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Annex E

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ICCVAM/NICEATM BG1Luc4E2 ER TA – Agonist Protocol

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**BG1LUC4E2 ER TA ASSAY
AGONIST PROTOCOL**

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

**Developed by:
Xenobiotic Detection Systems, Inc.
1601 E. Geer St., Suite S
Durham, NC 27704
12 March 2009**

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LIST OF ACRONYMS AND ABBREVIATIONS

144	13 mm test tube	13 x 100 mm glass test tubes
145	DMEM	Dulbecco's Modification of Eagle's Medium
146	DMSO	Dimethyl Sulfoxide
147	DMSO control	1% v/v dilution of DMSO in tissue culture media used as a
148		vehicle control
149	E2	17 β -estradiol
150	E2 reference standard	11 Point Serial Dilution of 17 β -estradiol reference standard for
151		the LUMI-CELL [®] ER agonist assay
152	EC ₅₀ value	Concentration that produces a half-maximal response as
153		calculated using the four parameter Hill function.
154	ER	Estrogen Receptor
155	Estrogen-free DMEM	DMEM (phenol red free) supplemented with 1%
156		Penicillin/Streptomycin, 2% L-Glutamine, and 5% Charcoal-
157		dextran treated FBS
158	FBS	Fetal Bovine Serum
159	G418	Gentamycin
160	Methoxychlor	<i>p,p'</i> -Methoxychlor
161	Methoxychlor control	3.13 μ g/mL Methoxychlor Weak Positive Control for the
162		BG1LUC4E2 ER TA Agonist Assay
163	RPMI	RPMI 1640 growth medium
164	TA	Transcriptional Activation
165	T25	25 cm ² tissue culture flask
166	T75	75 cm ² tissue culture flask
167	T150	150 cm ² tissue culture flask
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200

200 **1.0 PURPOSE**

201 This protocol is designed to evaluate coded test substances for potential estrogen receptor (ER) agonist
202 activity using the LUMI-CELL® ER assay.

203 **2.0 SPONSOR**

204 The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative
205 Toxicological Methods (NICEATM), P.O. Box 12233 Research Triangle Park, NC 27709

206

207 William S. Stokes, DVM, DACLAM
208 Rear Admiral, U.S. Public Health Service
209 Chief Veterinary Officer, USPHS
210 Director, NICEATM
211 National Institute of Environmental Health Sciences, NIH, DHHS
212 Bldg. 4401, Room 3129, MD EC-14
213 79 T.W. Alexander Drive
214 Research Triangle Park, NC 27709
215 Phone: 919-541-7997
216 Fax: 919-541-0947
217 Email: stokes@niehs.nih.gov

218

219 Raymond Tice, Ph.D.
220 Deputy Director, NICEATM
221 National Institute of Environmental Health Sciences
222 MD EC-17, P.O. Box 12233
223 Research Triangle Park, NC 27709
224 Phone: 919-541-4482
225 FAX: 919-541-0947
226 Email: tice@niehs.nih.gov

227

228 David Allen, Ph.D.
229 Principal Investigator
230 ILS, Inc./Contractor supporting NICEATM
231 National Institute of Environmental Health Sciences
232 MD EC-17, P.O. Box 12233
233 Research Triangle Park, NC 27709
234 Phone: 919-316-4664
235 FAX: 919-541-0947
236 Email: allen7@niehs.nih.gov

237

238 Frank Deal, M.S.

239 Staff Toxicologist
240 ILS, Inc./Contractor supporting NICEATM
241 National Institute of Environmental Health Sciences
242 MD EC-17, P.O. Box 12233
243 Research Triangle Park, NC 27709
244 Phone: 919-316-4587
245 FAX: 919-541-0947
246 Email: dealf@niehs.nih.gov

247
248 Patricia Ceger, M.S.
249 Project Coordinator/Technical Writer
250 ILS, Inc./Contractor supporting NICEATM
251 National Institute of Environmental Health Sciences
252 MD EC-17, P.O. Box 12233
253 Research Triangle Park, NC 27709
254 Phone: 919-316-4556
255 Fax: 919-541-0947
256 E-Mail: cegerp@niehs.nih.gov

257

258 **2.1 Substance Inventory and Distribution Management**

259 Cynthia Smith, Ph.D.
260 Chemistry Resources Group Leader
261 National Institute of Environmental Health Sciences
262 MD EC-06, P.O. Box 12233
263 Research Triangle Park, NC 27709
264 Phone: 919-541-3473

265 **3.0 DEFINITIONS**

- 266
- 267 • **Dosing Solution:** The test substance, control substance, or reference standard solution,
268 which is to be placed into the tissue culture wells for experimentation.
 - 269 • **Raw Data:** Raw data includes information that has been collected but not formatted or
270 analyzed, and consists of the following:
 - 271 ○ Data recorded in the Study Notebook
 - 272 ○ Computer printout of initial luminometer data
 - 273 ○ Other data collected as part of GLP compliance, e.g.:
 - 274 ■ Equipment logs and calibration records
 - 275 ■ Test substance and tissue culture media preparation logs
 - 276 ■ Cryogenic freezer inventory logs
 - 277 • **Soluble:** Test substance exists in a clear solution without visible cloudiness or
precipitate.

- 278 • **Study Notebook:** The study notebook contains recordings of all activities related to the
279 conduct of the BG1LUC4E2 ER TA agonist assay.
- 280 • **Test Substances:** Substances supplied to the testing laboratories that are coded and
281 distributed such that only the Project Officer, Study Management Team (SMT), and the
282 Substance Inventory and Distribution Management have knowledge of their true identity.
283 The test substances will be purchased, aliquoted, coded, and distributed by the Supplier
284 under the guidance of the NIEHS/NTP Project Officer and the SMT.

285 4.0 TESTING FACILITY AND KEY PERSONNEL¹

286 4.1 Testing Facility

287 Xenobiotic Detection Systems, Inc. (XDS), 1601 E. Geer St., Suite S, Durham, NC 27704

288 4.2 Key Personnel

- 289 • Study Director: John Gordon, Ph.D.
290 • Quality Assurance Director: Mr. Andrew

291 5.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

292 5.1 Test Substances

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

295 5.2 Controls

296 Controls for the ER agonist protocol are as follows:

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

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

302 *Positive control (p,p'-Methoxychlor [methoxychlor]):* Methoxychlor (CASRN 72-43-5), 3.13 µg/mL in
303 tissue culture media, used as a weak positive control.

304 6.0 OVERVIEW OF GENERAL PROCEDURES FOR AGONIST TESTING

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

308 Agonist range finder testing is conducted on 96-well plates using four concentrations of E2
309 (5.00×10^{-5} , 1.25×10^{-5} , 3.13×10^{-6} and 7.83×10^{-7} µg/mL) in duplicate as the reference standard and four

¹ Testing facility and personnel information are provided as an example.

310 replicate wells for the DMSO control. Range finder testing uses all wells of the 96-well plate to test six
311 substances as seven point 1:10 serial dilutions in duplicate.

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

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

E2 Concentrations ¹		
1.00 x 10 ⁻⁴	6.25 x 10 ⁻⁶	3.92 x 10 ⁻⁷
5.00 x 10 ⁻⁵	3.13 x 10 ⁻⁶	1.95 x 10 ⁻⁷
2.50 x 10 ⁻⁵	1.56 x 10 ⁻⁶	9.78 x 10 ⁻⁸
1.25 x 10 ⁻⁵	7.83 x 10 ⁻⁷	

317 ¹Concentrations are presented in µg/mL.

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

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

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

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

332
$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC_{50} - X)\text{HillSlope}}}$$

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

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

340 **6.1 Range Finder Testing**

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

345 **6.2 Comprehensive Testing**

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

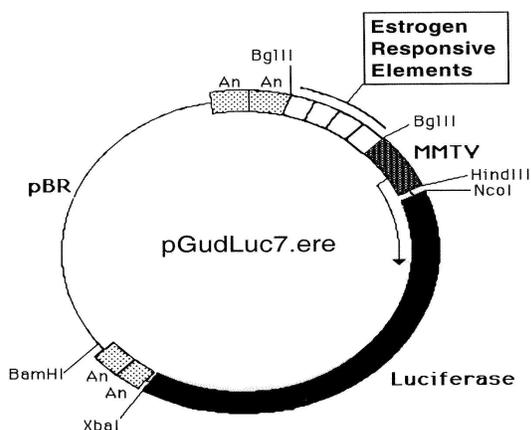
350 **7.0 MATERIALS FOR BG1LUC4E2 ER TA AGONIST TESTING**

351 This section provides the materials needed to conduct BG1LUC4E2 ER TA testing, with associated brand
352 names/vendors² in brackets.

