LUMI-CELL® ER ASSAY

ANTAGONIST 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 2770413

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LIST OF ACRONYMS AND ABBREVIATIONS

Acronym / Abbreviation	Definition
13 mm test tube	13 x 100 mm glass test tubes
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl Sulfoxide
DMSO Control	1% v/v dilution of DMSO in tissue culture media used as a vehicle control
E2	17β-estradiol
E2 Control	2.5 x 10^{-5} μg/mL E2 used as a control.
IC_{50} Value	Concentration that produces a half-maximal response as calculated using the four parameter Hill function.
ER	Estrogen Receptor
Estrogen-free DMEM	DMEM (phenol red free), supplemented with 1 % Penicillin/Streptomycin, 2 % L-Glutamine, and 5% Charcoal-dextran treated FBS
FBS	Fetal Bovine Serum
Flavone/E2 Control	25 μg/mL flavone + 2.5 x 10^{-5} μg/mL E2, used as a weak positive control.
G418	Gentamycin
Ral/E2 Reference Standard	Nine point dilution of raloxifene HCl + 2.5 x 10^{-5} 17β-estradiol reference standard for the LUMI-CELL® ER antagonist assay
RPMI	RPMI 1640 growth medium
TA	Transcriptional Activation

160	T25	25 cm^2 tissue culture flask
161	T75	75 cm^2 tissue culture flask
162	T150	150 cm^2 tissue culture flask
163		

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197 **1.0 PURPOSE**

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

200 **2.0 SPONSOR**

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

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

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

224

224 David Allen, Ph.D.
225 Principal Investigator
226 ILS, Inc./Contractor supporting NICEATM
227 National Institute of Environmental Health Sciences
228 MD EC-17, P.O. Box 12233
229 Research Triangle Park, NC 27709
230 Phone: 919-316-4587
231 FAX: 919-541-0947
232 Email: allen7@niehs.nih.gov
233
234 Frank Deal, M.S.
235 Staff Toxicologist
236 ILS, Inc./Contractor supporting NICEATM
237 National Institute of Environmental Health Sciences
238 MD EC-17, P.O. Box 12233
239 Research Triangle Park, NC 27709
240 Phone: 919-316-4587
241 FAX: 919-541-0947
242 Email: dealf@niehs.nih.gov
243
244 Patricia Ceger, M.S.
245 Project Coordinator/Technical Writer
246 ILS, Inc./Contractor supporting NICEATM
247 National Institute of Environmental Health Sciences
248 MD EC-17, P.O. Box 12233
249 Research Triangle Park, NC 27709
250 Phone: 919-316-4556
251 Fax: 919-541-0947
252 E-Mail: cegerp@niehs.nih.gov
253
254

2.1 Substance Inventory and Distribution Management

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

3.0 DEFINITIONS

- **Dosing Solution:** The test substance, control substance, or reference standard solution which is to be placed into the tissue culture wells for experimentation.
- **Raw Data:** Raw data includes information that has been collected but not formatted or analyzed, and consists of the following:
  - Data recorded in the Study Notebook
  - Computer printout of initial luminometer data
  - Other data collected as part of GLP compliance, e.g.:
    - Equipment logs and calibration records
    - Test substance and tissue culture media preparation logs
    - Cryogenic freezer inventory logs
- **Soluble:** Test substance exists in a clear solution without visible cloudiness or precipitate.
- **Study Notebook:** The study notebook contains recordings of all activities related to the conduct of the LUMI-CELL® ER TA antagonist assay.
- **Test Substances:** Substances supplied to the testing laboratories that are coded and distributed such that only the Project Officer, Study Management Team (SMT), and the Substance Inventory and Distribution Management have knowledge of their true identity. The test substances will be purchased, aliquoted,

281 coded, and distributed by the Supplier under the guidance of the NIEHS/NTP
282 Project Officer and the SMT.

283 4.0 TESTING FACILITY AND KEY PERSONNEL[1]

284 4.1 Testing Facility

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

286 4.2 Key Personnel

287 • Study Director: John Gordon, Ph.D.

288 • Quality Assurance Director: Mr. Carlos Daniel

289 5.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

290 5.1 Test Substances

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

293 5.2 Controls

294 Controls for the ER antagonist protocol are as follows:

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

297 *Ral/E2 reference standard for range finder testing*: Three concentrations (1.56×10^{-3},
298 3.91×10^{-4}, and 9.77×10^{-5} μg/mL) of raloxifene HCl (Ral), CASRN 84449-90-1, plus a fixed
299 concentration (2.5×10^{-5} μg/mL) of 17β-estradiol (E2), CASRN: 50-28-2, in duplicate wells.

300 *Ral/E2 reference standard for comprehensive testing*: A serial dilution of Ral plus a fixed
301 concentration (2.5×10^{-5} μg/mL) of E2 consisting of nine concentrations of Ral/E2 in duplicate
302 wells.

---

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

303 *E2 control*: *17β-estradiol*, 2.5 x 10^{-5} μg/mL E2 in tissue culture media used as a base line
304 negative control.

305 *Flavone/E2 Control*: Flavone, CASRN 525-82-6, 25 μg/mL, with 2.5 x 10^{-5} μg/mL E2 in tissue
306 culture media used as a weak positive control.

307 6.0 OVERVIEW OF GENERAL PROCEDURES FOR ANTAGONIST TESTING

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

311 Antagonist range finder testing is conducted on 96-well plates using three concentrations of
312 Ral/E2 (1.56 x 10^{-3}, 3.91 x 10^{-4}, and 9.77 x 10^{-5} μg/mL Ral) with 2.50 x 10^{-5} μg/mL E2) in
313 duplicate as the reference standard, with three replicate wells for the E2 and DMSO controls.

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

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

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

319 [1]Concentrations are presented in μg/mL.
320

321 Visual observations for cell viability are conducted for all experimental plates just prior to
322 LUMI-CELL® ER evaluation, as outlined in **Section 11.4**.

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

328 • A response is considered positive for antagonist activity when the average
329 adjusted RLU for a given concentration is less than the mean RLU value minus
330 three times the standard deviation for the E2 control.

331 • Any luminescence at or above this threshold is considered a negative response.

