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Appendix C

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BG1Luc ER TA – Antagonist Protocol

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**BG1Luc ER TA TEST METHOD
ANTAGONIST PROTOCOL**

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

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LIST OF ACRONYMS AND ABBREVIATIONS

35 13 mm test tube

13 x 100 mm glass test tubes

36 DMEM

Dulbecco's Modification of Eagle's Medium

37 DMSO

Dimethyl Sulfoxide

38 DMSO Control

1% v/v dilution of DMSO in tissue culture media
used as a vehicle control

39

40 E2

17 β -estradiol

41 E2 Control

2.5 x 10⁻⁵ μ g/mL E2 used as a control.42 IC₅₀ ValueConcentration that produces a half-maximal response as
calculated using the four parameter Hill function.

43

44 ER

Estrogen Receptor

45 Estrogen-free DMEM

DMEM (phenol red free), supplemented with 1 %
Penicillin/Streptomycin, 2 % L-Glutamine, and 5% Charcoal-
dextran treated FBS

46

47

48 FBS

Fetal Bovine Serum

49 Flavone/E2 Control

25 μ g/mL flavone + 2.5 x 10⁻⁵ μ g/mL E2,
used as a weak positive control.

50

51 G418

Gentamycin

52 Ral/E2 Reference Standard

Nine point dilution of raloxifene HCl + 2.5 x 10⁻⁵ 17 β -estradiol
reference standard for the LUMI-CELL[®] ER antagonist assay

53

54 RPMI

RPMI 1640 growth medium

55 TA

Transcriptional Activation

56 T25

25 cm² tissue culture flask

57 T75

75 cm² tissue culture flask

58 T150

150 cm² tissue culture flask

59

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60 1.0 PURPOSE

61 This protocol is designed to evaluate coded test substances for potential estrogen receptor (ER) antagonist
62 activity using the BG1Luc ER TA test method.

63 2.0 SPONSOR

64 (As Appropriate)

65 3.0 DEFINITIONS

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

85 4.0 TESTING FACILITY AND KEY PERSONNEL

86 4.1 Testing Facility

87 (As Appropriate)

88 4.2 Key Personnel

- 89 • Study Director: (As Appropriate)
- 90 • Quality Assurance Director: (As Appropriate)

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91 **5.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES**92 **5.1 Test Substances**

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

95 **5.2 Controls**

96 Controls for the ER antagonist protocol are as follows:

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

99 *Ral/E2 reference standard for range finder testing:* Three concentrations (1.56×10^{-3} ,
100 3.91×10^{-4} , and 9.77×10^{-5} $\mu\text{g/mL}$) of raloxifene HCl (Ral), CASRN 84449-90-1, plus a fixed
101 concentration (2.5×10^{-5} $\mu\text{g/mL}$) of 17 β -estradiol (E2), CASRN: 50-28-2, in duplicate wells.

102 *Ral/E2 reference standard for comprehensive testing:* A serial dilution of Ral plus a fixed concentration
103 (2.5×10^{-5} $\mu\text{g/mL}$) of E2 consisting of nine concentrations of Ral/E2 in duplicate wells.

104 *E2 control: 17 β -estradiol, 2.5×10^{-5} $\mu\text{g/mL}$ E2 in tissue culture media used as a base line negative
105 control.*

106 *Flavone/E2 Control:* Flavone, CASRN 525-82-6, 25 $\mu\text{g/mL}$, with 2.5×10^{-5} $\mu\text{g/mL}$ E2 in tissue culture
107 media used as a weak positive control.

108 **6.0 OVERVIEW OF GENERAL PROCEDURES FOR ANTAGONIST TESTING**

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

112 Antagonist range finder testing is conducted on 96-well plates using three concentrations of Ral/E2 (1.56
113 $\times 10^{-3}$, 3.91×10^{-4} , and 9.77×10^{-5} $\mu\text{g/mL}$ Ral) with 2.50×10^{-5} $\mu\text{g/mL}$ E2) in duplicate as the reference
114 standard, with three replicate wells for the E2 and DMSO controls.

115 Comprehensive testing is conducted on 96-well plates using nine concentrations of Ral/E2 in duplicate as
116 the reference standard (**Table 6-1**). Four replicate wells for the DMSO control, Flavone/E2 and E2
117 controls are included on each plate.

118 **Table 6-1 Concentrations of Ral/E2 Reference Standard Used for Comprehensive Testing**

Raloxifene Concentrations ¹	E2 Concentrations
1.25×10^{-2}	2.5×10^{-5}
6.25×10^{-3}	2.5×10^{-5}
3.13×10^{-3}	2.5×10^{-5}
1.56×10^{-3}	2.5×10^{-5}
7.81×10^{-4}	2.5×10^{-5}
3.91×10^{-4}	2.5×10^{-5}

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Raloxifene Concentrations ¹	E2 Concentrations
1.95 x 10 ⁻⁴	2.5 x 10 ⁻⁵
9.77 x 10 ⁻⁵	2.5 x 10 ⁻⁵
4.88 x 10 ⁻⁵	2.5 x 10 ⁻⁵

119 ¹Concentrations are presented in µg/mL.

120 Visual observations for cell viability are conducted for all experimental plates just prior to BG1LUC ER
121 TA evaluation, as outlined in **Section 11.4**.

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

- 127 • A response is considered positive for antagonist activity when the average adjusted RLU
128 for a given concentration is less than the mean RLU value minus three times the standard
129 deviation for the E2 control.
- 130 • Any luminescence at or above this threshold is considered a negative response.

131 For substances that are positive at one or more concentrations, the concentration of test substance that
132 causes a half-maximal response (the relative IC₅₀) is calculated using a Hill function analysis. The Hill
133 function is a four-parameter logistic mathematical model relating the substance concentration to the
134 response (typically following a sigmoidal curve) using the equation below

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

136 where Y = response (i.e., relative light units); X = the logarithm of concentration; Bottom = the minimum
137 response; Top = the maximum response; log IC₅₀ = 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 IC₅₀ parameters. See **Section 13.6.5** 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 16.0**.

143 **6.1 Range Finder Testing**

144 Antagonist range finding for coded substances consists of a seven-point 1:10 serial dilution using
145 duplicate wells per concentration. Concentrations for comprehensive testing are selected based on the
146 response observed in range finder testing. If necessary, a second range finder test can be conducted to
147 clarify the optimal concentration range to test (see **Section 14.0**).

148 **6.2 Comprehensive Testing**

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149 Comprehensive antagonist 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 15.0**).

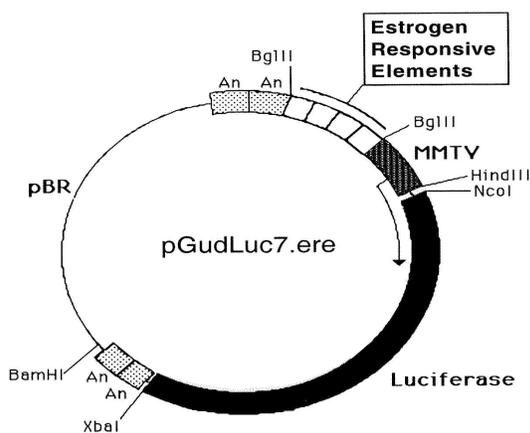
153 7.0 MATERIALS FOR BG1LUC ER TA ANTAGONIST TESTING

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 (**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 can be
 164 used.

- 165 • Analytical balance (Cat. No. 01-910-320)
- 166 • Berthold Orion 1 Microplate Luminometer [Berthold CatNo.: Orion 1 MPL3] or
 167 equivalent and dedicated computer
- 168 • Biological safety hood, class II, and stand (Cat. No. 16-108-99)
- 169 • Centrifuge (low speed, tabletop with swinging bucket rotor) (Cat. No. 04-978-50
 170 centrifuge, and 05-103B rotor)
- 171 • Combustion test kit (CO₂ monitoring) (Cat. No. 10-884-1)

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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- 172 • Drummond diaphragm pipetter (Cat. No. 13-681-15)
- 173 • Freezers, -20°C (Cat. No. 13-986-150), and -70°C (Cat. No. 13-990-86)
- 174 • Hand tally counter (Cat. No. 07905-6)
- 175 • Hemocytometer, cell counter (Cat. No. 02-671-5)
- 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 –10ml (Cat. No. 21-377-191), 2 –20 ml
- 181 (Cat. No. 21-377-287), 20 – 200 ml (Cat. No. 21-377-298), 200 - 1000 ml (Cat. No. 21-
- 182 377-195))
- 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 CO_2 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 SOPs.

191 7.3 Reference Standard, Controls, and Tissue Culture Supplies

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

195 Equivalent tissue culture media and sera from another commercial source can be used, but must first be
196 tested as described in **Section 17.0** to determine suitability for use in this test method.

197 The following are the necessary tissue culture reagents and possible sources based on their use in the pre-
198 validation studies:

- 199 • BackSeal-96/384, white adhesive bottom seal for 96-well and 384-well microplate
200 [Perkin-Elmer, Cat. No. 6005199]
- 201 • 17β -estradiol (CAS RN: 50-28-2) [Sigma-Aldrich, Cat. No. E8875]
- 202 • CellTiter-Glo[®] Luminescent Cell Viability Assay [Promega Cat. No. G7572]
- 203 • Cryovial, 2 mL (Corning Costar) [Fisher Scientific Cat. No. 03-374-21]

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- 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. 05-526C]
- 206 • DMSO, U.S.P. analytical grade. [Sigma-Aldrich, Cat. No. 34869-100ML]
- 207 • Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L glucose, with
- 208 sodium pyruvate, without phenol red or L-glutamine [Mediatech/Cellgro, Cat. No. 17-
- 209 205-CV]
- 210 • Fetal Bovine Serum [Mediatech/Cellgro Cat. No. MT 35-010-CV]
- 211 • Fetal Bovine Serum, charcoal/dextran treated, triple 0.1 µm sterile filtered [Hyclone, Cat.
- 212 No. SH30068.03]
- 213 • Flavone (CASRN: 525-82-6) [Sigma-Aldrich, Cat. No. F2003]
- 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 • Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 µg/mL streptomycin [Cellgro,
- 219 Cat. No. 30-001-CI].
- 220 • Phosphate buffered saline (PBS, 1X) without calcium and magnesium [Cellgro, Cat. No.
- 221 21-040-CV]
- 222 • Pipettes, serological: 2.0 mL [Sigma-Aldrich, Cat. No. P1736], 5.0 mL [Sigma-Aldrich,
- 223 Cat. No. P1986], 25 mL [Sigma-Aldrich, Cat. No. P2486]
- 224 • Raloxifene (CASRN 84449-90-1) [Sigma-Aldrich Cat. No. R1402]
- 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]; 75 cm²
- 227 (T75) [Fisher Cat. No. 10-126-37]; and 150 cm² (T150) [Fisher Cat. No. 10-126-34]
- 228 • Tissue culture plates (Corning-Costar): 96-well [Thomas Scientific Cat. No. 6916A05]
- 229 • Trypsin (10X), 2.5% in Hank's balanced salt solution (HBSS), without calcium and
- 230 magnesium, without phenol red [Cellgro, Cat. No. 25-054-CI].