353 **7.1 BG1Luc4E2 Cells:**

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

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



357

358 **7.2 Technical Equipment:**

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

- 362
- Analytical balance (Cat. No. 01-910-320)

- 363 • Berthold Orion 1 Microplate Luminometer [Berthold CatNo.: Orion 1 MPL3] or
- 364 equivalent and dedicated computer
- 365 • Biological safety hood, class II, and stand (Cat. No. 16-108-99)
- 366 • Centrifuge (low speed, tabletop with swinging bucket rotor) (Cat. No. 04-978-50
- 367 centrifuge, and 05-103B rotor)
- 368 • Combustion test kit (CO₂ monitoring) (Cat. No. 10-884-1)
- 369 • Drummond diaphragm pipetter (Cat. No. 13-681-15)
- 370 • Freezers, –20°C (Cat. No. 13-986-150), and –70°C (Cat. No. 13-990-86)
- 371 • Hand tally counter (Cat. No. 07905-6)
- 372 • Hemocytometer, cell counter (Cat. No. 02-671-5)
- 373 • Light microscope, inverted (Cat. No. 12-561-INV)
- 374 • Light microscope, upright (Cat. No. 12-561-3M)
- 375 • Liquid nitrogen flask (Cat. No. 11-675-92)
- 376 • Micropipetter, repeating (Cat. No. 21-380-9)
- 377 • Pipettors, air displacement, single channel (0.5 –10µl (Cat. No. 21-377-191), 2 –
- 378 20 µl (Cat. No. 21-377-287), 20 – 200 µl (Cat. No. 21-377-298), 200 - 1000 µl
- 379 (Cat. No. 21-377-195))
- 380 • Refrigerator/freezer (Cat. No. 13-986-106A)
- 381 • Shaker for 96-well plates (Cat. No. 14-271-9)
- 382 • Sodium hydroxide (Cat. No. 5318-500)
- 383 • Sonicating water bath (Cat. No. 15-335-30)
- 384 • Tissue culture incubator with CO₂ and temperature control (Cat. No. 11-689-4)
- 385 • Vacuum pump with liquid trap (side arm Erlenmeyer) (Cat. No. 01-092-29)
- 386 • Vortex mixer (Cat. No. 12-814)

387 Equipment should be maintained and calibrated as per GLP guidelines and individual laboratory
388 SOPs.

389 **7.3 Reference Standard, Controls, and Tissue Culture Supplies**

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

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

²Brand 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.

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

- 397 • BackSeal-96/384, white adhesive bottom seal for 96-well and 384-well microplate
398 [Perkin-Elmer, Cat. No. 6005199]
- 399 • 17 β -estradiol (CAS RN: 50-28-2) [Sigma-Aldrich, Cat. No. E8875]
- 400 • Cryovial, 2 mL (Corning Costar) [Fisher Scientific Cat. No. 03-374-21]
- 401 • Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 10009186R38]³
- 402 • Culture tube, 50 mL conical (Corning Costar) [Fisher Scientific Cat. No. 05-
403 526C]
- 404 • DMSO, U.S.P. analytical grade. [Sigma-Aldrich, Cat. No. 34869-100ML]
- 405 • Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L
406 glucose, with sodium pyruvate, without phenol red or L-glutamine
407 [Mediatech/Cellgro, Cat. No. 17-205-CV]
- 408 • Fetal Bovine Serum [Mediatech/Cellgro Cat. No. MT 35-010-CV]
- 409 • Fetal Bovine Serum, charcoal/dextran treated, triple 0.1 μ m sterile filtered
410 [Hyclone, Cat. No. SH30068.03]
- 411 • Gentamycin Sulfate (G418), 50 mg/mL [Mediatech/Cellgro Cat. No. 30-234-CR]
- 412 • L-glutamine, 29.2 mg/mL [Cellgro, Cat. No. 25005-CI]
- 413 • Luciferase Assay System (10-Pack) [Promega Cat. No. E1501]
- 414 • Lysis Solution 5X [Promega, Cat. No. E1531]
- 415 • Methoxychlor (CAS RN: 72-43-5) [Sigma-Aldrich, Cat. No. 49054]
- 416 • Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 μ g/mL streptomycin
417 [Cellgro, Cat. No. 30-001-CI].
- 418 • Phosphate buffered saline (PBS, 1X) without calcium and magnesium [Cellgro,
419 Cat. No. 21-040-CV]
- 420 • Pipettes, serological: 2.0 mL [Sigma-Aldrich, Cat. No. P1736], 5.0 mL [Sigma-
421 Aldrich, Cat. No. P1986], 25 mL [Sigma-Aldrich, Cat. No. P2486]
- 422 • RPMI 1640 medium, containing L-glutamine [Mediatech, Cat. No. 10-040-CV]
- 423 • Tissue culture flasks (Corning-Costar): 25 cm² (T25) [Fisher Cat. No. 10-126-28];
424 75 cm² (T75) [Fisher Cat. No. 10-126-37]; and 150 cm² (T150) [Fisher Cat. No.
425 10-126-34]
- 426 • Tissue culture plates (Corning-Costar): 96-well [Thomas Scientific Cat. No.
427 6916A05]

³ If glass tubes can not be obtained from Thomas Scientific, the preference is for flint glass, then lime glass, then borosilicate glass.

- 428 • Trypsin (10X), 2.5% in Hank’s balanced salt solution (HBSS), without calcium
429 and magnesium, without phenol red [Cellgro, Cat. No. 25-054-CI].

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

431 **8.0 PREPARATION OF TISSUE CULTURE MEDIA AND SOLUTIONS**

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

434 **8.1 RPMI 1640 Growth Medium (RPMI)**

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

437 Procedure for one 549 mL bottle:

- 438 1. Remove FBS from -70°C freezer, and Pen-Strep from -20°C freezer and allow to
439 equilibrate to room temperature.
440 2. Add 44 mL of FBS and 5 mL Pen-Strep to the bottle of RPMI 1640.
441 3. Label RPMI bottle as indicated in **Section 7.3**

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

444 **8.2 Estrogen-Free DMEM Medium**

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

447 Procedure for one 539 mL bottle:

- 448 1. Remove charcoal/dextran treated FBS from -70°C freezer, and L-glutamine and
449 Pen-Strep from -20°C freezer and allow to equilibrate to room temperature.
450 2. Add 24 mL of charcoal/dextran treated FBS, 10 mL L-glutamine, and 5 mL Pen-
451 Strep to one 500 mL bottle of DMEM.
452 3. Label estrogen-free DMEM bottle as indicated in **Section 7.3**

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

455 **8.3 1X Trypsin Solution**

456 1X Trypsin solution is prepared by dilution from a 10X premixed stock solution. The 10X stock
457 solution should be stored in 10 mL aliquots in a -20°C freezer.

458 Procedure for making 100 mL of 1X trypsin:

- 459 1. Remove a 10 mL aliquot of 10X trypsin from -20°C freezer and allow to
460 equilibrate to room temperature.
461 2. Aliquot 1 mL Trypsin (10X) along with 9 mL of 1X PBS into ten 15 mL sterile
462 centrifuge tubes.
463 3. Label 1X trypsin aliquots as indicated in **Section 7.3**
464 *1X Trypsin should be stored at -20°C.*

465 **8.4 1X Lysis Solution**

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

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

- 469 1. Thaw the 5X Promega Lysis solution and allow it to reach room temperature.
470 2. Remove 2 mL of 5X solution and place it in a 15 mL conical centrifuge tube.
471 3. Add 8 mL of distilled, de-ionized water to the conical tube.
472 4. Cap and shake gently until solutions are mixed.

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

474 **8.5 Reconstituted Luciferase Reagent**

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

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

479 To reconstitute luciferase reagent:

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

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

487 **9.0 OVERVIEW OF PROPAGATION AND EXPERIMENTAL PLATING OF** 488 **BG1LUC4E2 CELLS**

489 The BG1Luc4E2 (BG-1) cells are stored in liquid nitrogen in 2 mL cryovials. BG-1 cells are
490 grown as a monolayer in tissue culture flasks in a dedicated tissue culture incubator at 37°C ±
491 1°C, 90% ± 5% humidity, and 5.0% ± 1% CO₂/air. The cells should be examined, on a daily

492 basis during working days, under an inverted phase contrast microscope and any changes in
493 morphology and/or adhesive properties must be noted in the study notebook.

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

496 **9.1 Procedures for Thawing Cells and Establishing Tissue Cultures**

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

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

501 9.1.1 Thawing Cells

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

514 9.1.2 Establishing Tissue Cultures

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

- 517 1. Remove the T25 flask from the incubator.
- 518 2. Aspirate the RPMI, then add 5 mL 1X PBS, making sure that the cells are coated
519 with PBS.
- 520 3. Aspirate 1X PBS, then add 1 to 2 mL 1X trypsin to the T25 flask, gently swirling
521 the flask to coat all cells with the trypsin.
- 522 4. Place the flask in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
- 523 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the
524 hand.

- 525 6. Confirm cell detachment by examination under an inverted microscope. If cells
526 have not detached, return the flask to the incubator for an additional 2 minutes,
527 then hit the flask again.
- 528 7. After cells have detached, add 5 mL PBS, and transfer the suspended cells to a 50
529 mL centrifuge tube. Wash the flask one additional time with 5 mL PBS.
- 530 8. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular
531 digestion by residual trypsin.
- 532 9. Pellet the cells by centrifugation, as described in **Section 9.1.1**, and re-suspend the
533 cells in 10 mL RPMI medium.
- 534 10. Draw the pellet repeatedly through a 25 mL serological pipette to break up
535 clumps of cells
- 536 11. Transfer cells to a T75 flask, then place the flask in an incubator (see conditions
537 in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
538 When cells have reached 80% to 90% confluency, transfer them into a T150 flask by performing,
539 for example, the following steps:
- 540 12. Remove the T75 flask from the incubator, aspirate the old media and add 5 mL
541 1X PBS.
- 542 13. Aspirate 1X PBS, add 2 mL of 1X trypsin to the flask, and place it in an incubator
543 (see conditions in **Section 9.0**) for 5 to 10 min.
- 544 14. Repeat steps **5** through **11** in **Section 9.1.2**, re-suspending the pellet in 20 mL of
545 RPMI.
- 546 15. Transfer cells to a T150 flask and place it in the incubator (see conditions in
547 **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
- 548 16. Remove the T150 flask from the incubator.
- 549 17. Aspirate the RPMI and add 5 mL 1X PBS.
- 550 18. Aspirate 1X PBS and add 3 mL 1X trypsin to the T150 flask, making sure that the
551 cells are coated with the trypsin.
- 552 19. Incubate cells in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
- 553 20. Detach cells by hitting the side of the flask sharply against the palm or heel of the
554 hand.
- 555 21. Confirm cell detachment by examination under an inverted microscope. If cells
556 have not detached, return the flask to the incubator for an additional 2 minutes,
557 then hit the flask again.
- 558 22. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells
559 from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the
560 flask, swirl around the flask, and then transfer the PBS to the 50 mL conical tube.

