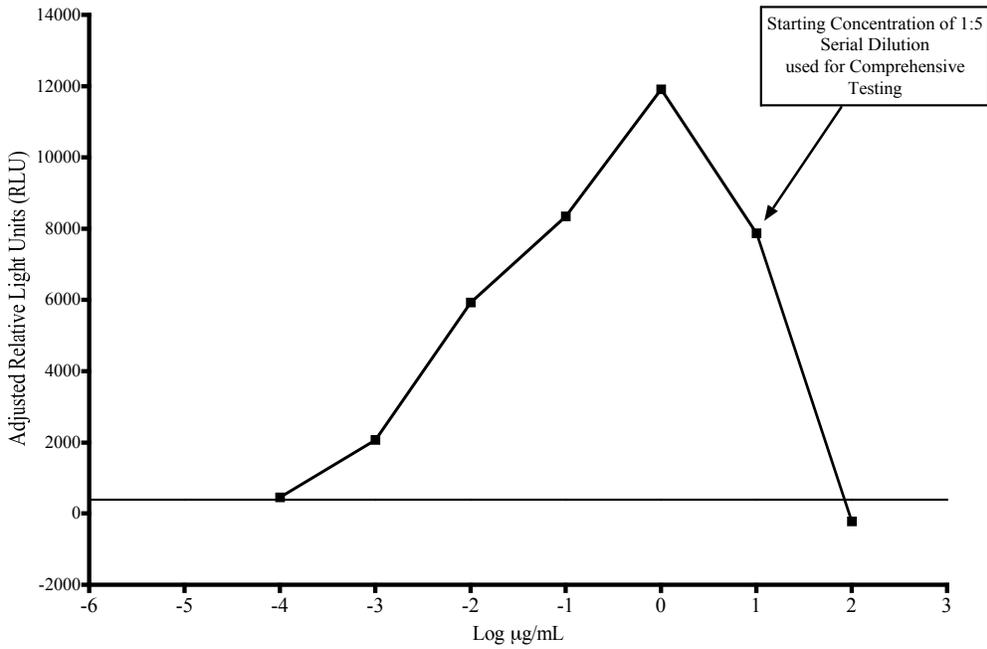


965

966 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

967

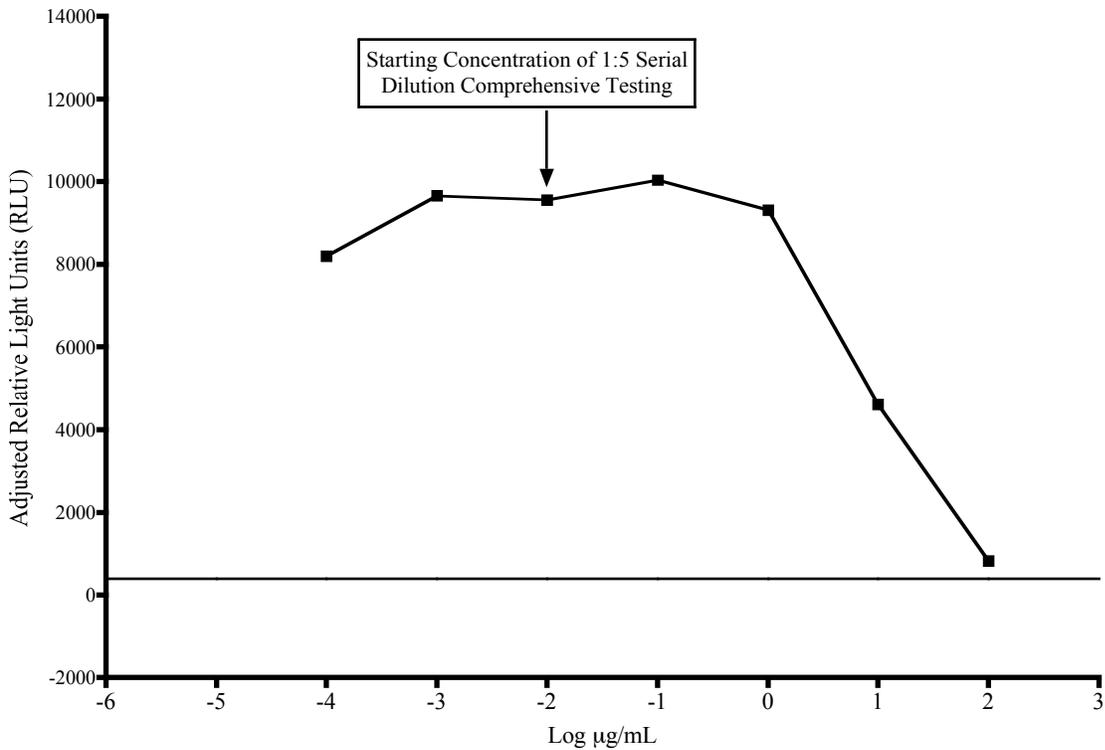
967 **Figure 12-5 Agonist Range Finder (example 4)**