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

232 **8.0 PREPARATION OF TISSUE CULTURE MEDIA AND SOLUTIONS**

233 All tissue culture media and media supplements must be quality tested before use in experiments (see
234 **Section 15.0**).

235 **8.1 RPMI 1640 Growth Medium (RPMI)**

²If 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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236 RPMI 1640 is supplemented with 0.9% Pen-Strep and 8.0% FBS to make RPMI growth medium (RPMI).

237 Procedure for one 549 mL bottle:

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

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

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

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

244 **8.2 Estrogen-Free DMEM Medium**

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

247 Procedure for one 539 mL bottle:

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

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

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

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

255 **8.3 1X Trypsin Solution**

256 1X Trypsin solution is prepared by dilution from a 10X premixed stock solution. The 10X stock solution
257 should be stored in 10 mL aliquots in a -20°C freezer.

258 Procedure for making 100 mL of 1X trypsin:

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

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

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

263 *1X Trypsin should be stored at -20°C.*

264 **8.4 1X Lysis Solution**

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

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

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

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

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

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

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

273 8.5 Reconstituted Luciferase Reagent

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

275 For long-term storage, unopened containers of the luciferase buffer and lyophilized luciferase substrate
276 can be stored at -70°C for up to six months.

277 To reconstitute luciferase reagent:

278 1. Remove luciferase buffer and luciferase substrate from -70°C freezer and allow them to
279 equilibrate to room temperature.

280 2. Add 10 mL of luciferase buffer solution to luciferase substrate container and swirl or
281 vortex to mix, the Luciferase substrate should readily go into solution.

282 3. Luciferase substrate should readily go into solution.

283 4. After solutions are mixed aliquot to a 15mL centrifuge tube.

284 5. Store complete solution at -20°C .

285 Reconstituted luciferase reagent is stable for 1 month at -20°C .

286 9.0 OVERVIEW OF PROPAGATION AND EXPERIMENTAL PLATING OF BG1Luc4E2 287 CELLS

288 BG1Luc4E2 cells are based on a continuous ovarian carcinoma cell line (BG-1 cells) that endogenously
289 express ER α and ER β and have been stably transfected with an ER responsive reporter gene (*luc*).

290 Although the cell line has proved to be stable over long-term passage *in vitro*, careful handling and the
291 use of quality cell culture materials is required to maintain the stability and integrity of the cell line.

292 Procedures specified in the Guidance on Good Cell Culture Practice (Coecke, 2005) should be followed
293 to assure the quality of all materials and methods in order to maintain the integrity, validity, and
294 reproducibility of any work conducted.

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

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

302 9.1 Procedures for Thawing Cells and Establishing Tissue Cultures

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

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305 All tissue culture media, media supplements, and tissue culture plasticware must be quality tested before
306 use in experiments (**Section 17.0**).

307 9.1.1 Thawing Cells

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

320 9.1.2 Establishing Tissue Cultures

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

- 323 1. Remove the T25 flask from the incubator.
- 324 2. Aspirate the RPMI, then add 5 mL 1X PBS, making sure that the cells are coated with
325 PBS.
- 326 3. Aspirate 1X PBS, then add 1 to 2 mL 1X trypsin to the T25 flask, gently swirling the
327 flask to coat all cells with the trypsin.
- 328 4. Place the flask in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
- 329 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.
- 330 6. Confirm cell detachment by examination under an inverted microscope. If cells have not
331 detached, return the flask to the incubator for an additional 2 minutes, then hit the flask
332 again.
- 333 7. After cells have detached, add 5 mL PBS, and transfer the suspended cells to a 50 mL
334 centrifuge tube. Wash the flask one additional time with 5 mL PBS.
- 335 8. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular digestion by
336 residual trypsin.
- 337 9. Pellet the cells by centrifugation, as described in **Section 9.1.1**, and re-suspend the cells
338 in 10 mL RPMI medium.
- 339 10. Draw the pellet repeatedly through a 25 mL serological pipette to break up clumps of
340 cells

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

372 **9.2 Ongoing Tissue Culture Maintenance, Conditioning in Estrogen-free Medium, and**
373 **Plating Cells for Experimentation**

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

376 To start the tissue culture maintenance and estrogen-free conditioning, split the two T150 culture flasks
377 into four T150 flasks. Two of these flasks will be used for continuing tissue culture and will use the

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- 378 RPMI media mentioned above. The other two flasks will be cultured in estrogen-free DMEM for
379 experimental use. Extra care must be taken to avoid contaminating the estrogen-free cells with RPMI.
- 380 1. Remove both T150 flasks from the incubator.
 - 381 2. Aspirate the medium and rinse the cells with 5 mL 1X PBS.
 - 382 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask to coat
383 all cells with the trypsin.
 - 384 4. Incubate cells in the incubator (see conditions in **Section 9.0**) for 5 to 10 min.
 - 385 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.
 - 386 6. Confirm cell detachment by examination under an inverted microscope. If cells have not
387 detached, return the flask to the incubator for an additional 2 minutes, then hit the flask
388 again.
 - 389 7. After cells have detached, add 5 mL 1X PBS to the first T150 flask and transfer the
390 suspended cells to the second T150 flask.
 - 391 8. Transfer the contents of both flasks to a 50 mL conical tube. Repeat step 7 with an
392 additional 5 mL 1X PBS and transfer to the 50 mL conical tube.
 - 393 9. Immediately add 20 mL estrogen-free DMEM to the 50 mL conical tube to inhibit further
394 cellular digestion by residual trypsin.
 - 395 10. Centrifuge at 1000 x g for eight minutes. If a pellet of cells has not formed, centrifuge for
396 an additional 5 minutes.
 - 397 11. Aspirate media from pellet and re-suspend it in 4 mL estrogen-free DMEM, drawing the
398 pellet repeatedly through a 1 mL serological pipette to break up clumps of cells.

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

401 9.2.1 Ongoing Tissue Culture Maintenance

- 402 1. Add 20 mL RPMI to two T150 flasks.
- 403 2. Add 220 μ L G418 to the RPMI in the T150 flasks
- 404 3. Add 1 mL of cell suspension from **Section 9.2 step 11** to each flask.
- 405 4. Place T150 flasks in tissue culture incubator (see conditions in **Section 9.0**) and grow to
406 80% to 90% confluence (approximately 48 to 72 hrs).
- 407 5. Tissue culture medium may need to be changed 24 hours after addition of G418 to
408 remove cells that have died because they do not express reporter plasmid.
- 409 6. G418 does not need to be added to the flasks a second time.
- 410 7. Repeat **Section 9.2 steps 1-11** for ongoing tissue culture maintenance.

411 9.2.2 Conditioning in Estrogen-free Medium

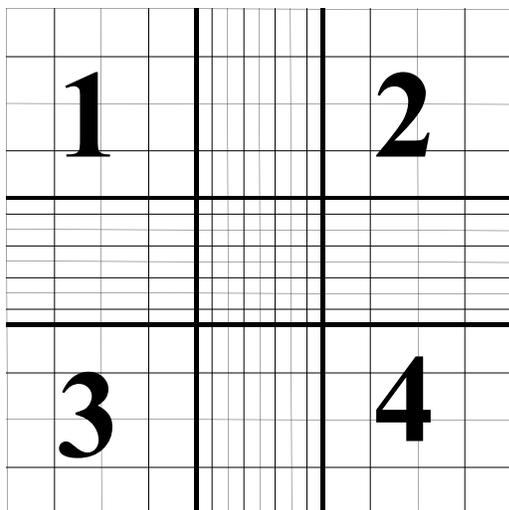
- 412 1. Add 20 mL estrogen-free DMEM to two T150 flasks.
- 413 2. Add 150 μ L G418 to the estrogen-free DMEM in the T150 flasks.

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- 414 3. Add 1 mL of cell suspension from **Section 9.2 step 11** to each flask.
- 415 4. Tissue culture medium may need to be changed 24 hours after addition of G418 to
- 416 remove cells that have died because they do not express reporter plasmid.
- 417 5. G418 does not need to be added to the flasks a second time.
- 418 6. Place the T150 flasks in the incubator (see conditions in **Section 9.0**) and grow to 80% to
- 419 90% confluence (approximately 48 to 72 hrs).
- 420 9.2.3 Plating Cells Grown in Estrogen-free DMEM for Experimentation
- 421 1. Remove the T150 flasks that have been conditioned in estrogen-free DMEM for 48 to 72
- 422 hours from the incubator.
- 423 2. Aspirate the medium, then rinse the cells with 5 mL 1X PBS.
- 424 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask to coat
- 425 all cells with the trypsin.
- 426 4. Place the flasks in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
- 427 5. Detach cells by hitting the side of the flask sharply against the palm or the heel of the
- 428 hand.
- 429 6. Confirm cell detachment by examination under an inverted microscope. If cells have not
- 430 detached, return the flask to the incubator for 2 additional minutes, then hit the flask
- 431 again.
- 432 7. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells from the
- 433 T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the flask, then
- 434 transfer to the 50 mL conical tube.
- 435 8. Immediately add 20 mL estrogen-free DMEM to each conical tube to inhibit further
- 436 cellular digestion by residual trypsin.
- 437 9. Centrifuge at 1000 x g for eight minutes. If a pellet of cells has not formed, centrifuge for
- 438 an additional 5 minutes.
- 439 10. Aspirate off the media from the pellet and re-suspend it in 20 mL DMEM, drawing the
- 440 pellet repeatedly through a 25 mL serological pipette to break up any clumps of cells.
- 441 11. Pipette 15 μ L of the cell suspension into the “v” shaped slot on the hemocytometer.
- 442 Ensure that the solution covers the entire surface area of the hemocytometer grid, and
- 443 allow cells to settle before counting.
- 444 12. Using 100x magnification, view the counting grid.
- 445 13. The counting grid on the hemocytometer consists of nine sections, four of which are
- 446 counted (upper left, upper right, lower left, and lower right, see **Figure 9-1**). Each section
- 447 counted consists of four by four grids. Starting at the top left and moving clockwise,
- 448 count all cells in each of the four by four grids. Some cells will be touching the outside
- 449 borders of the square, but only count those that touch the top and right borders of the

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450 square. This value is then used in the calculation below to get to the desired concentration
 451 of 200,000 cells/mL.

Figure 9-1 Hemocytometer Counting Grid.

452

453

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

454

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

455

Starting dilution: 20mL (for T150 flasks)

456

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

459 Example Calculation:

460

- Grids 1, 2, 3, and 4 are counted and provide the following data:

461

- 50, 51, 49, and 50: average number of cells per grid is equal to 50.

462

Cells/mL = 50 cells per grid $\div 10^{-4}$ mL volume of grid = 50×10^4 cells/mL (or 500,000 cells/mL)

463

Total # of Cells Harvested = 500,000 cells/mL $\times 20$ mL

464

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

465

Formula: (Concentration_{Final} \times Volume_{Final} = Concentration_{Initial} \times Volume_{Initial})

466

Concentration_{Final} = 200,000 cells/mL

467

Concentration_{Initial} = 500,000 cells/mL

468

Volume_{Initial} = 20 mL

469

Volume_{Final} – to be solved for.