- 561 23. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular
562 digestion by residual trypsin.
- 563 24. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge
564 for an additional 5 minutes.
- 565 25. Aspirate the media from the pellet and re-suspend it in 40 mL RPMI, drawing the
566 pellet repeatedly through a 25 mL serological pipette to break up any clumps of
567 cells.
- 568 26. Transfer 20 mL of cell suspension to each of two T150 flasks, place them in an
569 incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence
570 (approximately 48 to 72 hrs).

571 **9.2 Ongoing Tissue Culture Maintenance, Conditioning in Estrogen-free Medium,**
572 **and Plating Cells for Experimentation**

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

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

- 581 1. Remove both T150 flasks from the incubator.
- 582 2. Aspirate the medium and rinse the cells with 5 mL 1X PBS.
- 583 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask
584 to coat all cells with the trypsin.
- 585 4. Incubate cells in the incubator (see conditions in **Section 9.0**) for 5 to 10 min.
- 586 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the
587 hand.
- 588 6. Confirm cell detachment by examination under an inverted microscope. If cells
589 have not detached, return the flask to the incubator for an additional 2 minutes,
590 then hit the flask again.
- 591 7. After cells have detached, add 5 mL 1X PBS to the first T150 flask and transfer
592 the suspended cells to the second T150 flask.
- 593 8. Transfer the contents of both flasks to a 50 mL conical tube. Repeat step 7 with an
594 additional 5 mL 1X PBS and transfer to the 50 mL conical tube.
- 595 9. Immediately add 20 mL estrogen-free DMEM to the 50 mL conical tube to inhibit
596 further cellular digestion by residual trypsin.

597 10. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge
598 for an additional 5 minutes.

599 11. Aspirate media from pellet and re-suspend it in 4 mL estrogen-free DMEM,
600 drawing the pellet repeatedly through a 1 mL serological pipette to break up
601 clumps of cells.

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

604 9.2.1 Ongoing Tissue Culture Maintenance

- 605 1. Add 20 mL RPMI to two T150 flasks.
- 606 2. Add 220 µl G418 to the RPMI in the T150 flasks
- 607 3. Add 1 mL of cell suspension from **9.2 step 11** to each flask.
- 608 4. Place T150 flasks in tissue culture incubator (see conditions in **Section 9.0**) and
609 grow to 80% to 90% confluence (approximately 48 to 72 hrs).
- 610 5. Tissue culture medium may need to be changed 24 hours after addition of G418 to
611 remove cells that have died because they do not express reporter plasmid.
- 612 6. G418 does not need to be added to the flasks a second time.
- 613 7. Repeat **Section 9.2 steps 1-11** for ongoing tissue culture maintenance.

614 9.2.2 Conditioning in Estrogen-free Medium

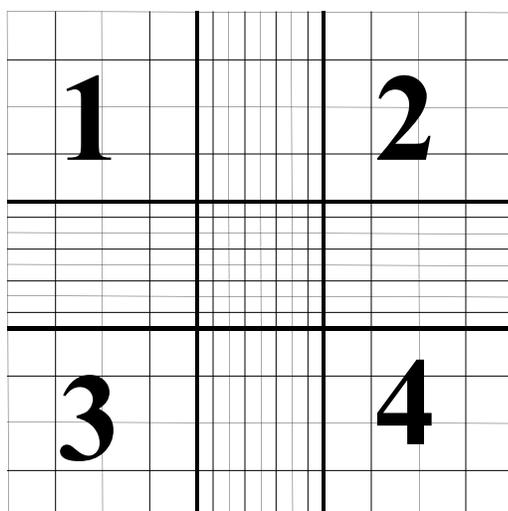
- 615 1. Add 20 mL estrogen-free DMEM to two T150 flasks.
- 616 2. Add 150 µL G418 to the estrogen-free DMEM in the T150 flasks.
- 617 3. Add 1 mL of cell suspension from **Section 9.2 step 11** to each flask.
- 618 4. Tissue culture medium may need to be changed 24 hours after addition of G418 to
619 remove cells that have died because they do not express reporter plasmid.
- 620 5. G418 does not need to be added to the flasks a second time.
- 621 6. Place the T150 flasks in the incubator (see conditions in **Section 9.0**) and grow to
622 80% to 90% confluence (approximately 48 to 72 hrs).

623 9.2.3 Plating Cells Grown in Estrogen-free DMEM for Experimentation

- 624 1. Remove the T150 flasks that have been conditioned in estrogen-free DMEM for
625 48 to 72 hours from the incubator.
- 626 2. Aspirate the medium, then rinse the cells with 5 mL 1X PBS.
- 627 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask
628 to coat all cells with the trypsin.
- 629 4. Place the flasks in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
- 630 5. Detach cells by hitting the side of the flask sharply against the palm or the heel of
631 the hand.

- 632 6. Confirm cell detachment by examination under an inverted microscope. If cells
633 have not detached, return the flask to the incubator for 2 additional minutes, then
634 hit the flask again.
- 635 7. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells
636 from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the
637 flask, gently swirl around the flask, and then transfer to the 50 mL conical tube.
- 638 8. Immediately add 20 mL estrogen-free DMEM to each conical tube to inhibit
639 further cellular digestion by residual trypsin.
- 640 9. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge
641 for an additional 5 minutes.
- 642 10. Aspirate the media from the pellet and re-suspend it in 20 mL DMEM, drawing
643 the pellet repeatedly through a 25 mL serological pipette to break up any clumps
644 of cells.
- 645 11. Pipette 15 μ L of the cell suspension into the “v” shaped slot on the
646 hemocytometer. Ensure that the solution covers the entire surface area of the
647 hemocytometer grid, and allow cells to settle before counting.
- 648 12. Using 100x magnification, view the counting grid.
- 649 13. The counting grid on the hemocytometer consists of nine sections, four of which
650 are counted (upper left, upper right, lower left, and lower right, see **Figure 9-1**).
651 Each section counted consists of four by four grids. Starting at the top left and
652 moving clockwise, count all cells in each of the four by four grids. Some cells
653 will be touching the outside borders of the square, but only count those that touch
654 the top and right borders of the square. This value is then used in the calculation
655 below to get to the desired concentration of 200,000 cells/mL.
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Figure 9-1 Hemocytometer Counting Grid.



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The volume of each square is 10^{-4} mL, therefore:
Cells/mL=(average number per grid) x 10^{-4} mL x 1/(starting dilution).
Starting dilution: 20 mL (for T150 flasks)

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

Example Calculation:

- Grids 1, 2, 3, and 4 are counted and provide the following data:
 - 50, 51, 49, and 50: average number of cells per grid is equal to 50.

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

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

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

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

Concentration_{Final} = 200,000 cells/mL

Concentration_{Initial} = 500,000 cells/mL

Volume_{Initial} = 20 mL

Volume_{Final} – to be solved for.

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

Solving for Volume_{Final} we find = 50 mL

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

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

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

692 **10.0 PREPARATION OF TEST SUBSTANCES**

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

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

700 **10.1 Determination of Test Substance Solubility**

- 701 1. Prepare a 100 mg/mL solution of the test substance in 100% DMSO in a 4 mL
702 conical tube.
- 703 2. Vortex to mix.
- 704 3. If the test substance does not dissolve at 100 mg/mL, prepare a 10 mg/mL
705 solution and vortex as above.
- 706 4. If the test substance does not dissolve at 10 mg/mL solution, prepare a 1 mg/mL
707 solution in a 4 mL conical tube and vortex as above.
- 708 5. If the test substance does not dissolve at 1 mg/mL, prepare a 0.1 mg/mL solution
709 in a 4 mL conical tube and vortex as above.
- 710 6. Continue testing, using 1/10 less substance in each subsequent attempt until test
711 substance is solubilized in DMSO.

712 Once the test substance has fully dissolved in 100% DMSO, the test substance is ready to be
713 used for BG1LUC4E2 ER TA testing.

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

716 **10.2 Preparation of Reference Standards, Control and Test Substances**

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

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

721 **10.2.1 Preparation of Reference Standard and Positive Control Stock Solutions**

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

725 10.2.1.1 *E2 Stock Solution*

726 The final concentration of the E2 stock solution is 1.0×10^{-2} $\mu\text{g/mL}$. Prepare the E2 stock as
727 shown in **Table 10-1**.