332 For substances that are positive at one or more concentrations, the concentration of test substance
333 that causes a half-maximal response (the relative IC_{50}) is calculated using a Hill function
334 analysis. The Hill function is a four-parameter logistic mathematical model relating the
335 substance concentration to the response (typically following a sigmoidal curve) using the
336 equation below

337 $$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{logIC50} - X)\text{HillSlope}}}$$

338 where Y = response (i.e., relative light units); X = the logarithm of concentration; Bottom = the
339 minimum response; Top = the maximum response; log IC_{50} = the logarithm of X as the response
340 midway between Top and Bottom; and HillSlope describes the steepness of the curve. The model
341 calculates the best fit for the Top, Bottom, HillSlope, and IC_{50} parameters. See **Section 13.6.5** for
342 more details.

343 Acceptance or rejection of a test is based on evaluation of reference standard and control results
344 from each experiment conducted on a 96-well plate. Results for these controls are compared to
345 historical results compiled in the historical database, as seen in **Section 16.0**.

346 **6.1 Range Finder Testing**

347 Antagonist range finding for coded substances consists of a seven-point 1:10 serial dilution using
348 duplicate wells per concentration. Concentrations for comprehensive testing are selected based

on the response observed in range finder testing. If necessary, a second range finder test can be conducted to clarify the optimal concentration range to test (see **Section 14.0**).

6.2 Comprehensive Testing

Comprehensive antagonist testing for coded substances consists of 11-point serial dilutions, with each concentration tested in triplicate wells of the 96-well plate. Three separate experiments are conducted for comprehensive testing on three separate days, except during Phases III and IV of the validation effort, in which comprehensive testing experiments are conducted once (see **Section 15.0**).

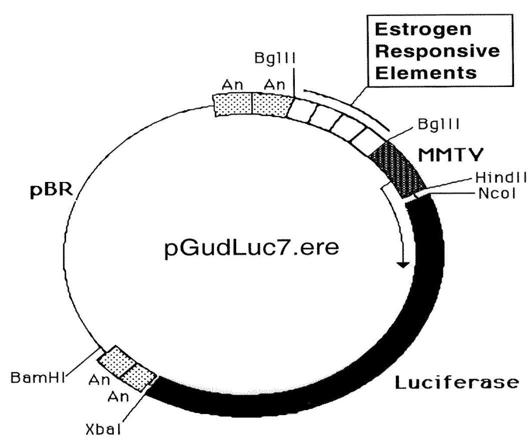
7.0 MATERIALS FOR LUMI-CELL® ER ANTAGONIST TESTING

This section provides the materials needed to conduct LUMI-CELL® ER testing, with associated brand names/vendors[2] in brackets.

7.1 BG1Luc4E2 Cells:

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

Figure 7-1 pGudLuc7.ERE Plasmid.



[2]Brand 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.

365 **7.2 Technical Equipment:**

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

369 - Analytical balance (Cat. No. 01-910-320)
370 - Berthold Orion 1 Microplate Luminometer [Berthold CatNo.: Orion 1 MPL3] or
371 equivalent and dedicated computer
372 - Biological safety hood, class II, and stand (Cat. No. 16-108-99)
373 - Centrifuge (low speed, tabletop with swinging bucket rotor) (Cat. No. 04-978-50
374 centrifuge, and 05-103B rotor)
375 - Combustion test kit (CO_2 monitoring) (Cat. No. 10-884-1)
376 - Drummond diaphragm pipetter (Cat. No. 13-681-15)
377 - Freezers, –20°C (Cat. No. 13-986-150), and –70°C (Cat. No. 13-990-86)
378 - Hand tally counter (Cat. No. 07905-6)
379 - Hemocytometer, cell counter (Cat. No. 02-671-5)
380 - Light microscope, inverted (Cat. No. 12-561-INV)
381 - Light microscope, upright (Cat. No. 12-561-3M)
382 - Liquid nitrogen flask (Cat. No. 11-675-92)
383 - Micropipetter, repeating (Cat. No. 21-380-9)
384 - Pipetters, air displacement, single channel (0.5 –10µl (Cat. No. 21-377-191), 2 –
385 20 µl (Cat. No. 21-377-287), 20 – 200 µl (Cat. No. 21-377-298), 200 - 1000 µl
386 (Cat. No. 21-377-195))
387 - Refrigerator/freezer (Cat. No. 13-986-106A)
388 - Shaker for 96-well plates (Cat. No. 14-271-9)
389 - Sodium hydroxide (Cat. No. 5318-500)

390 • Sonicating water bath (Cat. No. 15-335-30)

391 • Tissue culture incubator with CO_2 and temperature control (Cat. No. 11-689-4)

392 • Vacuum pump with liquid trap (side arm Erlenmeyer) (Cat. No. 01-092-29)

393 • Vortex mixer (Cat. No. 12-814)

394 Equipment should be maintained and calibrated as per GLP guidelines and individual laboratory
395 SOPs.

396 **7.3 Reference Standard, Controls, and Tissue Culture Supplies**

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

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

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

404 • BackSeal-96/384, white adhesive bottom seal for 96-well and 384-well microplate
405 [Perkin-Elmer, Cat. No. 6005199]

406 • 17 β-estradiol (CAS RN: 50-28-2) [Sigma-Aldrich, Cat. No. E8875]

407 • CellTiter-Glo® Luminescent Cell Viability Assay [Promega Cat. No. G7572]

408 • Cryovial, 2 mL (Corning Costar) [Fisher Scientific Cat. No. 03-374-21]

409 • Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 10009186R38][3]

410 • Culture tube, 50 mL conical (Corning Costar) [Fisher Scientific Cat. No. 05-
411 526C]

412 • DMSO, U.S.P. analytical grade. [Sigma-Aldrich, Cat. No. 34869-100ML]