470

Therefore: 200,000 cells/mL \times Volume_{Final} = 500,000 cells/mL $\times 20$ mL

471

Solving for Volume_{Final} we find = 50 mL

472

Therefore, add 30 mL of estrogen-free DMEM to the cell suspension for a total volume of 50 mL, which

473

will yield the desired concentration of 200,000 cells/mL for plating.

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- 474 14. This dilution scheme will give a concentration of 200,000 cells/mL. 200 mL of this cell
475 suspension is used for each well of a 96-well plate (i.e., 40,000 cells per well).
- 476 15. Remove a 96-well plate from its sterile packaging. Use a repeater pipetter to pipette 200
477 μ L of cell suspension into each well to be used for the testing of coded substances,
478 reference standard and controls (**note**: add 200 μ L of estrogen-free DMEM only to any
479 wells not being used for testing).
- 480 16. Incubate plate(s) in an incubator (see conditions in **Section 9.0**) for a minimum of 24
481 hours, but no longer than 48 hours before dosing.

482 Two T150 flasks containing cells at 80% to 90% confluence will typically yield sufficient cells to fill four
483 96-well plates (not including the perimeter wells).

484 **10.0 PREPARATION OF TEST SUBSTANCES**

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

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

492 **10.1 Determination of Test Substance Solubility**

- 493 1. Prepare a 200 mg/mL solution of the test substance in 100% DMSO in a 4 mL conical
494 tube.
- 495 2. Vortex to mix.
- 496 3. If the test substance does not dissolve at 200 mg/mL, prepare a 20 mg/mL solution and
497 vortex as above.
- 498 4. If the test substance does not dissolve at 20 mg/mL solution, prepare a 2 mg/mL solution
499 in a 4 mL conical tube and vortex as above.
- 500 5. If the test substance does not dissolve at 2 mg/mL, prepare a 0.2 mg/mL solution in a 4
501 mL conical tube and vortex as above.
- 502 6. Continue testing, using 1/10 less substance in each subsequent attempt until test
503 substance is solubilized in DMSO.

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

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

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508 **11.0 PREPARATION OF REFERENCE STANDARD, CONTROL AND TEST**
 509 **SUBSTANCE STOCK SOLUTIONS FOR RANGE FINDER AND COMPREHENSIVE**
 510 **TESTING**

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

513 **11.1 Preparation of Ral/E2 Stock Solutions**

514 E2 and raloxifene stocks are prepared separately and then combined into Ral/E2 stocks, which are then
 515 used to prepare dosing solutions in **Section 12**.

516 11.1.1 E2 Stock Solution

517 The final concentration of the E2 stock solution is 5.0×10^{-3} µg/mL. Prepare the E2 stock as shown in
 518 **Table 11-1**.

519 **Table 11-1 Preparation of E2 Stock Solution**

Step #	Action	DMSO	E2 Concentration
1	Make a 10 mg/mL stock solution in 100% DMSO in a 4mL vial.	-	10 mg/mL
2	Transfer 10 µL E2 solution from Step #1 to a new 4 mL vial.	Add 990 µL of 100% DMSO. Vortex to mix.	100 µg/mL
3	Transfer 10 µL E2 solution from Step #2 to a new 4mL vial.	Add 990 µL of 100% DMSO. Vortex to mix.	1 µg/mL
4	Transfer 100 µL E2 solution from Step #3 to a new glass container large enough to hold 15 mL.	Add 9.90 mL of 100% DMSO. Vortex to mix.	1.0×10^{-2} µg/mL
5	Transfer 5 mL E2 solution from Step #4 to a new glass container large enough to hold 15 mL	Add 5 mL of 100% DMSO. Vortex to mix.	5.0×10^{-3} µg/mL

520 11.1.2 Raloxifene Stock Solution

521 Prepare a 2.5 µg/mL raloxifene working stock solution as shown in **Table 11-2**.

522 **Table 11-2 Preparation of Raloxifene Stock Solution**

Step #	Action	DMSO	Raloxifene Concentration
1	Make a 10 mg/mL solution of raloxifene in a 4 mL glass vial.	-	1.0×10^4 µg/mL
2	Transfer 10 µL raloxifene solution from Step #1 to a new 4 mL vial.	Add 990 µL of 100% DMSO. Vortex to mix.	100 µg/mL
3	Transfer 150 µL raloxifene solution from Step #2 to a new 4 mL vial.	Add 2.850 mL of 100% DMSO. Vortex to mix.	5 µg/mL
4	Transfer 1.5 mL raloxifene solution from Step #3 to a new 13 mm test tube.	Add 1.5 mL of 100% DMSO. Vortex to mix.	2.5 µg/mL

523 **11.2 Ral/E2 Range Finder Testing Stock**

524 11.2.1 Raloxifene Dilutions

525 Number three 4 mL vials with the numbers 1 to 3 and use the raloxifene solution prepared in **Section**
 526 **11.1.2** to make raloxifene dilutions as shown **Table 11-3**.

527

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527 **Table 11-3 Preparation of Raloxifene Dilutions for Range Finder Testing**

Step #	Action	DMSO	Raloxifene Concentration
1	Transfer 250 µL of the 2.5 µg/mL raloxifene working stock solution to a 4 mL tube	Add 750 µL of 100% DMSO and vortex	6.25×10^{-1} µg/mL
2	Transfer 500 µL of the 6.25×10^{-1} µg/mL raloxifene solution to a 4 mL tube	Add 500 µL of 100% DMSO and vortex	3.13×10^{-1} µg/mL
3	Transfer 250 µL of the 3.13×10^{-1} µg/mL raloxifene solution to a 4 mL tube	Add 750 µL of 100% DMSO and vortex	7.81×10^{-2} µg/mL
4	Transfer 125 µL of the 7.81×10^{-2} µg/mL raloxifene solution to a 4 mL tube	Add 375 µL of 100% DMSO and vortex	1.95×10^{-2} µg/mL

528 11.2.2 Preparation of Ral/E2 Range Finder Working Stocks:

529 Label three 4 mL conical tubes with numbers 1 through 3 and add 500 µL of the 5×10^{-3} mg/mL E2
 530 solution prepared in **Section 11.1.1** to each tube. Add 500 µL of the 3.13×10^{-1} , 7.81×10^{-2} , and $1.95 \times$
 531 10^{-2} µg/mL raloxifene solutions prepared in **Section 11.2.1** to tubes 1, 2, and 3 respectively. Vortex each
 532 tube to mix. The final concentrations for raloxifene and E2 are listed in **Table 11-4**.

533 **Table 11-4 Concentrations of Raloxifene and E2 in the Ral/E2 Range Finder Working Stocks**

Tube #	Raloxifene (mg/ml)	E2 (mg/ml)
1	1.56×10^{-1}	2.5×10^{-3}
2	3.91×10^{-2}	2.5×10^{-3}
3	9.77×10^{-3}	2.5×10^{-3}

534 **11.3 Ral/E2 Comprehensive Testing Stock**535 11.3.1 Raloxifene Dilutions

536 Use the raloxifene solution prepared in **Section 11.1.2** to make a nine-point serial dilution of raloxifene as
 537 shown **Table 11-5**.

538 **Table 11-5 Preparation of Raloxifene Dilutions for Comprehensive Testing**

Step #	Action	DMSO	Discard	Raloxifene Concentration
1	Transfer 500 µL of the raloxifene working stock solution to a new 4 mL vial.	-	-	2.5 µg/mL
2	Transfer 500 µL of the raloxifene working stock solution to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	1.25 µg/mL
3	Transfer 500 µL raloxifene solution from Step #2 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	6.25×10^{-1} µg/mL
4	Transfer 500 µL raloxifene solution from Step #3 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	3.13×10^{-1} µg/mL
5	Transfer 500 µL raloxifene solution from Step #4 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	1.56×10^{-1} µg/mL
6	Transfer 500 µL raloxifene solution from Step #5 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	7.81×10^{-2} µg/mL
7	Transfer 500 µL raloxifene solution from Step #6 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	3.91×10^{-2} µg/mL
8	Transfer 500 µL raloxifene solution from Step #7 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	-	1.95×10^{-2} µg/mL
9	Transfer 500 µL raloxifene solution from Step #8 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.	Discard 500 µL from Tube #9	9.77×10^{-3} µg/mL

539 11.3.2 Preparation of Ral/E2 Comprehensive Testing Working Stocks:

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540 Add 500 μL of the 5×10^{-3} mg/mL E2 solution prepared in **Section 11.1.1** to each of the 9 raloxifene
 541 dilution vials (including the working stock solution in Tube #1). Vortex each tube to mix. The final
 542 concentrations for raloxifene and E2 are listed in **Table 11-6**.

543 **Table 11-6 Concentrations of Raloxifene and E2 in the Ral/E2 Working Stocks**

Tube #	Raloxifene (mg/mL)	E2 (mg/mL)
1	1.25	2.5×10^{-3}
2	6.25×10^{-1}	2.5×10^{-3}
3	3.13×10^1	2.5×10^{-3}
4	1.56×10^{-1}	2.5×10^{-3}
5	7.81×10^2	2.5×10^{-3}
6	3.91×10^{-2}	2.5×10^{-3}
7	1.95×10^{-2}	2.5×10^{-3}
8	9.77×10^{-3}	2.5×10^{-3}
9	4.88×10^{-3}	2.5×10^{-3}

544 **11.4 Flavone/E2 Stock Solution**

545 To prepare the flavone/E2 stock solution, proceed as follows:

- 546 1. Prepare 1 mL of 5 mg/mL flavone
- 547 2. Add 1 mL of 5×10^{-3} mg/mL E2 (prepared as in **Section 11.1.1**) to the 10 mg/mL flavone.
- 548 This will make a working solution of 2.5 mg/mL flavone with 2.5×10^{-3} mg/mL E2.

549 **12.0 PREPARATION OF REFERENCE STANDARD, CONTROL AND TEST**
 550 **SUBSTANCE DOSING SOLUTIONS FOR RANGE FINDER AND**
 551 **COMPREHENSIVE TESTING**

552 **12.1 Preparation of Reference Standard and Control Dosing Solutions - Range Finder Testing**

553 Range finder testing is conducted on 96-well plates using three concentrations of Ral/E2 in duplicate as
 554 the reference standard. Three replicate wells for the DMSO, and E2 controls are included on each plate.

555 All “dosing solutions” of test substance concentrations are to be expressed as $\mu\text{g/mL}$ in the study
 556 notebook and in all laboratory reports. Dosing solutions are to be used within 24 hours of preparation.

557 12.1.1 Preparation of Ral/E2 Reference Standard Range Finder Dosing Solutions

- 558 1. Label three 13 mm glass tubes with the numbers 1 to 3.
- 559 2. Add 6 μL Ral/E2 stock from tube #1 (**Section 11.2.2**) to 13 mm glass test tube #1.
- 560 3. Add 6 μL of Ral/E2 stock from tube #2 from **Section 11.2.2** to the 13 mm glass test tube
 561 labeled #2. Repeat for tube #3.
- 562 4. Add 600 μL of estrogen-free DMEM to each tube and vortex.