728 **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 $\mu\text{g/mL}$
3	Transfer 10 μL E2 solution from Step #2 to a new 4mL vial.	Add 990 μL of 100% DMSO. Vortex to mix.	1 $\mu\text{g/mL}$
4	Transfer 10 μL E2 solution from Step #3 to a 13 mm test tube to create the working solution.	Add 990 μL of 100% DMSO. Vortex to mix.	1.0×10^{-2} $\mu\text{g/mL}$

729

730 10.2.1.2 *Methoxychlor Stock Solution*

731 The final concentration of the methoxychlor stock solution is 313 $\mu\text{g/mL}$.

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

- 733 1. Make a 10 mg/mL stock solution of Methoxychlor in 100% DMSO in a 4 mL
734 vial.
- 735 2. Remove 94 μL of the methoxychlor solution and place it in a new 4 mL vial.
- 736 3. Add 2.906 mL of 100% DMSO to the 4mL vial and gently vortex to mix.

737 10.2.2 Preparation of Reference Standard and DMSO Control for Range Finder Testing

738 Range finder testing is conducted on 96-well plates using four concentrations of E2 in duplicate
739 as the reference standard. Four replicate wells are used for the DMSO control. All wells on the
740 96 well plate are used during range finder testing.

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

742 10.2.2.1 *Preparation of E2 Reference Standard for Range Finder Testing*

743 To make E2 dosing solutions:

- 744 1. label four 4 mL conical tubes with numbers 1 through 4 and place them in a tube
745 rack
- 746 2. label four 13 mm glass test tubes with numbers 1 through 4, place them in a tube
747 rack and add 600 μL of estrogen-free DMEM to each tube

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

749

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

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

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

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

755 10.2.2.2 *Preparation of DMSO Control for Range Finder Testing*

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

758 10.2.3 Preparation of Test Substance Dosing Solutions for Range Finder Testing

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

762 To make dosing solutions for coded substances:

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

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

768

768 **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

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

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

773
 774 Determination of whether a substance is positive in range finder testing and selection of starting
 775 concentrations for comprehensive testing will be discussed in **Section 12.0**.

776 10.2.4 Preparation of Reference Standard and Positive Control Dosing Solutions for
 777 Comprehensive Testing

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

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

782 10.2.4.1 *Preparation of E2 Reference Standard for Comprehensive Testing*

783 To make E2 dosing solutions:

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

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

789

789 **Table 10-4 Preparation of E2 Reference Standard Dosing Solution for**
 790 **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

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

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

795

796 10.2.4.2 *Preparation of Methoxychlor Control Dosing Solution for Comprehensive Testing*

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

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

799 10.2.4.3 *Preparation of DMSO Control Dosing Solution for Comprehensive Testing*

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

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

802 10.2.5 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

803 Comprehensive testing experiments are used to determine whether a substance possesses ER
 804 agonist activity in the BG1LUC4E2 ER TA test method. Agonist comprehensive testing for
 805 coded substances consists of either an 11 point 1:2 serial dilution or an 11 point 1:5 serial
 806 dilution, depending on the results from range finder testing (**see Section 12.0**) with each
 807 concentration tested in triplicate wells of the 96-well plate.

808 10.2.5.1 *Preparation of Test Substance 1:2 Serial Dilutions for*
 809 *Comprehensive Testing*

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

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

- 812 1. label eleven 4 mL conical tubes with numbers 1 through 11 and place them in a
813 tube rack
- 814 2. label eleven 13 mm glass test tubes with numbers 1 through 11, place them in a
815 tube rack and add 800 µL of estrogen-free DMEM to each tube

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

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

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

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

821

822 10.2.5.2 *Preparation of Test Substance 1:5 Serial Dilutions for Comprehensive*
823 *Testing*

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

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

- 826 3. label eleven 4 mL conical tubes with numbers 1 through 11 and place them in a
827 tube rack
- 828 4. label eleven 13 mm glass test tubes with numbers 1 through 11, place them in a
829 tube rack and add 800 µL of estrogen-free DMEM to each tube

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

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

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

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

835

836 **11.0 GENERAL PROCEDURES FOR THE TESTING OF CODED SUBSTANCES**

837 Range finder experiments are used to determine the concentrations of test substance to be used
838 during comprehensive testing. Comprehensive testing experiments are used to determine whether
839 a substance possesses ER agonist activity in the BG1LUC4E2 ER TA assay.

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

843 **11.1 Application of Reference Standard, Controls, and Test Substances**

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

853 11.1.1 Preparation of Excel® Data Analysis Template For Range Finder Testing

- 854 1. In Excel®, open a new “AgRFTemplate” and save it with the appropriate project
855 name as indicated in the NICEATM Style Guide.
- 856 2. Fill out the table at the top of the “Raw Data” worksheet with information
857 regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot.
858 Meas. Time/Well (s), etc. (**note**: this information can be permanently added to the
859 default template “AgRFTemplate” on a laboratory specific basis).
- 860 3. Add the following information regarding the assay to the “Compound Tracking”
861 worksheet.
- 862 ▪ Plate # - Enter the experiment ID or plate number into cell E1
 - 863 ▪ Cell Lot # - Enter the passage or lot number of the cells used for this
864 experiment into cell B5
 - 865 ▪ DMSO and Media Lot #'s – Enter the lot numbers for the DMSO and
866 Media in cells B6 and B7
 - 867 ▪ Test Substance Code – Enter the test substance codes into cells C13 to
868 C18
 - 869 ▪ Name: Enter the experimenter name into cell G6
 - 870 ▪ Date: Enter the experiment date in the format day\month\year into cell
871 G10
 - 872 ▪ Comments: - Enter any comments about the experiment in this box (e.g.,
873 plate contaminated)
- 874 4. Enter the following substance testing information to the “List” page:
- 875 ▪ Concentration – Type in the test substance concentration in µg/ml in
876 descending order.
 - 877 ▪ Also add any replicate-specific comments on this page (e.g, spilled tube,
878 etc.), in the comments section
 - 879 ▪ All of the remaining cells on the List tab should populate automatically.
 - 880 ▪ The “Template”, “Compound Mixing” and “Visual Inspection” tabs
881 should automatically populate with the information entered into the
882 Compound Tracking and List tabs.
- 883 5. Save the newly named project file.
- 884 6. Print out either the “List” or “Template” page for help with dosing the 96-well
885 plate. Sign and date the print out and store in study notebook.

886 11.1.2 Preparation of Excel® Data Analysis Template for Comprehensive Testing

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

- 889 2. Fill out the table at the top of the “Raw Data” worksheet with information
890 regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot.
891 Meas. Time/Well (s), etc. (**note**: this information can be permanently added to the
892 default template “AgCTTemplate” on a laboratory specific basis).
893
- 894 3. On the “Compound Tracking” tab, enter the following information:
895 ▪ Plate # - Enter the experiment ID or plate number into cell E1
896 ▪ Cell Lot # - Enter the passage or lot number of the cells used for this
897 experiment into cell C5
898 ▪ DMSO and Media Lot #'s – Enter the lot numbers for the DMSO and
899 Media in cells C6 and C7
900 ▪ Test Substance Code – Enter the test substance codes into cells C15 and
901 C16. Enter the test substance dilution into cells E25 and E26.
902 ▪ Name: Enter the experimenter name into cell G6
903 ▪ Date: Enter the experiment date in the format day\month\year into cell
904 G10
905 ▪ Comments: - Enter any comments about the experiment in this box (e.g.,
906 plate contaminated)
- 907 4. Enter substance testing concentrations to the “List” page. Also add any replicate-
908 specific comments on this page (e.g, spilled tube, etc.).
909 5. Save the newly named project file.
910 6. Print out either the “List” or “Template” page for help with dosing the 96-well
911 plate. Sign and date the print out and store in study notebook.

912 **11.2 Visual Evaluation of Cell Viability**

- 913 1. 19 to 24 hours after dosing the plate, remove the plate from the incubator and
914 remove the media from the wells by inverting the plate onto blotter paper. Gently
915 tap plate against the bench surface to remove residual liquid trapped in the wells.
916 2. Use a repeat pipetter to add 50 μ L 1X PBS to all wells. Immediately remove PBS
917 by inversion.
918 3. Using an inverted microscope, inspect all of the wells used in the 96-well plate
919 and record the visual observations using the scores in **Table 11-1**.
920

920 **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”

921 ¹Reference photomicrographs are provided in the BG1LUC4E2 ER TA Validation Study “Visual Observation Cell
922 Viability Manual.”

923

924 **11.3 Lysis of Cells for BG1LUC4E2 ER TA**

- 925 1. Apply the reflective white backing tape to the bottom of the 96-well plate (this
926 will increase the effectiveness of the luminometer).
- 927 2. Add 30 µL 1X lysis reagent to the assay wells and place the 96-well plate on an
928 orbital shaker for one minute.
- 929 3. Remove plate from shaker and measure luminescence (as described in **Section**
930 **11.4**).

931 **11.4 Measurement of Luminescence**

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

937 **11.5 Data Analysis**

938 BG1LUC4E2 ER TA uses an Excel[®] spreadsheet to collect and adjust the RLU values obtained
939 from the luminometer and a GraphPad Prism[®] template to analyze and graph data. The Excel[®]
940 spreadsheet subtracts background luminescence (average DMSO solvent control RLU value)
941 from test substance, reference standard and control RLU values. Plate induction is calculated
942 using these corrected RLU values. Test substance, reference standard, and control RLU values
943 are then adjusted relative to the highest E2 reference standard RLU value, which is set to 10,000.
944 After adjustment, values are transferred to GraphPad Prism[®] for data analysis and graphing.