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

413 - Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L
414 glucose, with sodium pyruvate, without phenol red or L-glutamine
415 [Mediatech/Cellgro, Cat. No. 17-205-CV]
416 - Fetal Bovine Serum [Mediatech/Cellgro Cat. No. MT 35-010-CV]
417 - Fetal Bovine Serum, charcoal/dextran treated, triple 0.1 μm sterile filtered
418 [Hyclone, Cat. No. SH30068.03]
419 - Flavone (CASRN: 525-82-6) [Sigma-Aldrich, Cat. No. F2003]
420 - Gentamycin Sulfate (G418), 50 mg/mL [Mediatech/Cellgro Cat. No. 30-234-CR]
421 - L-glutamine, 29.2 mg/mL [Cellgro, Cat. No. 25005-CI]
422 - Luciferase Assay System (10-Pack) [Promega Cat. No. E1501]
423 - Lysis Solution 5X [Promega, Cat. No. E1531]
424 - Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 μg/mL streptomycin
425 [Cellgro, Cat. No. 30-001-CI].
426 - Phosphate buffered saline (PBS, 1X) without calcium and magnesium [Cellgro,
427 Cat. No. 21-040-CV]
428 - Pipettes, serological: 2.0 mL [Sigma-Aldrich, Cat. No. P1736], 5.0 mL [Sigma-
429 Aldrich, Cat. No. P1986], 25 mL [Sigma-Aldrich, Cat. No. P2486]
430 - Raloxifene (CASRN 84449-90-1) [Sigma-Aldrich Cat. No. R1402]
431 - RPMI 1640 medium, containing L-glutamine [Mediatech, Cat. No. 10-040-CV]
432 - Tissue culture flasks (Corning-Costar): 25 cm^2 (T25) [Fisher Cat. No. 10-126-28];
433 75 cm^2 (T75) [Fisher Cat. No. 10-126-37]; and 150 cm^2 (T150) [Fisher Cat. No.
434 10-126-34]
435 - Tissue culture plates (Corning-Costar): 96-well [Thomas Scientific Cat. No.
436 6916A05]
437 - Trypsin (10X), 2.5% in Hank's balanced salt solution (HBSS), without calcium
438 and magnesium, without phenol red [Cellgro, Cat. No. 25-054-CI].

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

440 8.0 PREPARATION OF TISSUE CULTURE MEDIA AND SOLUTIONS

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

443 8.1 RPMI 1640 Growth Medium (RPMI)

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

446 Procedure for one 549 mL bottle:

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

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

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

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

453 8.2 Estrogen-Free DMEM Medium

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

456 Procedure for one 539 mL bottle:

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

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

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

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

464 **8.3 1X Trypsin Solution**

465 1X Trypsin solution is prepared by dilution from a 10X premixed stock solution. The 10X stock
466 solution should be stored in 10 mL aliquots in a -20°C freezer.

467 Procedure for making 100 mL of 1X trypsin:

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

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

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

473 *1X Trypsin should be stored at -20°C.*

474 **8.4 1X Lysis Solution**

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

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

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

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

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

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

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

483 **8.5 Reconstituted Luciferase Reagent**

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

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

488 To reconstitute luciferase reagent:

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

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

493 3. Luciferase substrate should readily go into solution.

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

495 5. Store complete solution at –20°C.

496 Reconstituted luciferase reagent is stable for 1 month at –20°C.

497 **9.0 OVERVIEW OF PROPAGATION AND EXPERIMENTAL PLATING OF**
498 **BG1Luc4E2 CELLS**

499 The BG1Luc4E2 (BG-1) cells are stored in liquid nitrogen in 2 mL cryovials. BG-1 cells are
500 grown as a monolayer in tissue culture flasks in a dedicated tissue culture incubator at 37ºC ±
501 1ºC, 90% ± 5% humidity, and 5.0% ± 1% CO_2/air. The cells should be examined on a daily basis
502 during working days under an inverted phase contrast microscope, and any changes in
503 morphology and adhesive properties must be noted in the study notebook.

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

506 **9.1 Procedures for Thawing Cells and Establishing Tissue Cultures**

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

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

511 9.1.1 Thawing Cells

512 1. Remove a cryovial of frozen BG-1 cells from the liquid nitrogen flask.

513 2. Facilitate rapid thawing by loosening the top slightly (do not remove top) to
514 release trapped gasses and retightening it. Roll vial between palms.

515 3. Use a micropipette to transfer cells to a 50 mL conical centrifuge tube.

516 4. Rinse cryovial twice with 1X PBS and add PBS rinse material to the conical tube.

517 5. Add 20 mL of RPMI to the conical tube.

518 6. Centrifuge at 1000 x *g* for eight min. If a pellet of cells has not formed, centrifuge
519 for an additional 5 minutes.

520 7. Aspirate media from pellet and re-suspend it in 5 mL RPMI, drawing the pellet
521 repeatedly through a 1.0 mL serological pipette to break up any clumps of cells.

522 8. Transfer cells to a T25 flask, place them in an incubator (see conditions in
523 **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

524 9.1.2 Establishing Tissue Cultures

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

527 1. Remove the T25 flask from the incubator.

528 2. Aspirate the RPMI, then add 5 mL 1X PBS, making sure that the cells are coated
529 with PBS.

530 3. Aspirate 1X PBS, then add 1 to 2 mL 1X trypsin to the T25 flask, gently swirling
531 the flask to coat all cells with the trypsin.

532 4. Place the flask in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.

533 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the
534 hand.

535 6. Confirm cell detachment by examination under an inverted microscope. If cells
536 have not detached, return the flask to the incubator for an additional 2 minutes,
537 then hit the flask again.

538 7. After cells have detached, add 5 mL PBS, and transfer the suspended cells to a 50
539 mL centrifuge tube. Wash the flask one additional time with 5 mL PBS.

540 8. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular
541 digestion by residual trypsin.

542 9. Pellet the cells by centrifugation, as described in **Section 9.1.1**, and re-suspend the
543 cells in 10 mL RPMI medium.

544 10. Draw the pellet repeatedly through a 25 mL serological pipette to break up
545 clumps of cells

546 11. Transfer cells to a T75 flask, then place the flask in an incubator (see conditions
547 in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

548 When cells have reached 80% to 90% confluency, transfer them into a T150 flask by performing,
549 for example, the following steps:

550 12. Remove the T75 flask from the incubator, aspirate the old media and add 5 mL
551 1X PBS.

552 13. Aspirate 1X PBS, add 2 mL of 1X trypsin to the flask, and place it in an incubator
553 (see conditions in **Section 9.0**) for 5 to 10 min.

554 14. Repeat **steps 5** through **11** in **Section 9.1.2,** re-suspending the pellet in 20 mL of
555 RPMI.

556 15. Transfer cells to a T150 flask and place it in the incubator (see conditions in
557 **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

558 16. Remove the T150 flask from the incubator.

559 17. Aspirate the RPMI and add 5 mL 1X PBS.

560 18. Aspirate 1X PBS and add 3 mL 1X trypsin to the T150 flask, making sure that the
561 cells are coated with the trypsin.

562 19. Incubate cells in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.

563 20. Detach cells by hitting the side of the flask sharply against the palm or heel of the
564 hand.