563 12.1.2 Preparation of DMSO Control Range Finder Dosing Solution

- 564 1. Add 8 μL of 100% DMSO to a 13 mm glass test tube.
- 565 2. Add 800 μL of estrogen-free DMEM to each tube and vortex.

566 12.1.3 Preparation of E2 Control Range Finder Dosing Solution

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- 567 1. Add 4 μL of the E2 stock from **Section 11.1.1** to a 13 mm glass test tube.
- 568 2. Add 4 μL of 100% DMSO to the tube.
- 569 3. Add 800 μL of estrogen-free DMEM to the tube and vortex to mix.

570 12.2 Preparation of Test Substance Dosing Solutions for Range Finder Testing

571 Range finder experiments are used to determine the concentrations of test substance to be used during
 572 comprehensive testing. Antagonist range finding for coded substances consists of seven-point 1:10 serial
 573 dilutions in duplicate.

574 To prepare test substance dosing solutions:

- 575 1. Label two sets of seven glass 13 mm test tubes with the numbers 1 through 7 and place
 576 them in a test tube rack. Perform a serial dilution of test substance as shown in **Table 12-**
 577 **1** using one set of tubes.

578 **Table 12-1 Preparation of Test Substance Serial Dilution for Range Finder Testing**

Tube #	100% DMSO	Test Substance ¹	Final Volume
1	-	100 μL of test substance solution from Section 10.1	100 μL
2	90 μL	10 μL of test substance solution from Section 10.1	100 μL
3	90 μL	10 μL from Tube #2	100 μL
4	90 μL	10 μL from Tube #3	100 μL
5	90 μL	10 μL from Tube #4	100 μL
6	90 μL	10 μL from Tube #5	100 μL
7	90 μL	10 μL from Tube #6	100 μL

579 ¹Vortex tubes #2 through 6 before removing test substance/DMSO solution to place in the next tube in the series.

- 580 2. Transfer test substance/DMSO solutions to the second set of labeled tubes and add E2 as
 581 shown in **Table 12-2**.

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

Tube Number	Test Substance	E2	Estrogen-free DMEM ³	Final Volume
1	Transfer 4 μL of test substance from Tube #1 in Section 12.2 step 1 to a new tube	Add 4 μL of the 5×10^{-3} $\mu\text{g}/\text{mL}$ E2 solution prepared in Section 11.1.1 . Vortex to mix.	800 μL	808 μL
2	Transfer 4 μL of test substance from Tube #2 to a new tube	Add 4 μL of the 5×10^{-3} $\mu\text{g}/\text{mL}$ E2 solution prepared in Section 11.1.1 . Vortex to mix.	800 μL	808 μL
3	Transfer 4 μL of test substance from Tube #3 to a new tube	Add 4 μL of the 5×10^{-3} $\mu\text{g}/\text{mL}$ E2 solution prepared in Section 11.1.1 . Vortex to mix.	800 μL	808 μL
4	Transfer 4 μL of test substance from Tube #4 to a new tube	Add 4 μL of the 5×10^{-3} $\mu\text{g}/\text{mL}$ E2 solution prepared in Section 11.1.1 . Vortex to mix.	800 μL	808 μL
5	Transfer 4 μL of test substance from Tube #5 to a new tube	Add 4 μL of the 5×10^{-3} $\mu\text{g}/\text{mL}$ E2 solution prepared in Section 11.1.1 . Vortex to mix.	800 μL	808 μL
6	Transfer 4 μL of test substance from Tube #6 to a new tube	Add 4 μL of the 5×10^{-3} $\mu\text{g}/\text{mL}$ E2 solution prepared in Section 11.1.1 . Vortex to mix.	800 μL	808 μL
7	Transfer 4 μL of test substance from Tube #7 to a new tube	Add 4 μL of the 5×10^{-3} $\mu\text{g}/\text{mL}$ E2 solution prepared in Section 11.1.1 . Vortex to mix.	800 μL	808 μL

583

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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 14.0**.

586 **12.3 Preparation of Reference Standard and Control Dosing Solutions for Comprehensive**
587 **Testing**

588 Comprehensive testing is conducted on 96-well plates using nine concentrations of Ral/E2 in duplicate as
589 the reference standard. Four replicate wells for the DMSO, E2 and flavone/E2 controls are included on
590 each plate.

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

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

594 12.3.1 Preparation of Ral/E2 Reference Standard Dosing Solutions for Comprehensive Testing

595 In preparation for making Ral/E2 1:2 serial dilutions, label two sets of nine glass 13 mm test tubes with
596 the numbers 1 through 9 and place them in a test tube rack. Tube number 1 will contain the highest
597 concentration of raloxifene (**Table 12-3**).

598 **Table 12-3 Preparation of Ral/E2 Reference Standard Dosing Solution**
599 **for Comprehensive Testing**

Tube Number	Ral/E2 Stock	Estrogen-free DMEM	Final Volume
1	6 µL of Tube #1 from Section 11.3.2	600 µL	606 µL
2	6 µL of Tube #2 from Section 11.3.2	600 µL	606 µL
3	6 µL of Tube #3 from Section 11.3.2	600 µL	606 µL
4	6 µL of Tube #4 from Section 11.3.2	600 µL	606 µL
5	6 µL of Tube #5 from Section 11.3.2	600 µL	606 µL
6	6 µL of Tube #6 from Section 11.3.2	600 µL	606 µL
7	6 µL of Tube #7 from Section 11.3.2	600 µL	606 µL
8	6 µL of Tube #8 from Section 11.3.2	600 µL	606 µL
9	6 µL of Tube #9 from Section 11.3.2	600 µL	606 µL

600 12.3.2 Preparation of DMSO Control Comprehensive Testing Dosing Solution

- 601 1. Add 10 µL of 100% DMSO to a 13 mm glass test tube.
602 2. Add 1000 µL of estrogen-free DMEM to the tube and vortex to mix.

603 12.3.3 Preparation of E2 Control Comprehensive Testing Dosing Solution

- 604 1. Add 5 µL of the E2 stock from **Section 11.1.1** to a 13 mm glass test tube.
605 2. Add 5 µL of 100% DMSO to the tube.
606 3. Add 1000 µL of estrogen-free DMEM to the tube and vortex to mix.

607 12.3.4 Preparation of Flavone/E2 Control Comprehensive Dosing Solution

- 608 1. Add 10 µL of flavone/E2 from **Section 11.4** to a 13 mm glass test tube.
609 2. Add 1000 µL of estrogen-free DMEM to the tube and vortex to mix.

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610 12.4 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

611 Comprehensive testing experiments are used to determine whether a substance possesses ER antagonist
 612 activity in the BG1Luc ER TA test method. Antagonist comprehensive testing for coded substances
 613 consists of either an 11-point 1:2 serial dilution, or an 11-point 1:5 serial dilution with each concentration
 614 tested in triplicate wells of the 96-well plate.

615 12.4.1 *Preparation of Test Substance 1:2 Serial Dilutions for*
 616 *Comprehensive Testing*

617 Start the 11-point serial dilution according to criteria in **Section 14.0**.

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

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

622 Prepare dilution of test substance as shown in **Table 12-4**.

623 **Table 12-4 Preparation of Test Substance 1:2 Serial Dilutions for Comprehensive Testing**

Tube Number	100% DMSO	Test Substance ¹	Discard	E2 Testing Stock	Estrogen-free DMEM ²	Final Volume
1	-	4 μ L of test substance solution from Section 10.2.4 step 1	-	4 μ L	800 μ L	808 μ L
2	4 μ L	4 μ L of test substance solution from Section 10.2.4 step 1	-	4 μ L	800 μ L	808 μ L
3	4 μ L	4 μ L from Tube #2	-	4 μ L	800 μ L	808 μ L
4	4 μ L	4 μ L from Tube #3	-	4 μ L	800 μ L	808 μ L
5	4 μ L	4 μ L from Tube #4	-	4 μ L	800 μ L	808 μ L
6	4 μ L	4 μ L from Tube #5	-	4 μ L	800 μ L	808 μ L
7	4 μ L	4 μ L from Tube #6	-	4 μ L	800 μ L	808 μ L
8	4 μ L	4 μ L from Tube #7	-	4 μ L	800 μ L	808 μ L
9	4 μ L	4 μ L from Tube #8	-	4 μ L	800 μ L	808 μ L
10	4 μ L	4 μ L from Tube #9	-	4 μ L	800 μ L	808 μ L
11	4 μ L	4 μ L from Tube #10	4 μ L	4 μ L	800 μ L	808 μ L

624 ¹Vortex tubes #2 through 10 before removing test substance/DMSO solution to place in the next tube in the series.

625 ²Vortex all tubes to mix media, test substance, and E2.

626 12.4.2 *Preparation of Test Substance 1:5 Serial Dilutions for*
 627 *Comprehensive Testing*

628 Start the 11-point serial dilution according to criteria in **Section 14.0**.

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

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

633 Prepare dilution of test substance as shown in **Table 12-5**.

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634 **Table 12-5 Preparation of Test Substance 1:5 Dilutions for Comprehensive Testing**

Tube Number	100% DMSO	Test Substance ¹	Discard	E2 Testing Stock	Estrogen-free DMEM ²	Final Volume
1	-	4 µL of test substance solution from Section 10.2.4 step 1	-	4 µL	800 µL	808 µL
2	16 µL	4 µL of test substance solution from Section 10.2.4 step 1	-	4 µL	800 µL	808 µL
3	16 µL	4 µL from Tube #2	-	4 µL	800 µL	808 µL
4	16 µL	4 µL from Tube #3	-	4 µL	800 µL	808 µL
5	16 µL	4 µL from Tube #4	-	4 µL	800 µL	808 µL
6	16 µL	4 µL from Tube #5	-	4 µL	800 µL	808 µL
7	16 µL	4 µL from Tube #6	-	4 µL	800 µL	808 µL
8	16 µL	4 µL from Tube #7	-	4 µL	800 µL	808 µL
9	16 µL	4 µL from Tube #8	-	4 µL	800 µL	808 µL
10	16 µL	4 µL from Tube #9	-	4 µL	800 µL	808 µL
11	16 µL	4 µL from Tube #10	20 µL	4 µL	800 µL	808 µL

635 ¹Vortex tubes #2 through 10 before removing test substance/DMSO solution to place in the next tube in the series.636 ²Vortex all tubes to mix media, test substance, and E2.637 **13.0 GENERAL PROCEDURES FOR THE TESTING OF CODED SUBSTANCES**

638 Range finder experiments are used to determine the concentrations of test substance to be used during
 639 comprehensive testing. Comprehensive testing experiments are used to determine whether a substance
 640 possesses ER antagonist activity in the BG1Luc ER TA test method.

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

644 **13.1 Application of Reference Standard, Control and Test Substances**

- 645 1. Remove the 96-well plates (from **Section 9.2.3 step 18**) from the incubator; inspect them
 646 using an inverted microscope. Only use plates in which the cells in all wells receive a
 647 score of 1 according to **Table 11-1**.
- 648 2. Remove medium by inverting the plate onto blotter paper. Gently tap plate against the
 649 bench surface to remove residual liquid trapped in the wells.
- 650 3. Add 200 µL of medium, reference standard, control or test substance to each well (see
 651 **Sections 14.0** and **15.0** for specific plate layouts).
- 652 4. Return plates to incubator (see **Section 9.0** for details) for 19 to 24 hours to allow
 653 maximal induction of luciferase activity in the cells.