968

969 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

970 **Figure 12-6 Agonist Range Finder (example 5)**

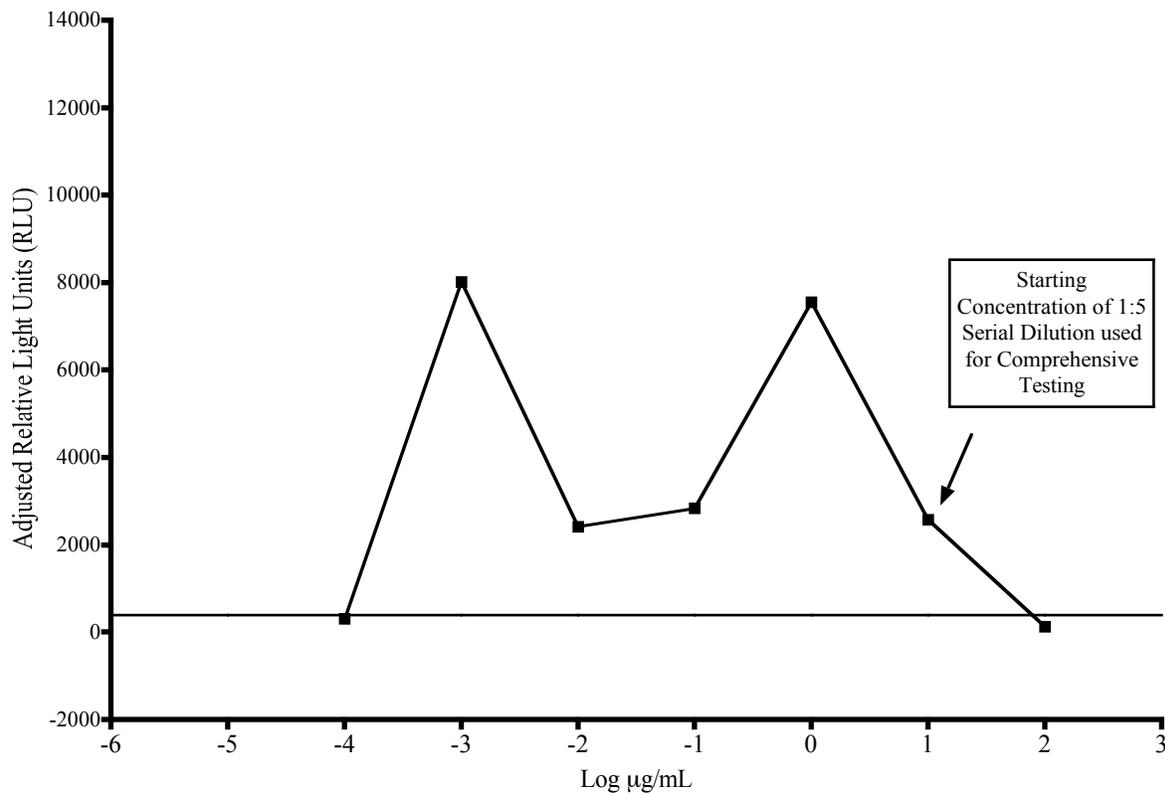


971

972 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

973

973 **Figure 12-7 Agonist Range Finder (example 6)**



974
975 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

976
977 **13.0 COMPREHENSIVE TESTING**

978 Agonist comprehensive testing for coded substances consists of 11 point serial dilutions (either
979 1:2 or 1:5 serial dilutions based on the starting concentration for comprehensive testing criteria in
980 **Section 12.0**) with each concentration tested in triplicate wells of the 96-well plate. **Figure 13-1**
981 contains a template for the plate layout to be used in agonist comprehensive testing.