565 21. Confirm cell detachment by examination under an inverted microscope. If cells
566 have not detached, return the flask to the incubator for an additional 2 minutes,
567 then hit the flask again.

568 22. After cells have detached, add 5mL 1X PBS and transfer the suspended cells from
569 the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the flask,
570 then transfer to the 50 mL conical tube.

571 23. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular
572 digestion by residual trypsin.

573 24. Centrifuge at 1000 x *g* for eight minutes. If a pellet of cells has not formed,
574 centrifuge for an additional 5 minutes.

575 25. Aspirate the media from the pellet and re-suspend it in 40 mL RPMI, drawing the
576 pellet repeatedly through a 25 mL serological pipette to break up any clumps of
577 cells.

578 26. Transfer 20 mL of cell suspension to each of two T150 flasks, place them in an
579 incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence
580 (approximately 48 to 72 hrs).

581 **9.2 Ongoing Tissue Culture Maintenance, Conditioning in Estrogen-free Medium,**
582 **and Plating Cells for Experimentation**

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

586
587 To start the tissue culture maintenance and estrogen-free conditioning, split the two T150 culture
588 flasks into four T150 flasks. Two of these flasks will be used for continuing tissue culture and
589 will use the RPMI media mentioned above. The other two flasks will be cultured in estrogen-free
590 DMEM for experimental use. Extra care must be taken to avoid contaminating the estrogen-free
591 cells with RPMI.

592 1. Remove both T150 flasks from the incubator.

593 2. Aspirate the medium and rinse the cells with 5 mL 1X PBS.

594 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask
595 to coat all cells with the trypsin.

596 4. Incubate cells in the incubator (see conditions in **Section 9.0**) for 5 to 10 min.

597 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the
598 hand.

599 6. Confirm cell detachment by examination under an inverted microscope. If cells
600 have not detached, return the flask to the incubator for an additional 2 minutes,
601 then hit the flask again.

602 7. After cells have detached, add 5 mL 1X PBS to the first T150 flask and transfer
603 the suspended cells to the second T150 flask.

604 8. Transfer the contents of both flasks to a 50 mL conical tube. Repeat step 7 with an
605 additional 5 mL 1X PBS and transfer to the 50 mL conical tube.

606 9. Immediately add 20 mL estrogen-free DMEM to the 50 mL conical tube to inhibit
607 further cellular digestion by residual trypsin.

608 10. Centrifuge at 1000 x *g* for eight minutes. If a pellet of cells has not formed,
609 centrifuge for an additional 5 minutes.

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

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

615 9.2.1 Ongoing Tissue Culture Maintenance

616 1. Add 20 mL RPMI to two T150 flasks.

617 2. Add 220 µL G418 to the RPMI in the T150 flasks

618 3. Add 1 mL of cell suspension from **Section 9.2 step 11** to each flask.

619 4. Place T150 flasks in tissue culture incubator (see conditions in **Section 9.0**) and
620 grow to 80% to 90% confluence (approximately 48 to 72 hrs).

621 5. Tissue culture medium may need to be changed 24 hours after addition of G418 to
622 remove cells that have died because they do not express reporter plasmid.

623 6. G418 does not need to be added to the flasks a second time.

624 7. Repeat **Section 9.2 steps 1-11** for ongoing tissue culture maintenance.

625 9.2.2 Conditioning in Estrogen-free Medium

626 1. Add 20 mL estrogen-free DMEM to two T150 flasks.

627 2. Add 150 μL G418 to the estrogen-free DMEM in the T150 flasks.

628 3. Add 1 mL of cell suspension from **Section 9.2 step 11** to each flask.

629 4. Tissue culture medium may need to be changed 24 hours after addition of G418 to
630 remove cells that have died because they do not express reporter plasmid.

631 5. G418 does not need to be added to the flasks a second time.

632 6. Place the T150 flasks in the incubator (see conditions in **Section 9.0**) and grow to
633 80% to 90% confluence (approximately 48 to 72 hrs).

634 9.2.3 Plating Cells Grown in Estrogen-free DMEM for Experimentation

635 1. Remove the T150 flasks that have been conditioned in estrogen-free DMEM for
636 48 to 72 hours from the incubator.

637 2. Aspirate the medium, then rinse the cells with 5 mL 1X PBS.

638 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask
639 to coat all cells with the trypsin.

640 4. Place the flasks in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.

641 5. Detach cells by hitting the side of the flask sharply against the palm or the heel of
642 the hand.

643 6. Confirm cell detachment by examination under an inverted microscope. If cells
644 have not detached, return the flask to the incubator for 2 additional minutes, then
645 hit the flask again.

646 7. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells
647 from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the
648 flask, then transfer to the 50 mL conical tube.

649 8. Immediately add 20 mL estrogen-free DMEM to each conical tube to inhibit
650 further cellular digestion by residual trypsin.

651 9. Centrifuge at 1000 x *g* for eight minutes. If a pellet of cells has not formed,
652 centrifuge for an additional 5 minutes.

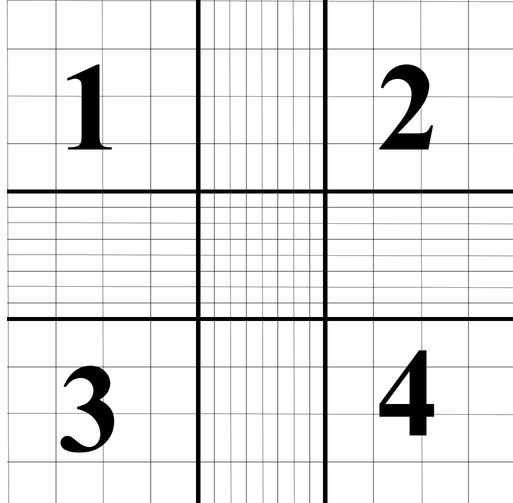
653 10. Aspirate off the media from the pellet and re-suspend it in 20 mL DMEM,
654 drawing the pellet repeatedly through a 25 mL serological pipette to break up any
655 clumps of cells.

656 11. Pipette 15 µL of the cell suspension into the "v" shaped slot on the
657 hemocytometer. Ensure that the solution covers the entire surface area of the
658 hemocytometer grid, and allow cells to settle before counting.

659 12. Using 100x magnification, view the counting grid.