654 **13.1.1 Preparation of Excel[®] Data Analysis Template For Range Finder Testing**

- 655 1. In Excel[®], open a new “AntRFTemplate” and save it with the appropriate project name as
 656 indicated in the NICEATM Style Guide.
- 657 2. Fill out the table at the top of the “Raw Data” worksheet with information regarding the
 658 Microplate reader used, Reading Direction, No. of Intervals, Tot. Meas. Time/Well (s),

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- 659 etc. (**note:** this information can be permanently added to the default template
660 “AntRFTemplate” on a laboratory specific basis).
- 661 3. Add the following information regarding the assay to the “Compound Tracking”
662 worksheet.
- 663 ▪ Plate # - Enter the experiment ID or plate number into cell E1
 - 664 ▪ Cell Lot # - Enter the passage or lot number of the cells used for this
665 experiment into cell B5
 - 666 ▪ DMSO and Media Lot #'s – Enter the lot numbers for the DMSO and Media in
667 cells B6 and B7
 - 668 ▪ Test Substance Code – Enter the test substance codes into cells C14 to C19
 - 669 ▪ Name: Enter the experimenter name into cell G6
 - 670 ▪ Date: Enter the experiment date in the format day\month\year into cell G10
 - 671 ▪ Comments: - Enter any comments about the experiment in this box (e.g., plate
672 contaminated)
- 673 4. Enter the following substance testing information to the “List” worksheet:
- 674 ▪ Concentration – Type in the test substance concentration in $\mu\text{g/ml}$ in
675 descending order.
 - 676 ▪ Any specific comments about the test substance or condition of the wells
677 should be entered into this sheet, in the comments section
 - 678 ▪ All of the remaining cells on the “List” worksheet should populate
679 automatically.
 - 680 ▪ The “Template”, “Compound Mixing” and “Visual Inspection” worksheet
681 should automatically populate with the information entered into the
682 “Compound Tracking” and “List” worksheet.
- 683 5. Save the newly named project file.
- 684 6. Print out either the “List” or “Template” worksheet for help with dosing the 96-well
685 plate. Sign and date the print out and store in study notebook.
- 686 13.1.2 Preparation of Excel® Data Analysis Template for Comprehensive Testing
- 687 1. In Excel®, open a new “AntCTTemplate” and save it with the appropriate project name as
688 indicated in the NICEATM Style Guide.
 - 689 2. Fill out the table at the top of the “Raw Data” worksheet with information regarding the
690 Microplate reader used, Reading Direction, No. of Intervals, Tot. Meas. Time/Well (s),
691 etc. (**note:** this information can be permanently added to the default template
692 “AntCTTemplate” on a laboratory specific basis).
 - 693 3. On the “Compound Tracking” worksheet, enter the following information:
694
 - Plate # - Enter the experiment ID or plate number into cell E1

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- 695 ▪ Cell Lot # - Enter the passage or lot number of the cells used for this
696 experiment into cell C5
- 697 ▪ DMSO and Media Lot #'s – Enter the lot numbers for the DMSO and Media in
698 cells C6 and C7
- 699 ▪ Test Substance Code – Enter the test substance codes into cells C15 and C16.
700 Enter the test substance dilution into cells D15 and D16.
- 701 ▪ Name: Enter the experimenter name into cell F6
- 702 ▪ Date: Enter the experiment date in the format day\month\year into cell G10
- 703 ▪ Comments: - Enter any comments about the experiment in this box (e.g., plate
704 contaminated)
- 705 4. Enter the following substance testing information to the “List” worksheet:
- 706 ▪ Concentration – Type in the test substance concentration in µg/ml in
707 descending order.
- 708 ▪ Any specific comments about the test substance or condition of the wells
709 should be entered into this sheet, in the comments section
- 710 ▪ All of the remaining cells on the “List” worksheet should populate
711 automatically.
- 712 ▪ The “Template”, “Compound Mixing” and “Visual Inspection” worksheet
713 should automatically populate with the information entered into the
714 “Compound Tracking” and “List” worksheet.
- 715 5. Save the newly named project file.
- 716 6. Print out either the “List” or “Template” worksheet for help with dosing the 96-well
717 plate. Sign and date the print out and store in study notebook.

718 13.2 Visual Evaluation of Cell Viability

- 719 1. 19 to 24 hours after dosing the plate, remove the plate from the incubator and remove the
720 media from the wells by inverting the plate onto blotter paper. Gently tap plate against
721 the bench surface to remove residual liquid trapped in the wells.
- 722 2. Use a repeat pipetter to add 50 µL 1X PBS to all wells. Immediately remove PBS by
723 inversion.
- 724 3. Using an inverted microscope, inspect all of the wells used in the 96-well plate and
725 record the visual observations using the scores in **Table 13-1**.
- 726

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726 **Table 13-1 Visual Observation Scoring**

Viability Score	Brief Description ¹
1	Normal Cell Morphology and Cell Density
2	Altered Cell Morphology and/or Small Gaps between Cells
3	Altered Cell Morphology and/or Large Gaps between Cells
4	Few (or no) Visible Cells
P	Wells containing precipitation are to be noted with "P"

727 ¹Reference photomicrographs are provided in the BG1LUC ER TA Validation Study "Visual Observation Cell
728 Viability Manual."

729 **13.3 Lysis of Cells for BG1Luc ER TA**

- 730 1. Apply the reflective white backing tape to the bottom of the 96-well plate (this will
731 increase the effectiveness of the luminometer).
- 732 2. Add 30µL 1X lysis reagent to the assay wells and place the 96-well plate on an orbital
733 shaker for one minute.
- 734 3. Remove plate from shaker and measure luminescence (as described in **Section 13.4**).

735 **13.4 Measurement of Luminescence**

736 Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and with
737 software that controls the injection volume and measurement interval. Light emission from each well is
738 expressed as relative light units (RLU) per well. The luminometer output is saved as raw data in an
739 Excel[®] spread sheet. A hard copy of the luminometer raw data should be signed, dated and stored in the
740 study notebook.

741 **13.5 Data Analysis**

742 BG1Luc ER TA uses an Excel[®] spreadsheet to collect and adjust the RLU values obtained from the
743 luminometer and a GraphPad Prism[®] template to analyze and graph data. Plate reduction is calculated
744 using unadjusted RLU values.

745 The Excel[®] spreadsheet subtracts background luminescence (average DMSO solvent control RLU value)
746 from test substance, reference standard and control RLU values. Test substance, reference standard, and
747 control RLU values are then adjusted relative to the highest Ral/E2 reference standard RLU value, which
748 is set to 10,000. After adjustment, values are transferred to GraphPad Prism[®] for data analysis and
749 graphing.

750 **13.5.1 Collection and Adjustment of Luminometer Data for Range Finder Testing**

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

- 753 1. Open the raw data file and the corresponding experimental Excel[®] spreadsheet from
754 **Section 13.1.1**.

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- 755 2. Copy the raw data using the Excel[®] copy function, then paste the copied data into cell
756 B19 of the “RAW DATA” tab in the experimental Excel[®] spreadsheet using the **Paste**
757 **Special – Values** command. This position corresponds to position A1 in the table labeled
758 Table 1 in this tab.
- 759 3. Examine the DMSO data in Table 1 of the Excel[®] spreadsheet to determine whether there
760 are any potential outliers. See **Section 13.5.3** for further explanation of outlier
761 determinations.
- 762 4. If an outlier is identified, perform the following steps to remove the outlier from
763 calculations:
- 764 ▪ correct the equation used to calculate DMSO background in Table 1 [e.g., if
765 outlier is located in cell F26, adjust the calculation in cell H40 to read
766 =AVERAGE(E26,G26)]
 - 767 ▪ then correct the equation used to calculate the average DMSO value in Table 2
768 [e.g., following the above example, adjust cell M42 to read
769 =AVERAGE(E38,G38)]
 - 770 ▪ then correct the equation used to calculate the standard deviation of the DMSO
771 value in Table 2 [e.g., following the above example, adjust cell M43 to read
772 =STDEV(E38,G38)]
- 773 5. Excel[®] will automatically subtract the background (the average DMSO control value)
774 from all of the RLU values in Table 1 and populate Table 2 with these adjusted values.
- 775 6. To calculate plate reduction, identify the cell containing the Ral/E2a replicate in Table 1,
776 plate row H that has the lowest RLU value (i.e., cell B26, C26, or D26).
- 777 7. Identify the cell containing the Ral/E2a replicate in Table 1, plate row H that has the
778 highest RLU value (i.e., cell B26, C26, or D26).
- 779 8. Click into cell D14 and enter the cell number from **Section 13.5.1 step 7** into the
780 numerator and the cell number from **step 6** into the denominator.
- 781 9. Identify the cell containing the Ral/E2b replicate in Table 1, plate row H that has the
782 lowest RLU value (i.e., cell K26, L26, or M26).
- 783 10. Identify the cell containing the Ral/E2b replicate in Table 1, plate row H that has the
784 highest RLU value (i.e., cell K26, L26, or M26).
- 785 11. Click into cell E14 and enter the cell number from **Section 13.5.1 step 10** into the
786 numerator and the cell number from **step 9** into the denominator.
- 787 12. Click on the “ER Antagonist Report” worksheet.
- 788 13. The data for the Ral/E2 reference standard, DMSO, and E2, replicates populate the left
789 portion (columns A-F) of the spreadsheet. The data is automatically placed into an Excel[®]
790 graph.
- 791 14. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell C2 of
792 “ER Antagonist Report” worksheet and check the formula contained within that cell. The

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

807 13.5.2 Collection and Adjustment of Luminometer Data for Comprehensive Testing

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

- 810 1. Open the raw data file and the corresponding experimental Excel[®] spreadsheet from
811 **Section 13.1.2**.
- 812 2. Copy the raw data using the Excel[®] copy function, then paste the copied data into cell
813 B14 of the “RAW DATA” tab in the experimental Excel[®] spreadsheet using the **Paste**
814 **Special – Values** command. This position corresponds to position A1 in the table labeled
815 Table 1 in this tab.
- 816 3. Examine the DMSO data in Table 1 of the Excel[®] spreadsheet to determine whether there
817 are any potential outliers. See **Section 13.5.3** for further explanation of outlier
818 determinations.
- 819 4. If an outlier is identified, perform the following steps to remove the outlier from
820 calculations:
 - 821 ▪ correct the equation used to calculate DMSO background in Table 1 [e.g., if
822 outlier is located in cell M14, adjust the calculation in cell H40 to read
823 =AVERAGE(M15:M17)]
 - 824 ▪ then correct the equation used to calculate the average DMSO value in Table 2
825 [e.g., following the above example, adjust cell M35 to read
826 =AVERAGE(M25:M27)]
 - 827 ▪ then correct the equation used to calculate the standard deviation of the DMSO
828 value in Table 2 [e.g., following the above example, adjust cell M36 to read
829 =STDEV(M25:M27)]