982

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982 **Figure 13-1 Agonist Comprehensive Test Plate Layout**

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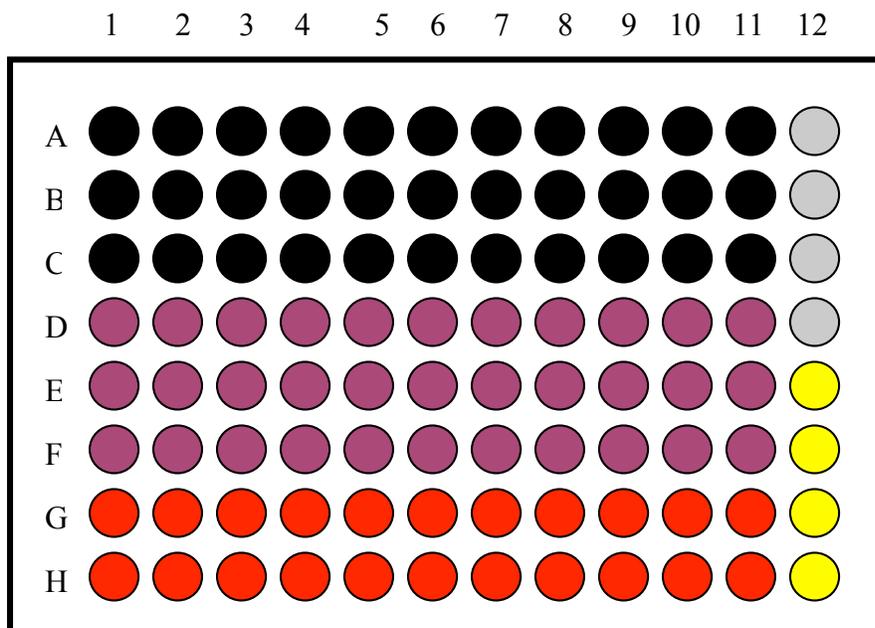
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1000

● **11 Point Duplicate E2 Reference Standard**

1001

○ **DMSO (Solvent Control)**

1002

● **Test Substance #1**

1003

● **Test Substance #2**

1004

● **Methoxychlor Control**

1005

1006 Evaluate whether comprehensive experiments have met acceptance criteria (see **Section 11.6.4**)
 1007 and graph the data as described in the NICEATM Prism[®] users guide.

- 1008
- 1009 • If the substance has been tested up to the limit dose or the maximum soluble dose,
 1010 without causing a significant decrease in cell viability, and there are no points on
 1011 the concentration curve that are greater than the mean plus three times the
 1012 standard deviation of the DMSO control, the substance is considered negative for
 1013 agonism
 - 1014 • If the substance has a positive response (See **Section 6.0**) at any concentration,
 the substance is considered positive for agonism.

1015 **14.0 COMPILATION OF THE HISTORICAL QUALITY CONTROL DATABASE**

1016 Historical databases are maintained in order to ensure that the assay is functioning properly.

1017 Historical databases are compiled using Excel[®] spreadsheets and are separate from the

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1018 spreadsheets used to collect the data for individual test plates. Reference standard and control
1019 data are used to develop and maintain the historical database and are used as quality controls to
1020 determine acceptance of individual test plates.

1021 The sources of the data needed to compile the historical database for the DMSO control are the
1022 experiment specific Excel® data collection and analysis spreadsheets used for BG1Luc ER TA
1023 agonist and antagonist testing (see **Section 11.5.2** of the BG1Luc ER TA agonist protocol and
1024 **Section 13.5.2** in the BG1Luc ER TA antagonist protocol).

1025 **14.1 DMSO Control**

1026 Open the combined agonist and antagonist BG1Luc ER TA historical database Excel®
1027 spreadsheet (LUMI_AgandAntQC.xls) and save under a new name using the Excel® “Save As”
1028 function, adding the laboratory designator to the file name (e.g., for Laboratory H, the new name
1029 would be HLUMI_AgandAntQC.xls). Enter the date and experiment name into worksheet
1030 columns A and B respectively. Enter the experimental mean DMSO control value (from cell H37
1031 in the RAW DATA worksheet of the agonist and antagonist Excel® data collection and analysis
1032 spreadsheet) into worksheet column C. Acceptance or rejection of the plate DMSO control data
1033 for range finding and comprehensive testing is based on whether the mean plate DMSO RLU
1034 value falls within 2.5 times the standard deviation of the DMSO value in the historical database
1035 (columns G and H in the DMSO worksheet).

1036 **15.0 QUALITY TESTING OF MATERIALS**

1037 All information pertaining to the preparation and testing of media, media supplements, and other
1038 materials should be recorded in the Study Notebook.

1039 **15.1 Tissue Culture Media**

1040 Each lot of tissue culture medium must be tested in a single growth flask of cells before use in
1041 ongoing tissue culture or experimentation (**note:** each bottle within a given lot of
1042 Charcoal/Dextran treated FBS must be tested separately).