660 13. The counting grid on the hemocytometer consists of nine sections, four of which
661 are counted (upper left, upper right, lower left, and lower right, see **Figure 9-1**).
662 Each section counted consists of four by four grids. Starting at the top left and
663 moving clockwise, count all cells in each of the four by four grids. Some cells
664 will be touching the outside borders of the square, but only count those that touch
665 the top and right borders of the square. This value is then used in the calculation
666 below to get to the desired concentration of 200,000 cells/mL.

Figure 9-1 Hemocytometer Counting Grid.



667

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

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

670 **Starting dilution: 20mL (for T150 flasks)**

671

672 Harvested cells for a T150 flask are suspended in 20 mL of estrogen-free DMEM and sampled

673 for determination of concentration of cells/mL.

674

675 Example Calculation:

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

677 - 50, 51, 49, and 50: average number of cells per grid is equal to 50.

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

679 cells/mL)

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

681 Desired Concentration (or Concentration $_{Final}$)= 200,000 cells/mL

682 Formula: (Concentration $_{Final}$ x Volume $_{Final}$ = Concentration $_{Initial}$ x Volume $_{Initial}$)

683 Concentration $_{Final}$ = 200,000 cells/mL

684 Concentration $_{Initial}$ = 500,000 cells/mL

685 Volume $_{Initial}$ = 20 mL

686 Volume $_{Final}$ – to be solved for.

687 Therefore: 200,000 cells/mL x Volume $_{Final}$ = 500,000 cells/mL x 20 mL

688 Solving for Volume $_{Final}$ we find = 50 mL

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

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

691 14. This dilution scheme will give a concentration of 200,000 cells/mL. 200 µL of

692 this cell suspension is used for each well of a 96-well plate (i.e., 40,000 cells per

693 well).

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

698 16. Incubate plate(s) in an incubator (see conditions in **Section 9.0**) for a minimum of
699 24 hours, but no longer than 48 hours before dosing.

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

702 **10.0 PREPARATION OF TEST SUBSTANCES**

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

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

710 **10.1 Determination of Test Substance Solubility**

711 1. Prepare a 200 mg/mL solution of the test substance in 100% DMSO in a 4 mL
712 conical tube.

713 2. Vortex to mix.

714 3. If the test substance does not dissolve at 200 mg/mL, prepare a 20 mg/mL
715 solution and vortex as above.

716 4. If the test substance does not dissolve at 20 mg/mL solution, prepare a 2 mg/mL
717 solution in a 4 mL conical tube and vortex as above.

718 5. If the test substance does not dissolve at 2 mg/mL, prepare a 0.2 mg/mL solution
719 in a 4 mL conical tube and vortex as above.

720 6. Continue testing, using 1/10 less substance in each subsequent attempt until test
721 substance is solubilized in DMSO.

722 Once the test substance has fully dissolved in 100% DMSO, the test substance is ready to be
723 used for LUMI-CELL® ER testing.

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

726 **11.0 PREPARATION OF REFERENCE STANDARD, CONTROL AND TEST**
727 **SUBSTANCE STOCK SOLUTIONS FOR RANGE FINDER AND**
728 **COMPREHENSIVE TESTING**

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

731 **11.1 Preparation of Ral/E2 Stock Solutions**

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

734 11.1.1 E2 Stock Solution

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

737 **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 x 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 x 10^{-3} µg/mL

738 11.1.2 Raloxifene Stock Solution

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

740 **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

741

742 **11.2 Ral/E2 Range Finder Testing Stock**

743 11.2.1 Raloxifene Dilutions

744 Number three 4 mL vials with the numbers 1 to 3 and use the raloxifene solution prepared in

745 **Section 11.1.2** to make raloxifene dilutions as shown **Table 11-3**.

746 **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

747

748 11.2.2 Preparation of Ral/E2 Range Finder Working Stocks:

749 Label three 4 mL conical tubes with numbers 1 through 3 and add 500 µL of the 5×10^{-3} µg/mL

750 E2 solution prepared in **Section 11.1.1** to each tube. Add 500 µL of the 3.13×10^{-1}, 7.81×10^{-2} ,

751 and 1.95×10^{-2} μg/mL raloxifene solutions prepared in **Section 11.2.1** to tubes 1, 2, and 3
752 respectively. Vortex each tube to mix. The final concentrations for raloxifene and E2 are listed in
753 **Table 11-4**.

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

Tube #	Raloxifene (μg/ml)	E2 (μg/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}

756

757 **11.3 Ral/E2 Comprehensive Testing Stock**

758 11.3.1 Raloxifene Dilutions
759 Use the raloxifene solution prepared in **Section 11.1.2** to make a nine-point serial dilution of
760 raloxifene as shown **Table 11-5**.

761 **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

Step #	Action	DMSO	Discard	Raloxifene Concentration
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

762

763 11.3.2 Preparation of Ral/E2 Comprehensive Testing Working Stocks:

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

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

Tube #	Raloxifene (µg/mL)	E2 (µg/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}

768

769 **11.4 Flavone/E2 Stock Solution**

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

771 1. Prepare 1 mL of 5 mg/mL flavone

772 2. Add 1 mL of the 5×10^{-3} µg/mL E2 (prepared as in **Section 11.1.1**) to the 10
773 mg/mL flavone. This will make a working solution of 2.5 mg/mL flavone with
774 2.5×10^{-3} µg/mL E2.

775 **12.0 PREPARATION OF REFERENCE STANDARD, CONTROL AND TEST**
776 **SUBSTANCE DOSING SOLUTIONS FOR RANGE FINDER AND**
777 **COMPREHENSIVE TESTING**

778 **12.1 Preparation of Reference Standard and Control Dosing Solutions for Range**
779 **Finder Testing**

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

783 All "dosing solutions" of test substance concentrations are to be expressed as μg/mL in the study
784 notebook and in all laboratory reports.

785 Dosing solutions are to be used within 24 hours of preparation.

786 12.1.1 Preparation of Ral/E2 Reference Standard Range Finder Dosing Solutions
787 1. Label three 13 mm glass tubes with the numbers 1 to 3.
788 2. Add 6 μL of Ral/E2 stock from tube #1 from **Section 11.2.2** to the 13 mm glass
789 test tube labeled #1.
790 3. Add 6 μL of Ral/E2 stock from tube #2 from **Section 11.2.2** to the 13 mm glass
791 test tube labeled #2. Repeat for tube #3.
792 4. Add 600 μL of estrogen-free DMEM to each tube and vortex.

793 12.1.2 Preparation of DMSO Control Range Finder Dosing Solution
794 1. Add 8 μL of 100% DMSO to a 13 mm glass test tube.
795 2. Add 800 μL of estrogen-free DMEM to each tube and vortex.