945 **11.5.1 Collection and Adjustment of Luminometer Data for Range Finder Testing**

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

- 948 1. Open the raw data file and the corresponding experimental Excel® spreadsheet
949 from **Section 11.1.1**.
- 950 2. Copy the raw data using the Excel® copy function, then paste the copied data into
951 cell B19 of the “RAW DATA” tab in the experimental Excel® spreadsheet using
952 the **Paste Special – Values** command. This position corresponds to position A1 in
953 the table labeled Table 1 in this tab.
- 954 3. Examine the DMSO data in Table 1 of the Excel® spreadsheet to determine
955 whether there are any potential outliers. See **Section 11.6.2** for further explanation
956 of outlier determinations.
- 957 4. If an outlier is identified, perform the following steps to remove the outlier from
958 calculations:
- 959 ▪ correct the equation used to calculate DMSO background in Table 1 [e.g.,
960 if outlier is located in cell F26, adjust the calculation in cell H40 to read
961 =AVERAGE(G26:I26)]
 - 962 ▪ then correct the equation used to calculate the average DMSO value in
963 Table 2 [e.g., following the above example, adjust cell M42 to read
964 =AVERAGE(G26:I26)]
 - 965 ▪ then correct the equation used to calculate the standard deviation of the
966 DMSO value in Table 2 [e.g., following the above example, adjust cell
967 M43 to read =STDEV(G36:I36)]
- 968 5. Excel® will automatically subtract the background (the average DMSO control
969 value) from all of the RLU values in Table 1 and populate Table 2 with these
970 adjusted values.
- 971 6. To calculate plate induction, identify the cell containing the E2a replicate in Table
972 1, plate row H that has the highest RLU value (i.e., cell B26, C26, D26, or E26).
- 973 7. Click into cell D14 and enter the cell number from the previous step into the
974 numerator.
- 975 8. Identify the cell containing the E2b replicate in Table 1, plate row H that has the
976 highest RLU value (i.e., cell J26, K26, L26, or M26).
- 977 9. Click into cell E14 and enter the cell number from the previous step into the
978 numerator.
- 979 10. Click on the “ER Agonist Report” worksheet.
- 980 11. The data for the E2 reference standard, methoxychlor, and DMSO replicates
981 populate the left portion (columns A – F) of the spreadsheet. The data is
982 automatically placed in an Excel® graph.
- 983 12. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell
984 D2 of “ER Agonist Report” tab and check the formula contained within that cell.

985 The divisor should be the cell number of the cell containing the highest Mean E2
986 RLU value ((i.e., cell A16, A17, A18, or A19).

987 13. Open the “Visual Observation Scoring” worksheet. Enter the visual observation
988 scores for each well on the 96-well plate. This data will be linked to the “ER
989 Agonist Report” worksheet.

990 14. After the testing results have been evaluated and reviewed for quality control,
991 enter the following information into the Compound Tracking worksheet:

- 992 ▪ Enter pass/fail results for plate reference standard and control parameters
993 into the Plate Pass/Fail Table
- 994 ▪ Enter information from the testing of coded substances into the Testing
995 Results Table
- 996 ▪ Reviewer Name – Enter the name of the person who Reviewed\QC’ed the
997 data into cell A34
- 998 ▪ Date – Enter the date on which the data was reviewed into cell D34

999 11.5.2 Collection and Adjustment of Luminometer Data for Comprehensive Testing

1000 The following steps describe the procedures required to populate the Excel® spreadsheet that has
1001 been configured to collect and adjust the RLU values obtained from the luminometer.

- 1002 1. Open the raw data file and the corresponding experimental Excel® spreadsheet
1003 from **Section 11.1.2**.
- 1004 2. Copy the raw data using the Excel® copy function, then paste the copied data into
1005 cell B16 of the “RAW DATA” worksheet in the experimental Excel® spreadsheet
1006 using the **Paste Special – Values** command. This position corresponds to position
1007 A1 in the table labeled Table 1 in this worksheet.
- 1008 3. Fill out the table at the top of the “Raw Data” worksheet with information
1009 regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot.
1010 Meas. Time/Well (s), etc. If desired, this information can be added to the
1011 Laboratory Template File.
- 1012 4. Examine the DMSO data in Table 1 of the Excel® spreadsheet to determine
1013 whether there are any potential outliers. See **Section 11.6.2** for further explanation
1014 of outlier determinations.
- 1015 5. If an outlier is identified, perform the following steps to remove the outlier from
1016 calculations:
 - 1017 ▪ correct the equation used to calculate DMSO background in Table 1[e.g.,
1018 if outlier is located in cell M17, adjust the calculation in cell H37 to read
1019 =AVERAGE(M16,M18:M19)]

- 1020 ▪ then correct the equation used to calculate the DMSO mean and SD
1021 values [e.g., following the above example, adjust cell M39 to read
1022 = AVERAGE(M28,M30:M31), and adjust cell M40 to read
1023 =STDEV(M28,M30:M31)]
- 1024 6. Excel® will automatically subtract the background (the average DMSO control
1025 value) from all of the RLU values in Table 1 and populate Table 2 with these
1026 adjusted values.
- 1027 7. To calculate plate induction, identify the cell in containing the E2 replicate in
1028 Table 1, plate row G that has the highest RLU value.
- 1029 8. Click into cell D11 and enter the cell number from the previous step into the
1030 numerator.
- 1031 9. Identify the cell containing the E2 replicate in plate row H that has the highest
1032 RLU value.
- 1033 10. Click into cell E11 and enter the cell number from the previous step into the
1034 numerator.
- 1035 11. Open the “ER Agonist Report” worksheet.
- 1036 12. The data for the E2 reference standard, methoxychlor, and DMSO replicates
1037 populate the left portion (columns A – E) of the spreadsheet. The data is
1038 automatically placed in an Excel® graph.
- 1039 13. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell
1040 E2 of “ER Agonist Report” tab and check the formula contained within that cell.
1041 The divisor should be the cell number of the cell containing the highest Avg E2
1042 RLU value (cells A16 through A26).
- 1043 14. Open the “Visual Observation Scoring” worksheet. Enter the visual observation
1044 scores for each well on the 96-well plate. This data will be linked to the “ER
1045 Agonist Report” worksheet.
- 1046 15. Copy the data from the “ER Agonist Report” worksheet into GraphPad Prism® for
1047 the calculation of EC₅₀ values and to graph experimental results as indicated in the
1048 NICEATM Prism® Users Guide.
- 1049 16. After the testing results have been evaluated and reviewed for quality control,
1050 enter the following information into the Compound Tracking worksheet:
- 1051 ▪ Enter pass/fail results for plate reference standard and control parameters
1052 into the Plate Pass/Fail Table
- 1053 ▪ Enter information from the testing of coded substances into the Testing
1054 Results Table
- 1055 ▪ Reviewer Name – Enter the name of the person who Reviewed\QC’ed the
1056 data into cell A32

1057 ▪ Date – Enter the date on which the data was reviewed into cell D32

1058 11.5.3 Determination of Outliers

1059 The Study Director will use good statistical judgment for determining “unusable” wells that will
1060 be excluded from the data analysis and will provide an explanation in the study notebook for any
1061 excluded data. This judgment for data acceptance will include Q-test analysis.

1062 The formula for the Q test is:

1063 Outlier – Nearest Neighbor

Range (Highest – Lowest)

1064 where the outlier is the value proposed for exclusion, the nearest neighbor is the value closest to
1065 the outlier, and the range is the range of the three values (Q values for samples sizes from 3 to 10
1066 are provided in **Table 11-2**). For example, if the value of this ratio is greater than 0.94 (the Q
1067 value for the 90% confidence interval for a sample size of three) or 0.76 (the Q value for the 90%
1068 confidence interval for a sample size of four), the outlier may be excluded from data analysis.

1069 **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

1070
1071 For E2 reference standard replicates (sample size of two), any adjusted RLU value for a replicate
1072 at a given concentration of E2 is considered and outlier if its value is more than 20% above or
1073 below the adjusted RLU value for that concentration in the historical database.

1074 11.5.4 Acceptance Criteria

1075 11.5.4.1 *Range Finder Testing*

1076 Acceptance or rejection of a test is based on evaluation of reference standard and control results
1077 from each experiment conducted on a 96-well plate. Results are compared to quality controls
1078 (QC) for these parameters derived from the historical database, which are summarized below.

1079 • Induction: Plate induction, as measured by dividing the averaged highest E2
1080 reference standard RLU value by the averaged DMSO control RLU value, must
1081 be greater than three-fold.

1082 • DMSO control results: Solvent control RLU values must be within 2.5 times the
1083 standard deviation of the historical solvent control mean RLU value.

1084 An experiment that fails either acceptance criterion will be discarded and repeated.

1085 11.5.4.2 *Comprehensive testing*

1086 Acceptance or rejection of a test is based on evaluation of reference standard and control results
1087 from each experiment conducted on a 96-well plate. Results are compared to quality controls
1088 (QC) for these parameters derived from the historical database, which are summarized below.

1089 • Induction: Plate induction, as measured by dividing the averaged highest E2
1090 reference standard RLU value by the averaged DMSO control RLU value, must
1091 be greater than three-fold.