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- 830 5. Excel[®] will automatically subtract the background (the average DMSO control value)
831 from all of the RLU values in Table 1 and populate Table 2 with these adjusted values.
- 832 6. To calculate plate reduction, identify the cell containing the Ral/E2 replicate in plate row
833 G that has the lowest RLU value.
- 834 7. Identify the cell containing the Ral/E2 replicate in plate row G that has the highest RLU
835 value.
- 836 8. Click into cell D14 and enter the cell number from **Section 13.5.2 step 7** into the
837 numerator and the cell number from **step 6** into the denominator.
- 838 9. Identify the cell containing the Ral/E2 replicate in plate row H that has the lowest RLU
839 value.
- 840 10. Identify the cell containing the Ral/E2 replicate in plate row H that has the highest RLU
841 value.
- 842 11. Click into cell E14 and enter the cell number from **Section 13.5.2 step 10** into the
843 numerator and the cell number from **step 9** into the denominator.
- 844 12. Click on the “ER Antagonist Report” worksheet.
- 845 13. The data for the Ral/E2 reference standard, DMSO, E2, and Flavone/E2 replicates
846 populate the left portion (columns A-E) of the spreadsheet. The data is automatically
847 placed into an Excel[®] graph.
- 848 14. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell D2 of
849 “ER Antagonist Report” worksheet and check the formula contained within that cell. The
850 divisor should be the cell number of the cell containing the highest averaged Ral/E2 RLU
851 value (column A).
- 852 15. Open the “Visual Observation Scoring” worksheet. Enter the visual observation scores
853 for each well on the 96-well plate. This data will be linked to the “ER Antagonist Report”
854 worksheet.
- 855 16. Copy the data into GraphPad Prism[®] for the calculation of IC₅₀ values and to graph
856 experimental results as indicated in the NICEATM Prism[®] Users Guide.
- 857 17. After the testing results have been evaluated and reviewed for quality control, enter the
858 following information into the Compound Tracking worksheet:
- 859 ▪ Enter pass/fail results for plate reference standard and control parameters into the
860 Plate Pass/Fail Table
 - 861 ▪ Enter information from the testing of coded substances into the Testing Results
862 Table
 - 863 ▪ Reviewer Name – Enter the name of the person who Reviewed\QC’ed the data into
864 cell A34
 - 865 ▪ Date – Enter the date on which the data was reviewed into cell D32

866 13.5.3 Determination of Outliers

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

870 The formula for the Q test is:

871 Outlier – Nearest Neighbor

Range (Highest – Lowest)

872 where the outlier is the value proposed for exclusion, the nearest neighbor is the value closest to the
 873 outlier, and the range is the range of the three values (Q values for samples sizes from 3 to 10 are
 874 provided in Table 13-2). For example, if the value of this ratio is greater than 0.94 (the Q value for the
 875 90% confidence interval for a sample size of three) or 0.76 (the Q value for the 90% confidence interval
 876 for a sample size of four), the outlier may be excluded from data analysis.

877 **Table 13-2 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

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

881 13.5.4 Acceptance Criteria

882 13.5.4.1 *Range Finder Testing*

883 Acceptance or rejection of a range finder test is based on reference standard and solvent control results
 884 from each experiment conducted on a 96-well plate.

- 885 • Reduction: Plate reduction, as measured by dividing the averaged highest Ral/E2
 886 reference standard RLU value by the averaged DMSO control RLU value, must be
 887 greater than three-fold.
- 888 • E2 control results: E2 control RLU values must be within 2.5 times the standard
 889 deviation of the historical E2 control mean RLU value (**See Section 16.1**).
- 890 • DMSO control results: DMSO control RLU values must be within 2.5 times the standard
 891 deviation of the historical solvent control mean RLU value (see **Section 16.2**).

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

893

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894 13.5.4.2 *Comprehensive Testing*

895 Acceptance or rejection of a test is based on evaluation of reference standard and control results from
896 each experiment conducted on a 96-well plate. Results are compared to quality controls (QC) for these
897 parameters derived from the historical database (see **Section 16.5**), which are summarized below.

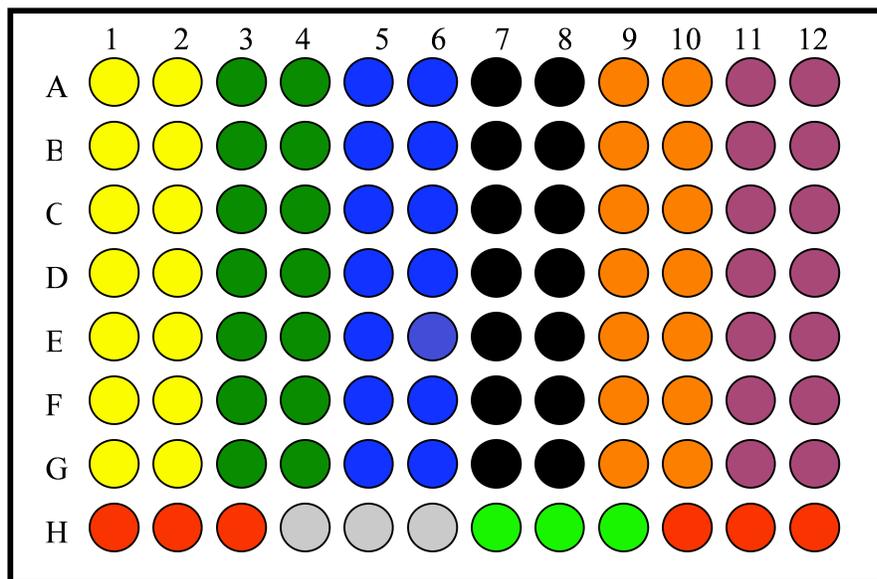
- 898 • Reduction: Plate reduction, as measured by dividing the averaged highest Ral/E2
899 reference standard RLU value by the averaged lowest Ral/E2 control RLU value, must be
900 greater than three-fold.
- 901 • DMSO control results: DMSO control RLU values must be within 2.5 times the standard
902 deviation of the historical solvent control mean RLU value (see **Section 16.5**).
- 903 • Reference standard results: The Ral/E2 reference standard concentration-response curve
904 should be sigmoidal in shape and have at least three values within the linear portion of
905 the concentration-response curve.
- 906 • E2 control results: E2 control RLU values must be within 2.5 times the standard
907 deviation of the historical E2 control mean RLU value.
- 908 • Positive control results: Flavone/E2 control RLU values must be less than the E2 control
909 mean minus three times the standard deviation from the E2 control mean.

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

911 **14.0 RANGE FINDER TESTING**

912 Antagonist range finding for coded substances consists of seven point, 1:10 serial dilutions tested in
913 duplicate wells of the 96-well plate. **Figure 14-1** contains a template for the plate layout used in
914 antagonist range finder testing.

915



-  **Three Point Ral/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**
-  **E2 Control**

915 **Figure 14-1** Antagonist Range Finder Plate Layout

916

917 Evaluate whether range finder experiments have met acceptance criteria (see **Section 13.6.3**).

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

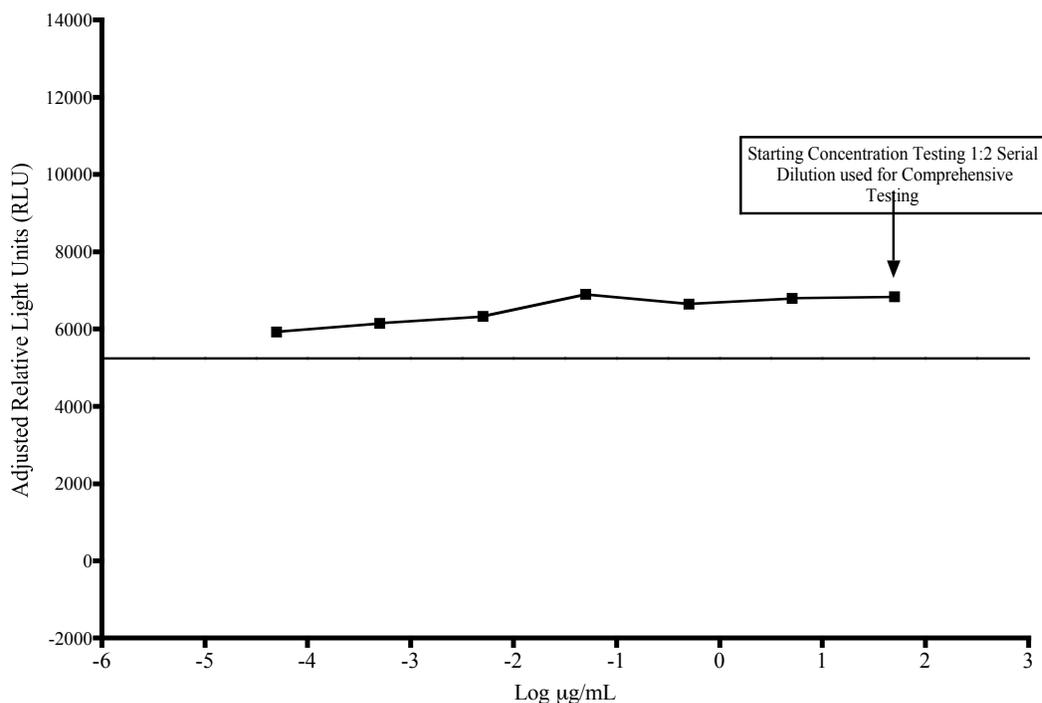
- 919 • If results in the range finder test suggest that the test substance is negative for antagonist
- 920 activity (i.e., if there are no points on the test substance concentration curve that are less
- 921 than the mean minus three times the standard deviation of the E2 control, see **Figure 14-**
- 922 **2**), comprehensive testing will be conducted using an 11-point 1:2 serial dilution using

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- 923 the maximum soluble concentration of test substance as the with the limit dose as the
924 starting concentration.
- 925 • If results in the range finder test suggest that the test substance is negative for agonist
926 activity (i.e., if there are no points on the test substance concentration curve that are
927 greater than the mean plus three times the standard deviation of the DMSO control), and
928 the higher concentrations in the range finder are cytotoxic, comprehensive testing will be
929 conducted using an 11 point 1:2 serial dilution with the lowest cytotoxic concentration as
930 the starting concentration (see **Figure 14-3**).
 - 931 • If results in the range finder test suggest that the test substance is positive for antagonist
932 activity (i.e., if there are points on the test substance concentration curve that are less than
933 the mean minus three times the standard deviation of the E2 control), the top
934 concentration to be used for the 11-point dilution scheme in comprehensive testing
935 should be one of the following:
 - 936 – The concentration giving the lowest adjusted RLU value in the range finder
 - 937 – The maximum soluble concentration (See **Figure 14-2**)
 - 938 – The lowest cytotoxic concentration (See **Figure 14-3** for a related example).
- 939 The 11-point dilution scheme will be based on either a 1:2 or 1:5 serial or dilution
940 according to the following criteria:
- 941 – An 11-point 1:2 serial dilution should be used if the resulting concentration range
942 (note: an 11-point 1:2 serial dilution will cover a range of concentrations over
943 approximately three orders of magnitude [three logs]) will encompass the full
944 range of responses based on the concentration response curve generated in the
945 range finder test (see **Figure 14-4**).
 - 946 – If the concentration range that would be generated with the 1:2 serial dilution
947 will not encompass the full range of responses based on the concentration
948 response curve in the range finder test (see **Figure 14-5**), an 11-point 1:5 serial
949 dilution should be used instead.
 - 950 • If a substance exhibits a biphasic concentration response curve in the range finder test
951 (see **Figure 14-6**), both phases should also be resolved in comprehensive testing. In this
952 case, two peaks could potentially be used to identify the top concentration to be used for
953 the 11-point dilution scheme in comprehensive testing. In order to resolve both curves,
954 the top concentration should be based on the peak associated with the higher
955 concentration and the top dose one log concentration higher than the concentration giving
956 the lowest adjusted RLU value in the range finder. An 11-point 1:5 serial dilution should
957 be used.
- 958