796 12.1.3 Preparation of E2 Control Range Finder Dosing Solution
797 1. Add 4 μL of the E2 stock from **Section 11.1.1** to a 13 mm glass test tube.
798 2. Add 4 μL of 100% DMSO to the tube.
799 3. Add 800 μL of estrogen-free DMEM to the tube and vortex to mix.

800 **12.2 Preparation of Test Substance Dosing Solutions for Range Finder Testing**

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

804 To prepare test substance dosing solutions:

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

808 **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

809 [1]Vortex tubes #2 through 6 before removing test substance/DMSO solution to place in the next tube in the series.
810
811 2. Transfer test substance/DMSO solutions to the second set of labeled tubes and
812 add E2 as shown in **Table 12-2**.

813 **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 x 10^{-3} µg/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 x 10^{-3} µg/mL E2 solution prepared in **Section 11.1.1** Vortex to mix.	800 µL	808 µL

Tube Number	Test Substance	E2	Estrogen-free DMEM[3]	Final Volume
3	Transfer 4 µL of test substance from Tube #3 to a new tube	Add 4 µL of the 5×10^{-3} µg/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} µg/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} µg/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} µg/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} µg/mL E2 solution prepared in **Section 11.1.1**. Vortex to mix.	800 µL	808 µL

814

815 Determination of whether a substance is positive in range finder testing and selection of starting
816 concentrations for comprehensive testing will be discussed in **Section 14.0**.

817 12.3 Preparation of Reference Standard and Control Dosing Solutions for 818 Comprehensive Testing

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

822 All "dosing solutions" of test substance concentrations are to be expressed as µg/mL in the study
823 notebook and in all laboratory reports.

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

825

825 12.3.1 Preparation of Ral/E2 Reference Standard Dosing Solutions for Comprehensive
826 Testing

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

830 **Table 12-3 Preparation of Ral/E2 Reference Standard Dosing Solution**
831 **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

832

833 12.3.2 Preparation of DMSO Control Comprehensive Testing Dosing Solution

834 1. Add 10 µL of 100% DMSO to a 13 mm glass test tube.

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

836 12.3.3 Preparation of E2 Control Comprehensive Testing Dosing Solution

837 1. Add 5 µL of the E2 stock from **Section 11.1.1** to a 13 mm glass test tube.

838 2. Add 5 µL of 100% DMSO to the tube.

839 3. Add 1000 µL of estrogen-free DMEM to the tube and vortex to mix.

840 12.3.4 Preparation of Flavone/E2 Control Comprehensive Dosing Solution

841 1. Add 10 µL of flavone/E2 from **Section 11.4** to a 13 mm glass test tube.

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

843 **12.4 Preparation of Test Substance Dosing Solutions for Comprehensive Testing**

844 Comprehensive testing experiments are used to determine whether a substance possesses ER
845 antagonist activity in the LUMI-CELL® ER test method. Antagonist comprehensive testing for
846 coded substances consists of either an 11-point 1:2 serial dilution, or an 11-point 1:5 serial
847 dilution with each concentration tested in triplicate wells of the 96-well plate.

848 12.4.1 *Preparation of Test Substance 1:2 Serial Dilutions for*
849 *Comprehensive Testing*

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

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

852 1. label eleven 4 mL conical tubes with numbers 1 through 11 and place them in a
853 tube rack

854 2. label eleven 13 mm glass test tubes with numbers 1 through 11, place them in a
855 tube rack and add 800 µL of estrogen-free DMEM to each tube

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

857

857 **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 µL of test substance solution from **Section 10.2.4 step 1**	-	4 µL	800 µL	808 µL
2	4 µL	4 µL of test substance solution from **Section 10.2.4 step 1**	-	4 µL	800 µL	808 µL
3	4 µL	4 µL from Tube #2	-	4 µL	800 µL	808 µL
4	4 µL	4 µL from Tube #3	-	4 µL	800 µL	808 µL
5	4 µL	4 µL from Tube #4	-	4 µL	800 µL	808 µL
6	4 µL	4 µL from Tube #5	-	4 µL	800 µL	808 µL
7	4 µL	4 µL from Tube #6	-	4 µL	800 µL	808 µL
8	4 µL	4 µL from Tube #7	-	4 µL	800 µL	808 µL
9	4 µL	4 µL from Tube #8	-	4 µL	800 µL	808 µL
10	4 µL	4 µL from Tube #9	-	4 µL	800 µL	808 µL
11	4 µL	4 µL from Tube #10	4 µL	4 µL	800 µL	808 µL

858 [1]Vortex tubes #2 through 10 before removing test substance/DMSO solution to place in the next tube in the series.
859 [2]Vortex all tubes to mix media, test substance, and E2.
860

861 12.4.2 *Preparation of Test Substance 1:5 Serial Dilutions for*
862 *Comprehensive Testing*

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

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

865 1. label eleven 4 mL conical tubes with numbers 1 through 11 and place them in a
866 tube rack

867 2. label eleven 13 mm glass test tubes with numbers 1 through 11, place them in a
868 tube rack and add 800 µL of estrogen-free DMEM to each tube

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

870

870 **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

871 [1]Vortex tubes #2 through 10 before removing test substance/DMSO solution to place in the next tube in the series.
872 [2]Vortex all tubes to mix media, test substance, and E2.
873

874 13.0 GENERAL PROCEDURES FOR THE TESTING OF CODED SUBSTANCES

875 Range finder experiments are used to determine the concentrations of test substance to be used
876 during comprehensive testing. Comprehensive testing experiments are used to determine whether
877 a substance possesses ER antagonist activity in the LUMI-CELL® ER test method.

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

881 13.1 Application of Reference Standard, Control and Test Substances

882 1. Remove the 96-well plates (from **Section 9.2.3 step 18)** from the incubator;
883 inspect them using an inverted microscope. Only use plates in which the cells in
884 all wells receive a score of 1 according to **Table 11-1**.

885 2. Remove medium by inverting the plate onto blotter paper. Gently tap plate against
886 the bench surface to remove residual liquid trapped in the wells.

887 3. Add 200 μL of medium, reference standard, control or test substance to each well
888 (see **Sections 14.0** and **15.0** for specific plate layouts).

889 4. Return plates to incubator (see **Section 9.0** for details) for 19 to 24 hours to allow
890 maximal induction of luciferase activity in the cells.