1092 • Reference standard results: The E2 reference standard concentration-response
1093 curve should be sigmoidal in shape and have at least three values within the linear
1094 portion of the concentration-response curve.

1095 • DMSO control results: DMSO control RLU values must be within 2.5 times the
1096 standard deviation of the historical solvent control mean RLU value.

1097 • Positive control results: Methoxychlor control RLU values must be above the line
1098 representing the DMSO mean plus three times the standard deviation from the
1099 DMSO mean.

1100 An experiment that fails any single acceptance criterion will be discarded and repeated.

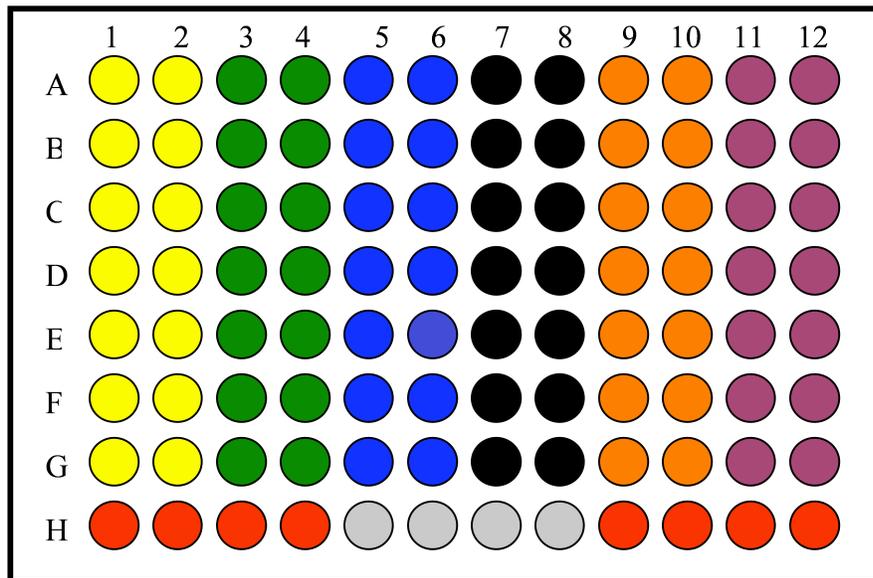
1101

1102 **12.0 RANGE FINDER TESTING**

1103 Agonist range finding for coded substances consists of seven point, 1:10 serial dilutions, with
1104 each concentration tested in duplicate wells of the 96-well plate. **Figure 12-1** contains a template
1105 for the plate layout to be used in agonist range finder testing.

1106

1106 **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**

1107

1108 Evaluate whether range finder experiments have met the acceptance criteria

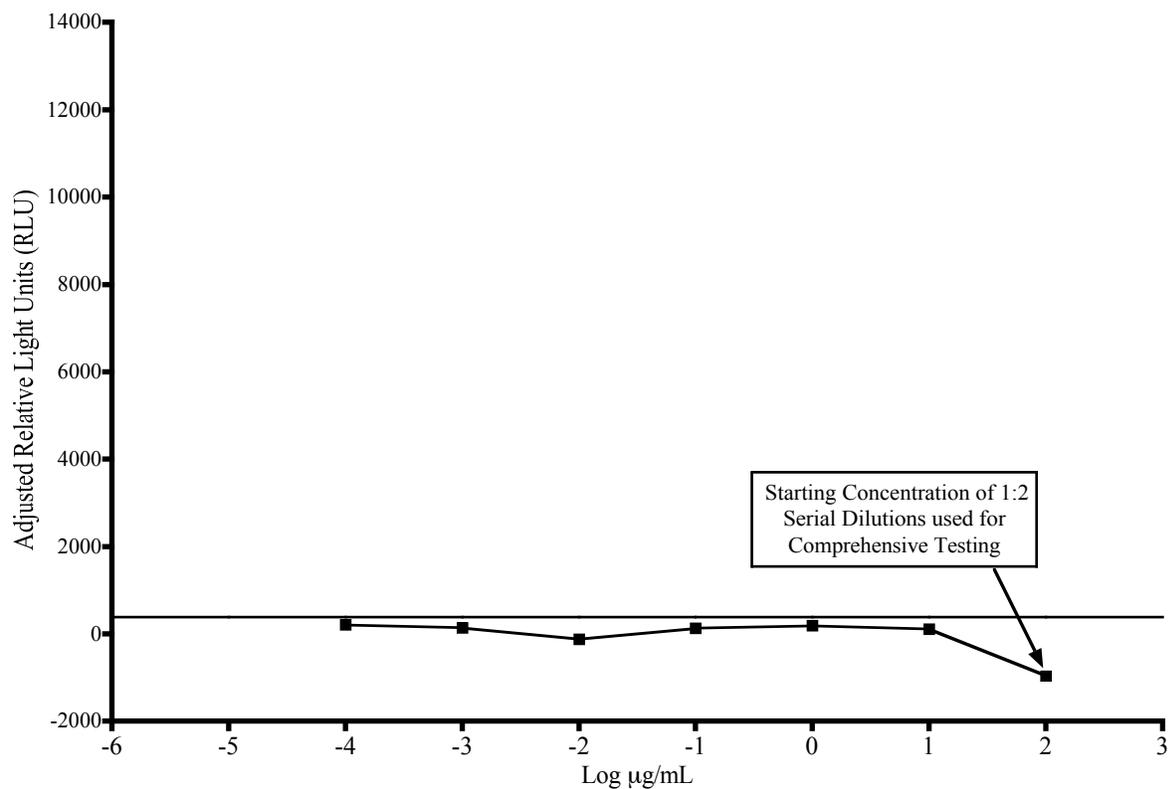
1109 (see **Section 11.5.4.1**).

1110 To determine starting concentrations for comprehensive testing use the following criteria:

- 1111 • If results in the range finder test suggest that the test substance is negative for
- 1112 agonist activity (i.e., if there are no points on the test substance concentration
- 1113 curve that are greater than the mean plus three times the standard deviation of the
- 1114 DMSO control, see **Figure 12-2**), comprehensive testing will be conducted using
- 1115 an 11 point 1:2 serial dilution starting at the maximum soluble concentration.

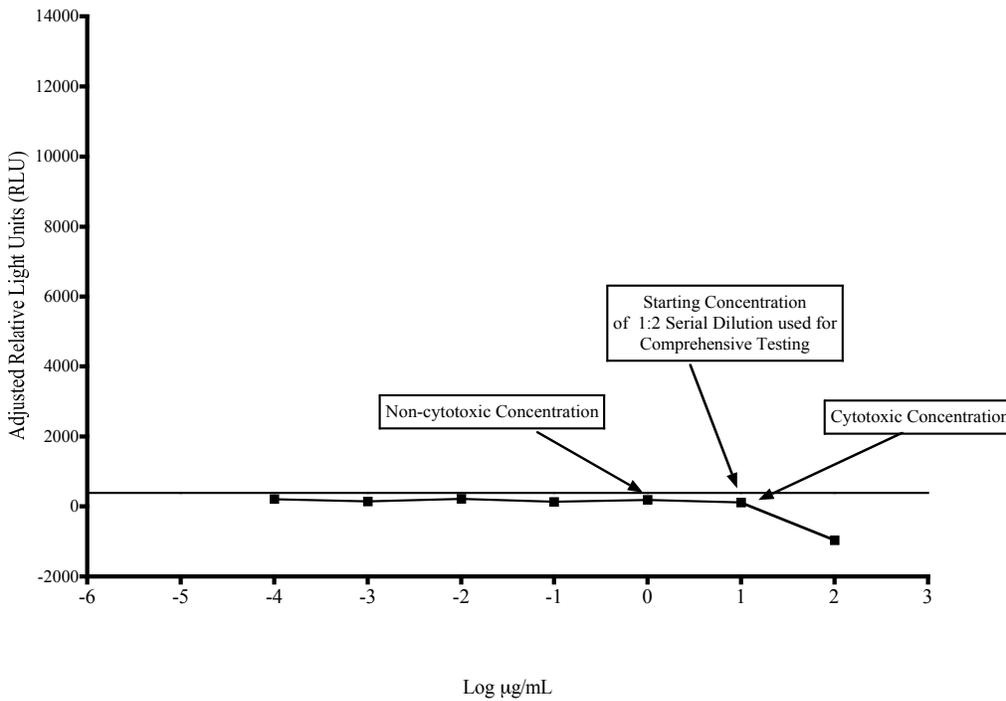
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- 1147
- If results in the range finder test suggest that the test substance is negative for agonist activity (i.e., if there are no points on the test substance concentration curve that are greater than the mean plus three times the standard deviation 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**.