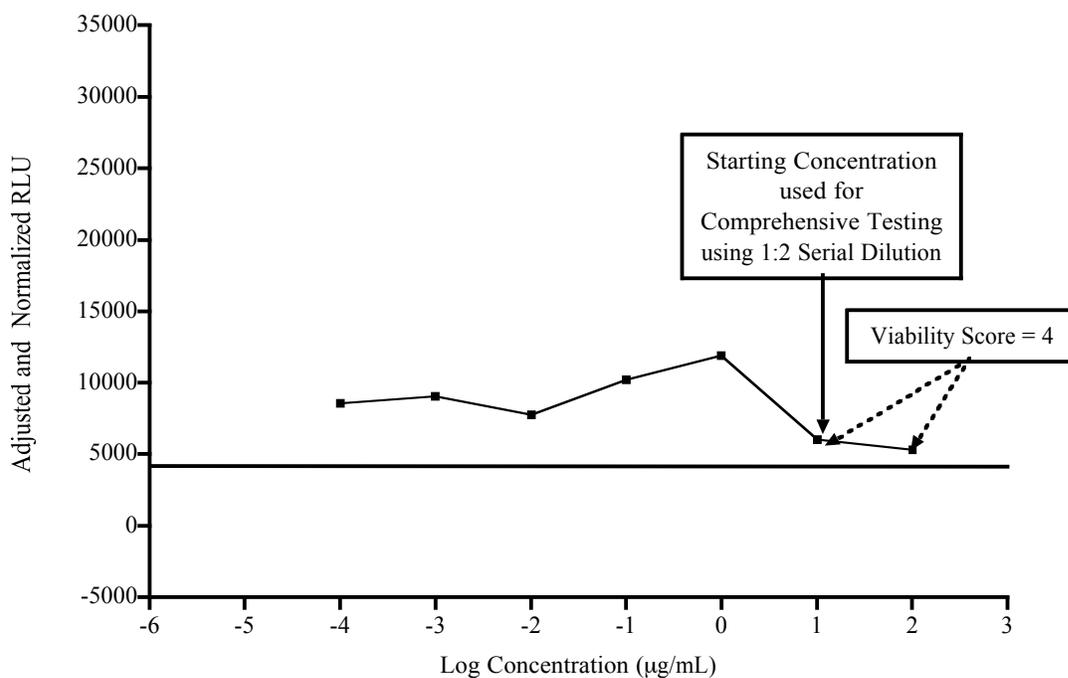
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958 **Figure 14-2 Antagonist Range Finder (example 1)**



959
960 The solid horizontal line represents the mean minus three times the standard deviation of the E2 control.

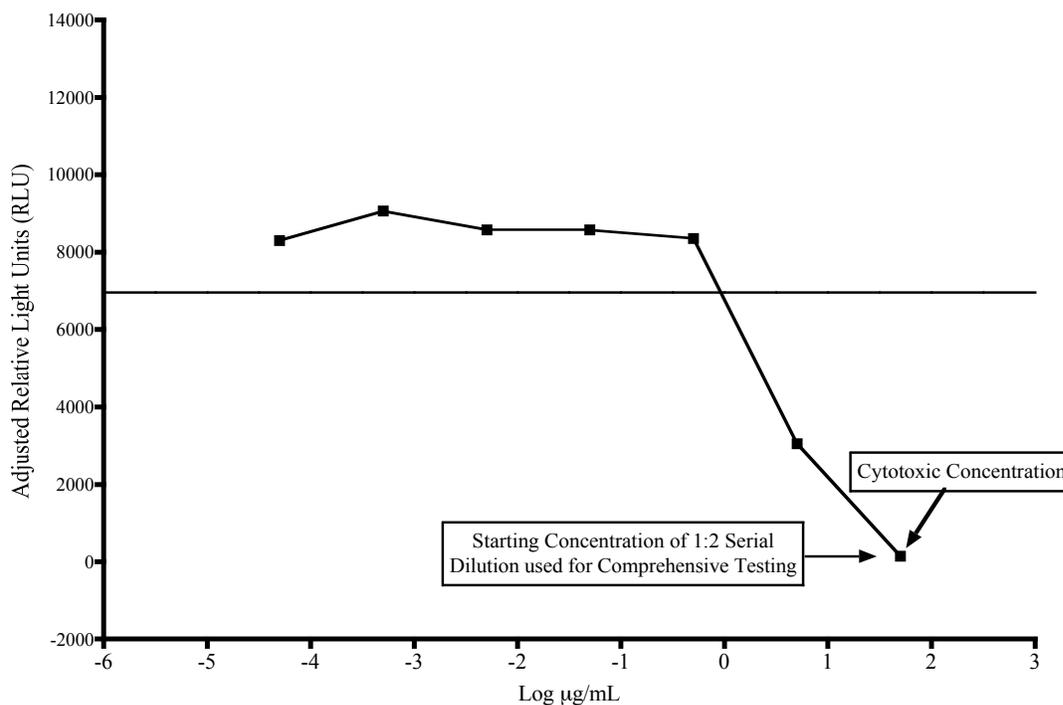
961 **Figure 14-3 Antagonist Range Finder (example 2)**



962
963 The solid horizontal line represents the mean minus three times the standard deviation of the E2 control.

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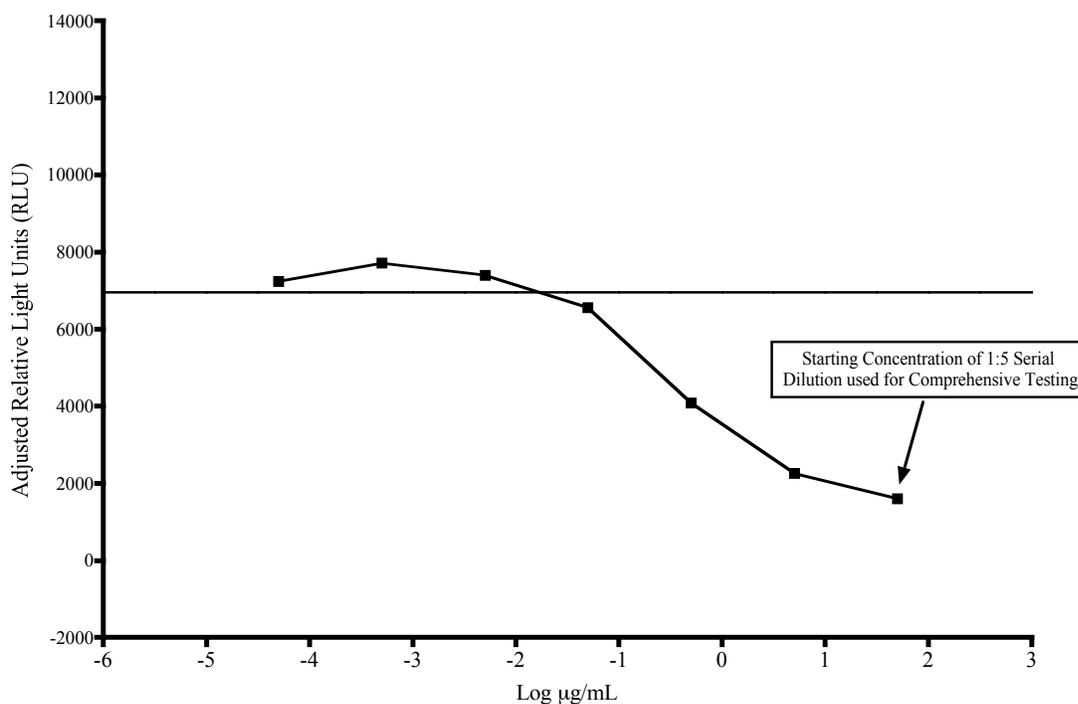
964 **Figure 14-4 Antagonist Range Finder (example 3)**



965

966 The solid horizontal line represents the mean minus three times the standard deviation of the E2 control.

967 **Figure 14-5 Antagonist Range Finder (example 4)**

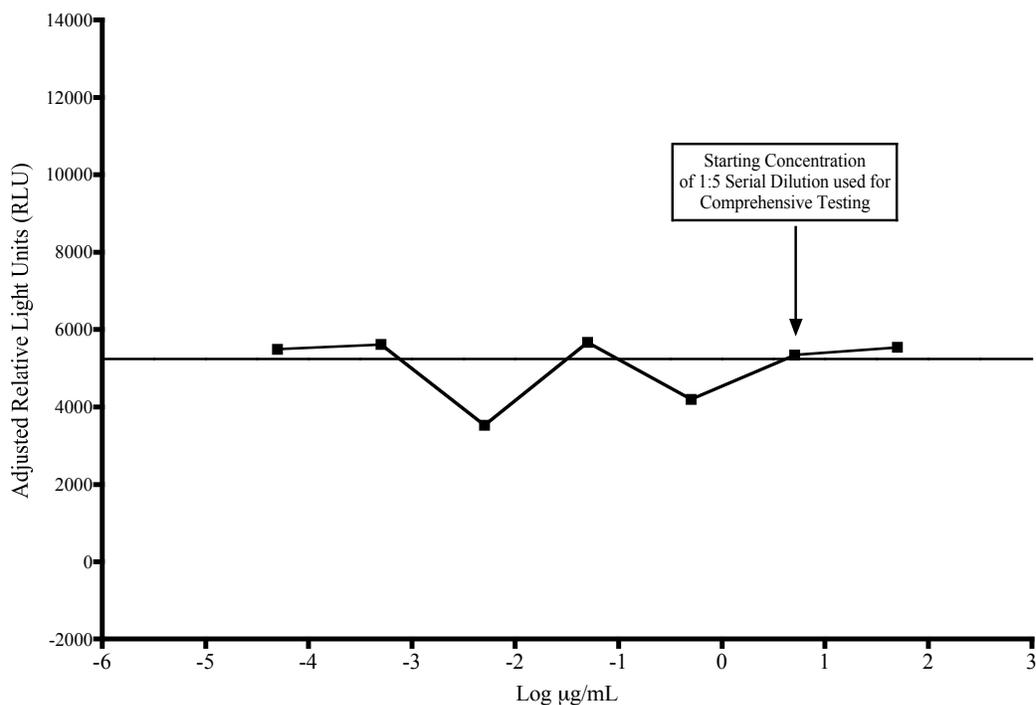


968

969 The solid horizontal line represents the mean minus three times the standard deviation of the E2 control.

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970 **Figure 14-6 Antagonist Range Finder (example 5)**



971
 972 The solid horizontal line represents the mean minus three times the standard deviation of the E2 control.
 973

974 **15.0 COMPREHENSIVE TESTING**

975 Antagonist comprehensive testing for coded substances consists of 11 point, 1:2 serial dilutions, with
 976 each concentration tested in triplicate wells of the 96-well plate. **Figure 15-1** contains a template for the
 977 plate layout to be used in antagonist comprehensive testing.