891 13.1.1 Preparation of Excel® Data Analysis Template For Range Finder Testing

892 1. In Excel®, open a new "AntRFTemplate" and save it with the appropriate project
893 name as indicated in the NICEATM Style Guide.

894 2. Fill out the table at the top of the "Raw Data" worksheet with information
895 regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot.
896 Meas. Time/Well (s), etc. (**note**: this information can be permanently added to the
897 default template "AntRFTemplate" on a laboratory specific basis).

898 3. Add the following information regarding the assay to the "Compound Tracking"
899 worksheet.

900 ▪ Plate # - Enter the experiment ID or plate number into cell E1

901 ▪ Cell Lot # - Enter the passage or lot number of the cells used for this
902 experiment into cell B5

903 ▪ DMSO and Media Lot #'s – Enter the lot numbers for the DMSO and
904 Media in cells B6 and B7

905 ▪ Test Substance Code – Enter the test substance codes into cells C14 to
906 C19

907 ▪ Name: Enter the experimenter name into cell G6

908 ▪ Date: Enter the experiment date in the format day\month\year into cell
909 G10

910 ▪ Comments: - Enter any comments about the experiment in this box (e.g.,
911 plate contaminated)

912 4. Enter the following substance testing information to the "List" worksheet:

913 ▪ Concentration – Type in the test substance concentration in µg/ml in
914 descending order.

915 ▪ Any specific comments about the test substance or condition of the wells
916 should be entered into this sheet, in the comments section

917 ▪ All of the remaining cells on the "List" worksheet should populate
918 automatically.

919 ▪ The "Template", "Compound Mixing" and "Visual Inspection"
920 worksheet should automatically populate with the information entered
921 into the "Compound Tracking" and "List" worksheet.

922 5. Save the newly named project file.

923 6. Print out either the "List" or "Template" worksheet for help with dosing the 96-
924 well plate. Sign and date the print out and store in study notebook.

925 13.1.2 Preparation of Excel® Data Analysis Template for Comprehensive Testing

926 1. In Excel®, open a new "AntCTTemplate" and save it with the appropriate project
927 name as indicated in the NICEATM Style Guide.

928 2. Fill out the table at the top of the "Raw Data" worksheet with information
929 regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot.
930 Meas. Time/Well (s), etc. (**note**: this information can be permanently added to the
931 default template "AntCTTemplate" on a laboratory specific basis).

932 3. On the "Compound Tracking" worksheet, enter the following information:

933 ▪ Plate # - Enter the experiment ID or plate number into cell E1

934 ▪ Cell Lot # - Enter the passage or lot number of the cells used for this
935 experiment into cell C5

936 ▪ DMSO and Media Lot #'s – Enter the lot numbers for the DMSO and
937 Media in cells C6 and C7

938 ▪ Test Substance Code – Enter the test substance codes into cells C15 and
939 C16. Enter the test substance dilution into cells D15 and D16.

940 ▪ Name: Enter the experimenter name into cell F6

941 ▪ Date: Enter the experiment date in the format day\month\year into cell
942 G10

943 ▪ Comments: - Enter any comments about the experiment in this box (e.g.,
944 plate contaminated)

945 4. Enter the following substance testing information to the "List" worksheet:

946 ▪ Concentration – Type in the test substance concentration in μg/ml in
947 descending order.

948 ▪ Any specific comments about the test substance or condition of the wells
949 should be entered into this sheet, in the comments section

950 ▪ All of the remaining cells on the "List" worksheet should populate
951 automatically.

952 ▪ The "Template", "Compound Mixing" and "Visual Inspection"
953 worksheet should automatically populate with the information entered
954 into the "Compound Tracking" and "List" worksheet.

955 5. Save the newly named project file.

956 6. Print out either the "List" or "Template" worksheet for help with dosing the 96-
957 well plate. Sign and date the print out and store in study notebook.

958 **13.2 Visual Evaluation of Cell Viability**

959 1. 19 to 24 hours after dosing the plate, remove the plate from the incubator and
960 remove the media from the wells by inverting the plate onto blotter paper. Gently
961 tap plate against the bench surface to remove residual liquid trapped in the wells.

962 2. Use a repeat pipetter to add 50 μL 1X PBS to all wells. Immediately remove PBS
963 by inversion.

964 3. Using an inverted microscope, inspect all of the wells used in the 96-well plate
965 and record the visual observations using the scores in **Table 13-1**.

966

966 **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"

967 [1]Reference photomicrographs are provided in the LUMI-CELL® ER Validation Study "Visual Observation Cell
968 Viability Manual."

969

970 **13.3 Lysis of Cells for LUMI-CELL® ER**

971 1. Apply the reflective white backing tape to the bottom of the 96-well plate (this
972 will increase the effectiveness of the luminometer).

973 2. Add 30µL 1X lysis reagent to the assay wells and place the 96-well plate on an
974 orbital shaker for one minute.

975 3. Remove plate from shaker and measure luminescence (as described in **Section**
976 **13.4**).

977 **13.4 Measurement of Luminescence**

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

983 **13.5 Data Analysis**

984 LUMI-CELL® ER uses an Excel® spreadsheet to collect and adjust the RLU values obtained
985 from the luminometer and a GraphPad Prism® template to analyze and graph data. Plate
986 reduction is calculated using unadjusted RLU values.

987 The Excel® spreadsheet subtracts background luminescence (average DMSO solvent control
988 RLU value) from test substance, reference standard and control RLU values. Test substance,
989 reference standard, and control RLU values are then adjusted relative to the highest Ral/E2

990 reference standard RLU value, which is set to 10,000. After adjustment, values are transferred to
991 GraphPad Prism® for data analysis and graphing.

992 13.5.1 Collection and Adjustment of Luminometer Data for Range Finder Testing

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

995 1. Open the raw data file and the corresponding experimental Excel® spreadsheet
996 from **Section 13.1.1**.

997 2. Copy the raw data using the Excel® copy function, then paste the copied data into
998 cell B19 of the "RAW DATA" tab in the experimental Excel® spreadsheet using
999 the **Paste Special – Values** command. This position corresponds to position A1 in
1000 the table labeled Table 1 in this tab.

1001 3. Examine the DMSO data in Table 1 of the Excel® spreadsheet to determine
1002 whether there are any potential outliers. See **Section 13.5.3** for further explanation
1003 of outlier determinations.