1147 **Figure 12-2 Agonist Range Finder (example 1)**



1148
1149 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.
1150
1151

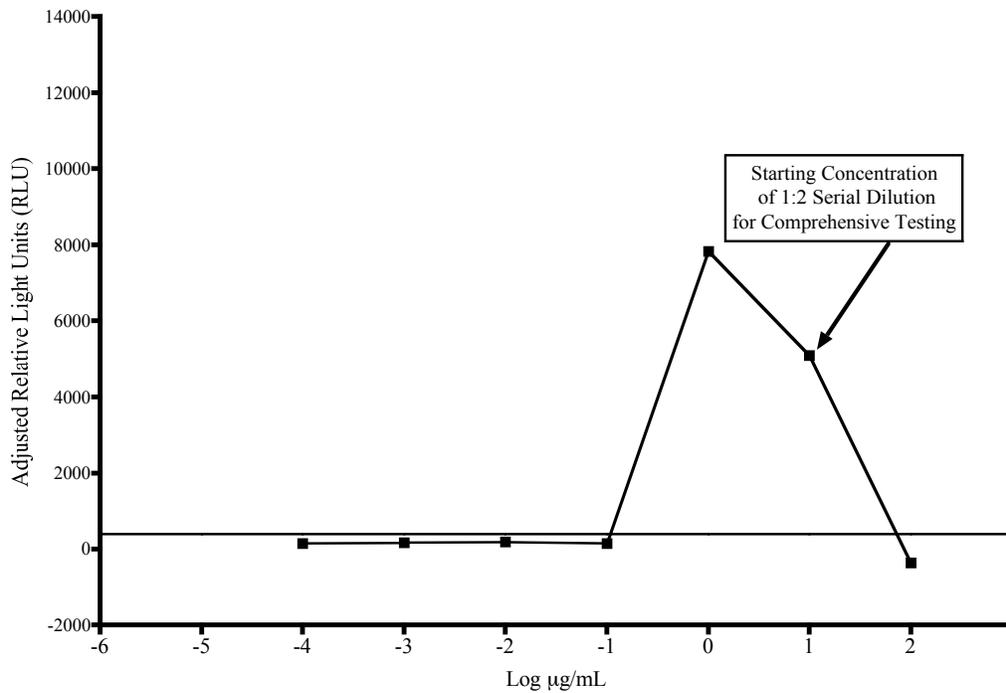
1151 **Figure 12-3 Agonist Range Finder (example 2)**



1152

1153 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

1154 **Figure 12-4 Agonist Range Finder (example 3)**

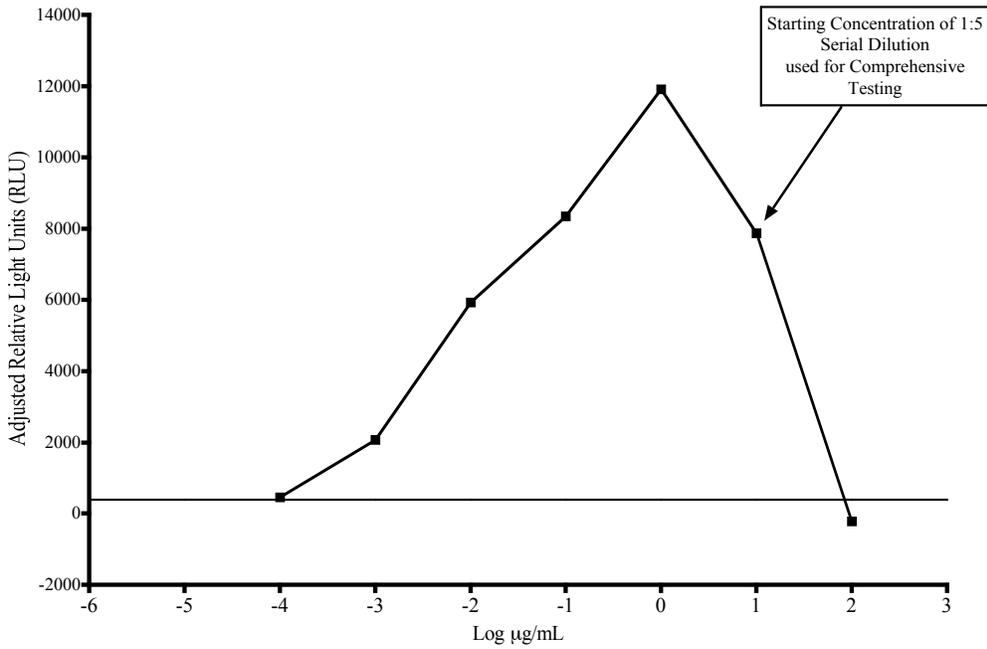


1155

1156 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

1157

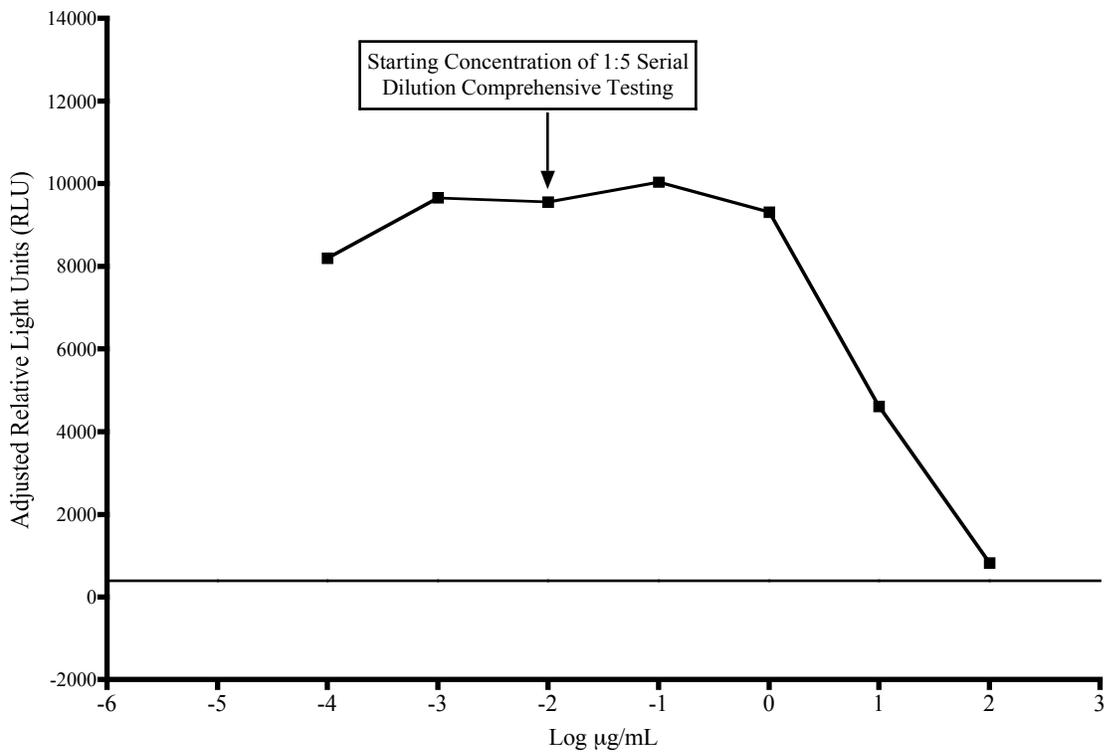
1157 **Figure 12-5 Agonist Range Finder (example 4)**



1158

1159 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

1160 **Figure 12-6 Agonist Range Finder (example 5)**

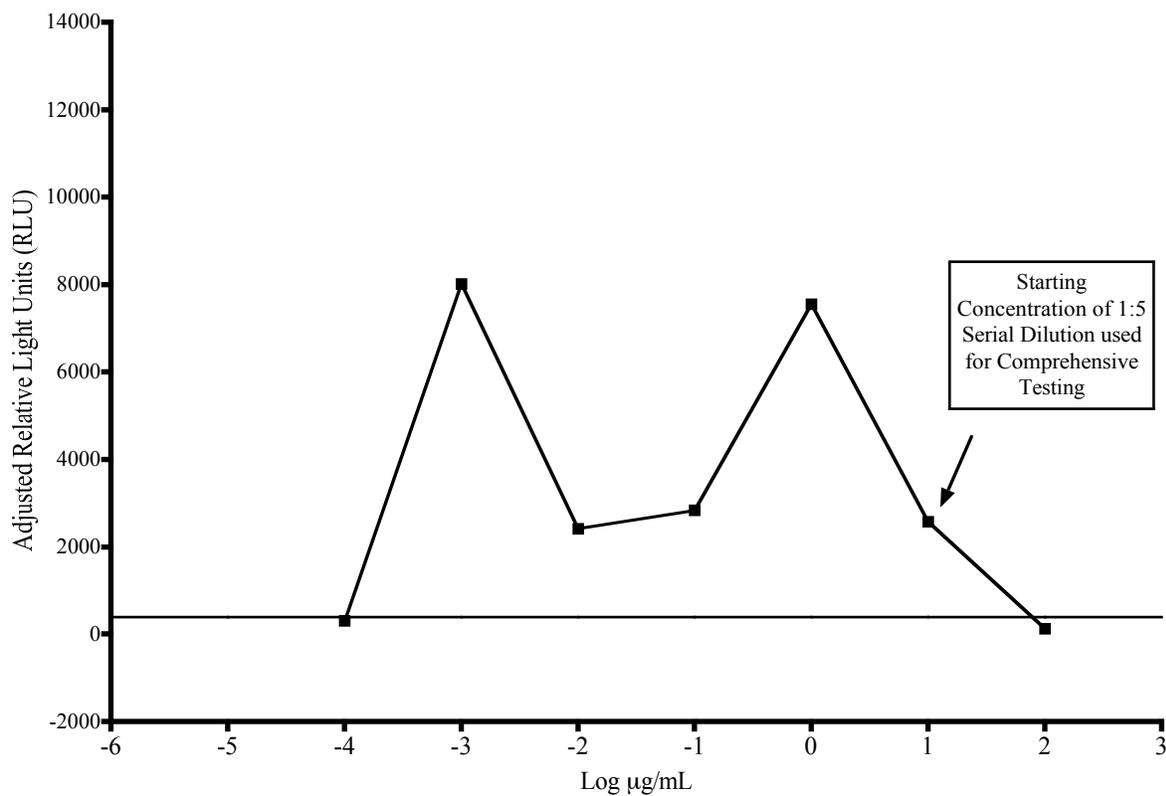


1161

1162 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

1163

1163 **Figure 12-7 Agonist Range Finder (example 6)**



1164
1165 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.
1166

1167 **13.0 COMPREHENSIVE TESTING**

1168 Agonist comprehensive testing for coded substances consists of 11 point serial dilutions (either
1169 1:2 or 1:5 serial dilutions based on the starting concentration for comprehensive testing criteria in
1170 **Section 12.0**) with each concentration tested in triplicate wells of the 96-well plate. **Figure 13-1**
1171 contains a template for the plate layout to be used in agonist comprehensive testing.
1172