978
 979

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979 **Figure 15-1 Antagonist Comprehensive Test Plate Layout**

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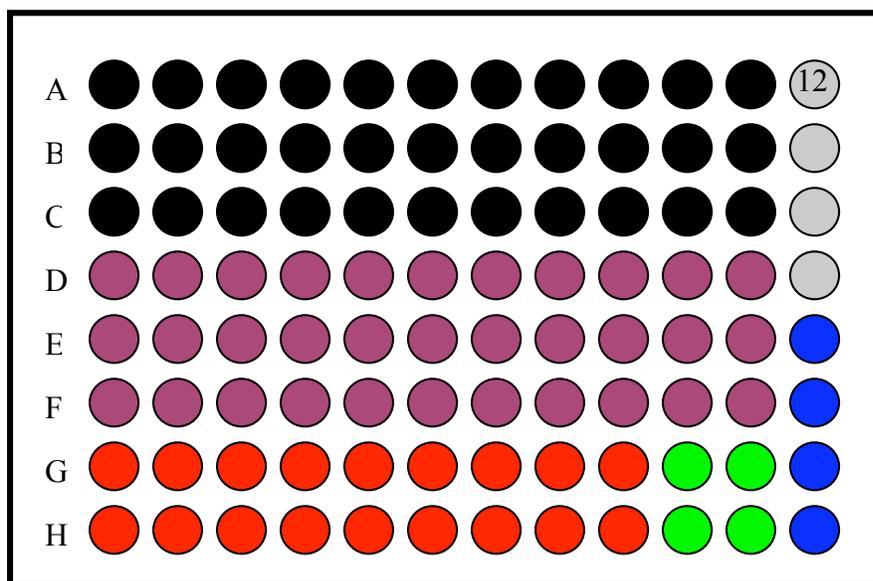
1011

1012

1013

1014

1015



● **9 Point Duplicate Ral/E2 Reference Standard**

● **DMSO (Solvent Control)**

● **Test Substance #1**

● **Test Substance #2**

● **E2 Control**

● **Flavone Control**

Evaluate whether comprehensive experiments have met acceptance criteria (see **Section 13.6.3**) and graph the data as described in the NICEATM Prism[®] users guide.

- If the substance has been tested up to the limit dose or the maximum soluble dose without causing a significant decrease in cell viability, and there are no points on the concentration curve that are less than the mean minus three times the standard deviation of the E2 control, the substance is considered negative for antagonism.
- If the substance has been tested up to the limit dose and there are points on the concentration curve that are less than the mean minus three times the standard deviation of the E2 control, but cell viability has a visual inspection score of 2 or greater, at all points falling below the E2 line, the substance is considered negative for antagonism.
- If there are points on the test substance concentration curve that are less than the mean minus three times the standard deviation of the E2 control that do not cause a visual inspection score of 2 or greater, the substance is positive for antagonism.

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- 1016 – Points in the test substance concentration curve that cause a visual inspection
1017 score of 2 or greater, are not included in data analyses.

1018 **16.0 COMPILATION OF THE HISTORICAL QUALITY CONTROL DATABASE**

1019 Historical databases are maintained in order to ensure that the assay is functioning properly. Historical
1020 databases are compiled using Excel® spreadsheets and are separate from the spreadsheets used to collect
1021 the data for individual test plates. Reference standard and control data is used to develop and maintain the
1022 historical database and are used as quality controls to determine acceptance of individual test plates.

1023 The sources of data needed to compile the historical database for the E2 control and flavone/E2 control
1024 values are the experiment specific Excel® data collection and analysis spreadsheets (see **Section 13.5.2**)
1025 used for BG1Luc ER TA antagonist testing. The sources of the data needed to compile the historical
1026 database for the DMSO control are the experiment specific Excel® data collection and analysis
1027 spreadsheets used for BG1Luc ER TA antagonist and agonist testing (see **Section 13.5.2** of the BG1Luc
1028 ER TA antagonist protocol and **Section 11.5.2** in the BG1LUC ER TA agonist protocol).

1029 **16.1 E2 Control**

1030 Open the BG1Luc ER TA antagonist specific historical database Excel® spreadsheet
1031 (LUMI_AgandAntQC.xls) and save under a new name using the Excel® “Save As” function, adding the
1032 laboratory designator to the file name (e.g., for Laboratory H, new name = HLUMI_AgandAntQC.xls).
1033 Open the E2 Control worksheet and enter the date and experiment name into worksheet columns A and B
1034 respectively. Enter the experimental mean adjusted E2 control value (from cell D37 in the ER Antagonist
1035 Report worksheet of the Excel® data collection and analysis spreadsheet) into the Antagonist E2 control
1036 worksheet, column C. Acceptance or rejection of plate E2 control data for comprehensive testing is based
1037 on whether the mean plate E2 RLU value falls within 2.5 times the standard deviation of the E2 value in
1038 the historical database (columns G and H in the E2 Control worksheet).

1039 **16.2 DMSO**

1040 Open the combined agonist and antagonist BG1Luc ER TA historical database Excel® spreadsheet
1041 (LUMI_AgandAntQC.xls) and save under a new name using the Excel® “Save As” function, adding the
1042 laboratory designator to the file name (e.g., for Laboratory H, new name = HLUMI_AgandAntQC.xls).
1043 Enter the date and experiment name into worksheet columns A and B respectively. Enter the experimental
1044 mean DMSO control value (from cell H37 in the RAW DATA worksheet of the agonist and antagonist
1045 Excel® data collection and analysis spreadsheet) into worksheet column C. Acceptance or rejection of the
1046 plate DMSO control data for range finding and comprehensive testing is based on whether the mean plate
1047 DMSO RLU value falls within 2.5 times the standard deviation of the DMSO value in the historical
1048 database (columns G and H in the DMSO worksheet).

1049 **17.0 QUALITY TESTING OF MATERIALS**

1050 All information pertaining to the preparation and testing of media, media supplements, and other
1051 materials should be recorded in the Study Notebook.

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1052 **17.1 Tissue Culture Media**

1053 Each lot of tissue culture medium must be tested in a single growth flask of cells before use in ongoing
1054 tissue culture or experimentation (**note:** each bottle within a given lot of Charcoal/Dextran treated FBS
1055 must be tested separately).

- 1056 1. Every new lot of media (RPMI and DMEM) and media components (FBS,
1057 Charcoal/Dextran treated FBS, and L-glutamine) must first be tested on the BG1Luc ER
1058 TA prior to being used in any GLP acceptable assays.
- 1059 2. Add 4 μ L of DMSO (previously tested) into four separate 13 mm tubes.
- 1060 3. Add 400 mL media (to be tested) to 13 mm tube.
- 1061 4. Dose an experimental plate as in **Section 12.0**, treating the media being tested as a test
1062 substance.
- 1063 5. Analyze 96-well plate as described in **Section 12.0**, comparing the data from the DMSO
1064 controls made using previously tested tissue culture media to the new media being tested.
- 1065 6. Use the agonist historical database to determine if the new media with DMSO lies within
1066 2.5 standard deviations of the mean for the media. If the RLU values for the new media
1067 with DMSO lie within 2.5 standard deviations of the DMSO mean from the historical
1068 database, the new lot of media is acceptable. If the RLU values for the new media with
1069 DMSO do not lie within 2.5 standard deviations of the DMSO mean from the historical
1070 database, the new lot may not be used in the assay.
- 1071 7. Note date and lot number in study notebook.
- 1072 8. If the new bottle passes quality testing as described in **Section 15.1 step 6**, apply the
1073 media to a single flask cells and observe the cells growth and morphology over the
1074 following 2 to 3 days. If there is no change in growth or morphology, the new media is
1075 acceptable for use.

1076 **17.2 G418**

- 1077 1. New lots of G418 must first be tested on the LUMI-CELL[®] ER assay prior to being used
1078 in any GLP acceptable assays.
- 1079 2. Add 220 μ L of G418 (previously tested) to a single flask containing cells growing in
1080 RPMI.
- 1081 3. Add 220 μ L of G418 (to be tested) to a different flask containing cells growing in RPMI.
- 1082 4. Observe cellular growth and morphology in both tissue culture flasks over a 48 to 72
1083 hour period. If there are no differences in observed growth rate and morphology between
1084 the two flasks, the new G418 lot is acceptable.
- 1085 5. If cellular growth is decreased, or the cells exhibit abnormal morphology, the new lot of
1086 G418 is not acceptable.
- 1087 6. Note date and lot number in study book.

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1088 **17.3 DMSO**

- 1089 1. Every new bottle of DMSO must be tested on the BG1Luc ER TA prior to use in any
1090 GLP acceptable assays.
- 1091 2. Add 4 μ L of DMSO (to be tested) into four separate 13 mm tubes.
- 1092 3. Add 400 mL media (previously tested) the same tubes.
- 1093 4. Dose an experimental plate as in **Section 15.0**, treating the media being tested as a test
1094 substance.
- 1095 5. Analyze 96-well plate as described in **Section 15.0**, comparing the data from the DMSO
1096 controls made using previously tested tissue culture media to the new media being tested.
- 1097 6. Use the agonist historical database to determine if media with new DMSO lies within 2.5
1098 standard deviations of the DMSO mean from historical database. If the RLU values for
1099 the media with new DMSO lie within 2.5 standard deviations of the DMSO mean from
1100 the historical database, the new lot of DMSO is acceptable. If the RLU values for media
1101 with new DMSO do not lie within 2.5 standard deviations of the DMSO mean from
1102 historical database, the new lot may not be used in the assay.
- 1103 7. Note the date, lot number, and bottle number in study book.
- 1104 8. If no DMSO has been previously tested, test several bottles as described in **Section 15.3**,
1105 and determine whether any of the bottles of DMSO have a higher average RLU than the
1106 other bottle(s) tested. Use the DMSO with the lowest average RLU for official
1107 experiments.

1108 **17.4 Plastic Tissue Culture Materials**

- 1109 1. Grow one set of cells, plate them for experiments on plastic ware from the new lot and
1110 one set of cells in the plastic ware from a previous lot, and dose them with E2 reference
1111 standard and controls.
- 1112 2. Perform the BG1Luc ER TA experiment with both sets of cells.
- 1113 3. If all of the analysis falls within acceptable QC criteria, then the new manufacturer's
1114 products may be used.

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1118 ICCVAM. 2001. Guidance Document on Using *In Vitro* Data to Estimate *In Vivo* Starting Doses for
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1121 guidance/iv_guide.pdf](http://iccvam.niehs.nih.gov/methods/invidocs/guidance/iv_guide.pdf) [accessed 31 August 2006]

1122 Coecke, S., Guidance on Good Cell Culture Practice. 2005. ATLA 33, 261-287.

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