- 1043 1. Every new lot of media (RPMI and DMEM) and media components (FBS,
1044 Charcoal/Dextran treated FBS, and L-glutamine) must first be tested on the
1045 BG1Luc ER TA prior to being used in any GLP acceptable assays.
- 1046 2. Add 4 µL of DMSO (previously tested) into four separate 13 mm tubes.
- 1047 3. Add 400 µL media (to be tested) to the same tubes.
- 1048 4. Dose an experimental plate as in **Section 12.0**, treating the media being tested as a
1049 test substance.
- 1050 5. Analyze 96-well plate as described in **Section 12.0**, comparing the data from the
1051 DMSO controls made using previously tested tissue culture media to the new
1052 media being tested.

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- 1053 6. Use the agonist historical database to determine if the new media with DMSO lies
1054 within 2.5 standard deviations of the mean for the media. If the RLU values for
1055 the new media with DMSO lie within 2.5 standard deviation of the mean for the
1056 historical data on DMSO, the new lot of media is acceptable. If the RLU values
1057 for the new media with DMSO do not lie within 2.5 standard deviations of the
1058 DMSO mean from historical database, the new lot may not be used in the assay.
1059 7. Note date and lot number in study notebook.
1060 8. If the new bottle passes quality testing as described in **Section 15.1 step 6**, apply
1061 the media to a single flask of cells and observe cell growth and morphology over
1062 the following 2 – 3 days. If there is no change in growth or morphology, the new
1063 media is acceptable for use.

1064 **15.2 G418:**

- 1065 1. New lots of G418 must first be tested on the LUMI-CELL[®] ER assay prior to
1066 being used in any GLP acceptable assays.
1067 2. Add 220 µL of G418 (previously tested) to a single flask containing cells growing
1068 in RPMI.
1069 3. Add 220 µL of G418 (to be tested) to a different flask containing cells growing in
1070 RPMI.
1071 4. Observe cellular growth and morphology in both tissue culture flasks over a 48 to
1072 72 hour period. If there are no differences in observed growth rate and
1073 morphology between the two flasks, the new G418 lot is acceptable.
1074 5. If cellular growth is decreased, or the cells exhibit abnormal morphology, the new
1075 lot of G418 is not acceptable.
1076 6. Note date and lot number in study book.

1077 **15.3 DMSO**

- 1078 1. Every new bottle of DMSO must be tested on the LUMI-CELL[®] ER assay prior
1079 to use in any GLP acceptable assays.
1080 2. Add 4 µL of DMSO (to be tested) into four separate 13 mm tubes.
1081 3. Add 400 µL media (previously tested) to the same tubes.
1082 4. Dose an experimental plate as in **Section 12.0**, treating the DMSO containing
1083 media being tested as a test substance.
1084 5. Analyze 96-well plate as described in **Section 12.0**, comparing the data from the
1085 DMSO controls made using previously tested tissue culture media.
1086 6. Use the agonist historical database to determine if media with new DMSO lies
1087 within 2.5 standard deviations of the DMSO mean from historical database. If the
1088 RLU values for the media with new DMSO lie within 2.5 standard deviations of

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1089 the DMSO mean from the historical database, the new lot of DMSO is acceptable.
1090 If the RLU values for media with new DMSO do not lie within 2.5 standard
1091 deviations of the DMSO mean from historical database, the new lot may not be
1092 used in the assay.

1093 7. Note the date, lot number, and bottle number in study book.

1094 8. If no DMSO has been previously tested, test several bottles as described in
1095 **Section 15.3**, and determine whether any of the bottles of DMSO have a lower
1096 average RLU than the other bottle(s) tested. Use the DMSO with the lowest
1097 average RLU for official experiments.

1098 **15.4 Plastic Tissue Culture Materials**

1099 1. Grow one set of cells, plate them for experiments on plastic ware from the new lot
1100 and one set of cells in the plastic ware from a previous lot, and dose them with E2
1101 reference standard and controls.

1102 2. Perform the BG1Luc ER TA experiment with both sets of cells.

1103 3. If all of the analysis falls within acceptable QC criteria, then the new
1104 manufacturer's products may be used.

1105 **16.0 REFERENCES**

1106 Eli Lilly and Company and National Institutes of Health Chemical Genomics Center. 2005.

1107 Assay Guidance Manual Version 4.1. Bethesda, MD: National Institutes of Health. Available:
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1112 guidance/iv_guide.pdf](http://iccvam.niehs.nih.gov/methods/invidocs/guidance/iv_guide.pdf) [accessed 31 August 2006]

1113 Coecke, S., Guidance on Good Cell Culture Practice. 2005. ATLA 33, 261-287.

1114