1172 **Figure 13-1 Agonist Comprehensive Test Plate Layout**

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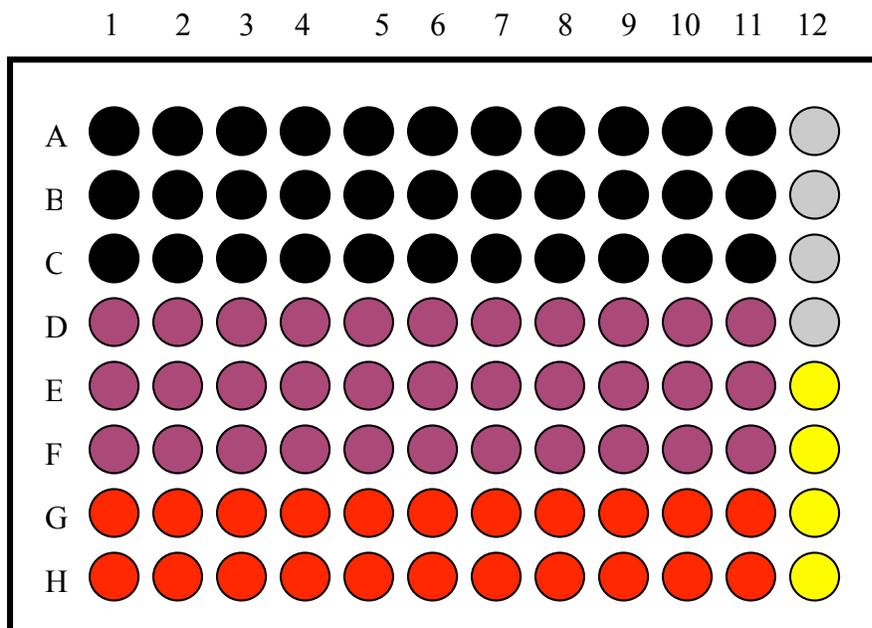
1191

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1195

- 11 Point Duplicate E2 Reference Standard
- DMSO (Solvent Control)
- Test Substance #1
- Test Substance #2
- Methoxychlor Control

1196

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

1198

1199

1200

1201

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1203

1204

- 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 greater than the mean plus three times the standard deviation of the DMSO control, the substance is considered negative for agonism
- If the substance has a positive response (See **Section 6.0**) at any concentration, the substance is considered positive for agonism.

1205

14.0 COMPILATION OF THE HISTORICAL QUALITY CONTROL DATABASE

1206

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

1207

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

1208 spreadsheets used to collect the data for individual test plates. Reference standard and control
1209 data are used to develop and maintain the historical database and are used as quality controls to
1210 determine acceptance of individual test plates.

1211 The sources of the data needed to compile the historical database for the DMSO control are the
1212 experiment specific Excel® data collection and analysis spreadsheets used for BG1LUC4E2 ER
1213 TA agonist and antagonist testing (see **Section 11.5.2** of the BG1LUC4E2 ER TA agonist
1214 protocol and **Section 13.5.2** in the BG1LUC4E2 ER TA antagonist protocol).

1215 **14.1 DMSO Control**

1216 Open the combined agonist and antagonist BG1LUC4E2 ER TA historical database Excel®
1217 spreadsheet (LUMI_AgandAntQC.xls) and save under a new name using the Excel® “Save As”
1218 function, adding the laboratory designator to the file name (e.g., for Laboratory H, the new name
1219 would be HLUMI_AgandAntQC.xls). Enter the date and experiment name into worksheet
1220 columns A and B respectively. Enter the experimental mean DMSO control value (from cell H37
1221 in the RAW DATA worksheet of the agonist and antagonist Excel® data collection and analysis
1222 spreadsheet) into worksheet column C. Acceptance or rejection of the plate DMSO control data
1223 for range finding and comprehensive testing is based on whether the mean plate DMSO RLU
1224 value falls within 2.5 times the standard deviation of the DMSO value in the historical database
1225 (columns G and H in the DMSO worksheet).

1226 **15.0 QUALITY TESTING OF MATERIALS**

1227 All information pertaining to the preparation and testing of media, media supplements, and other
1228 materials should be recorded in the Study Notebook.

1229 **15.1 Tissue Culture Media**

1230 Each lot of tissue culture medium must be tested in a single growth flask of cells before use in
1231 ongoing tissue culture or experimentation (**note:** each bottle within a given lot of
1232 Charcoal/Dextran treated FBS must be tested separately).

- 1233 1. Every new lot of media (RPMI and DMEM) and media components (FBS,
1234 Charcoal/Dextran treated FBS, and L-glutamine) must first be tested on the
1235 LUMI-CELL® ER assay prior to being used in any GLP acceptable assays.
- 1236 2. Add 4 µL of DMSO (previously tested) into four separate 13 mm tubes.
- 1237 3. Add 400 µL media (to be tested) to the same tubes.
- 1238 4. Dose an experimental plate as in **Section 12.0**, treating the media being tested as a
1239 test substance.
- 1240 5. Analyze 96-well plate as described in **Section 12.0**, comparing the data from the
1241 DMSO controls made using previously tested tissue culture media to the new
1242 media being tested.

- 1243 6. Use the agonist historical database to determine if the new media with DMSO lies
1244 within 2.5 standard deviations of the mean for the media. If the RLU values for
1245 the new media with DMSO lie within 2.5 standard deviation of the mean for the
1246 historical data on DMSO, the new lot of media is acceptable. If the RLU values
1247 for the new media with DMSO do not lie within 2.5 standard deviations of the
1248 DMSO mean from historical database, the new lot may not be used in the assay.
1249 7. Note date and lot number in study notebook.
1250 8. If the new bottle passes quality testing as described in **Section 15.1 step 6**, apply
1251 the media to a single flask of cells and observe cell growth and morphology over
1252 the following 2 – 3 days. If there is no change in growth or morphology, the new
1253 media is acceptable for use.

1254 **15.2 G418:**

- 1255 1. New lots of G418 must first be tested on the LUMI-CELL[®] ER assay prior to
1256 being used in any GLP acceptable assays.
1257 2. Add 220 µL of G418 (previously tested) to a single flask containing cells growing
1258 in RPMI.
1259 3. Add 220 µL of G418 (to be tested) to a different flask containing cells growing in
1260 RPMI.
1261 4. Observe cellular growth and morphology in both tissue culture flasks over a 48 to
1262 72 hour period. If there are no differences in observed growth rate and
1263 morphology between the two flasks, the new G418 lot is acceptable.
1264 5. If cellular growth is decreased, or the cells exhibit abnormal morphology, the new
1265 lot of G418 is not acceptable.
1266 6. Note date and lot number in study book.

1267 **15.3 DMSO**

- 1268 1. Every new bottle of DMSO must be tested on the LUMI-CELL[®] ER assay prior
1269 to use in any GLP acceptable assays.
1270 2. Add 4 µL of DMSO (to be tested) into four separate 13 mm tubes.
1271 3. Add 400 µL media (previously tested) to the same tubes.
1272 4. Dose an experimental plate as in **Section 12.0**, treating the DMSO containing
1273 media being tested as a test substance.
1274 5. Analyze 96-well plate as described in **Section 12.0**, comparing the data from the
1275 DMSO controls made using previously tested tissue culture media.
1276 6. Use the agonist historical database to determine if media with new DMSO lies
1277 within 2.5 standard deviations of the DMSO mean from historical database. If the
1278 RLU values for the media with new DMSO lie within 2.5 standard deviations of

1279 the DMSO mean from the historical database, the new lot of DMSO is acceptable.
1280 If the RLU values for media with new DMSO do not lie within 2.5 standard
1281 deviations of the DMSO mean from historical database, the new lot may not be
1282 used in the assay.

1283 7. Note the date, lot number, and bottle number in study book.

1284 8. If no DMSO has been previously tested, test several bottles as described in
1285 **Section 15.3**, and determine whether any of the bottles of DMSO have a lower
1286 average RLU than the other bottle(s) tested. Use the DMSO with the lowest
1287 average RLU for official experiments.

1288 **15.4 Plastic Tissue Culture Materials**

1289 1. Grow one set of cells, plate them for experiments on plastic ware from the new lot
1290 and one set of cells in the plastic ware from a previous lot, and dose them with E2
1291 reference standard and controls.

1292 2. Perform the BG1LUC4E2 ER TA experiment with both sets of cells.

1293 3. If all of the analysis falls within acceptable QC criteria, then the new
1294 manufacturer's products may be used.

1295 **16.0 REFERENCES**

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