1004 4. If an outlier is identified, perform the following steps to remove the outlier from
1005 calculations:

1006 ▪ correct the equation used to calculate DMSO background in Table 1
1007 [e.g., if outlier is located in cell F26, adjust the calculation in cell H40 to
1008 read =AVERAGE(E26,G26)]

1009 ▪ then correct the equation used to calculate the average DMSO value in
1010 Table 2 [e.g., following the above example, adjust cell M42 to read
1011 =AVERAGE(E38,G38)]

1012 ▪ then correct the equation used to calculate the standard deviation of the
1013 DMSO value in Table 2 [e.g., following the above example, adjust cell
1014 M43 to read =STDEV(E38,G38)]

1015 5. Excel® will automatically subtract the background (the average DMSO control
1016 value) from all of the RLU values in Table 1 and populate Table 2 with these
1017 adjusted values.

1018 6. To calculate plate reduction, identify the cell containing the Ral/E2a replicate in
1019 Table 1, plate row H that has the lowest RLU value (i.e., cell B26, C26, or D26).

1020 7. Identify the cell containing the Ral/E2a replicate in Table 1, plate row H that has
1021 the highest RLU value (i.e., cell B26, C26, or D26).

1022 8. Click into cell D14 and enter the cell number from **Section 13.5.1 step 7** into the
1023 numerator and the cell number from **step 6** into the denominator.

1024 9. Identify the cell containing the Ral/E2b replicate in Table 1, plate row H that has
1025 the lowest RLU value (i.e., cell K26, L26, or M26).

1026 10. Identify the cell containing the Ral/E2b replicate in Table 1, plate row H that has
1027 the highest RLU value (i.e., cell K26, L26, or M26).

1028 11. Click into cell E14 and enter the cell number from **Section 13.5.1 step 10** into the
1029 numerator and the cell number from **step 9** into the denominator.

1030 12. Click on the "ER Antagonist Report" worksheet.

1031 13. The data for the Ral/E2 reference standard, DMSO, and E2, replicates populate
1032 the left portion (columns A-F) of the spreadsheet. The data is automatically
1033 placed into an Excel® graph.

1034 14. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell
1035 C2 of "ER Antagonist Report" worksheet and check the formula contained within
1036 that cell. The divisor should be the cell number of the cell containing the highest
1037 averaged Ral/E2 RLU value (column A).

1038 15. Open the "Visual Observation Scoring" worksheet. Enter the visual observation
1039 scores for each well on the 96-well plate. This data will be linked to the "ER
1040 Antagonist Report" worksheet.

1041 16. After the testing results have been evaluated and reviewed for quality control,
1042 enter the following information into the Compound Tracking worksheet:

1043 - Enter pass/fail results for plate reference standard and control parameters
1044 into the Plate Pass/Fail Table

1045 ▪ Enter information from the testing of coded substances into the Testing
1046 Results Table

1047 ▪ Reviewer Name – Enter the name of the person who Reviewed\QC'ed the
1048 data into cell A34

1049 ▪ Date – Enter the date on which the data was reviewed into cell D34

1050

1051 13.5.2 Collection and Adjustment of Luminometer Data for Comprehensive Testing

1052 The following steps describe the procedures required to populate the Excel® spreadsheet that has
1053 been configured to collect and adjust the RLU values obtained from the luminometer.

1054 1. Open the raw data file and the corresponding experimental Excel® spreadsheet
1055 from **Section 13.1.2**.

1056 2. Copy the raw data using the Excel® copy function, then paste the copied data into
1057 cell B14 of the "RAW DATA" tab in the experimental Excel® spreadsheet using
1058 the **Paste Special – Values** command. This position corresponds to position A1 in
1059 the table labeled Table 1 in this tab.

1060 3. Examine the DMSO data in Table 1 of the Excel® spreadsheet to determine
1061 whether there are any potential outliers. See **Section 13.5.3** for further explanation
1062 of outlier determinations.

1063 4. If an outlier is identified, perform the following steps to remove the outlier from
1064 calculations:

1065 ▪ correct the equation used to calculate DMSO background in Table 1[e.g.,
1066 if outlier is located in cell M14, adjust the calculation in cell H40 to read
1067 =AVERAGE(M15:M17)]

1068 ▪ then correct the equation used to calculate the average DMSO value in
1069 Table 2 [e.g., following the above example, adjust cell M35 to read
1070 =AVERAGE(M25:M27)]

1071 ▪ then correct the equation used to calculate the standard deviation of the
1072 DMSO value in Table 2 [e.g., following the above example, adjust cell
1073 M36 to read =STDEV(M25:M27)]

1074 5. Excel® will automatically subtract the background (the average DMSO control
1075 value) from all of the RLU values in Table 1 and populate Table 2 with these
1076 adjusted values.

1077 6. To calculate plate reduction, identify the cell containing the Ral/E2 replicate in
1078 plate row G that has the lowest RLU value.

1079 7. Identify the cell containing the Ral/E2 replicate in plate row G that has the highest
1080 RLU value.

1081 8. Click into cell D14 and enter the cell number from **Section 13.5.2 step 7** into the
1082 numerator and the cell number from **step 6** into the denominator.

1083 9. Identify the cell containing the Ral/E2 replicate in plate row H that has the lowest
1084 RLU value.

1085 10. Identify the cell containing the Ral/E2 replicate in plate row H that has the highest
1086 RLU value.

1087 11. Click into cell E14 and enter the cell number from **Section 13.5.2 step 10** into the
1088 numerator and the cell number from **step 9** into the denominator.

1089 12. Click on the "ER Antagonist Report" worksheet.

1090 13. The data for the Ral/E2 reference standard, DMSO, E2, and Flavone/E2 replicates
1091 populate the left portion (columns A-E) of the spreadsheet. The data is
1092 automatically placed into an Excel® graph.

1093 14. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell
1094 D2 of "ER Antagonist Report" worksheet and check the formula contained within
1095 that cell. The divisor should be the cell number of the cell containing the highest
1096 averaged Ral/E2 RLU value (column A).

1097 15. Open the "Visual Observation Scoring" worksheet. Enter the visual observation
1098 scores for each well on the 96-well plate. This data will be linked to the "ER
1099 Antagonist Report" worksheet.

1100 16. Copy the data into GraphPad Prism® for the calculation of IC_{50} values and to
1101 graph experimental results as indicated in the NICEATM Prism® Users Guide.

1102 17. After the testing results have been evaluated and reviewed for quality control,
1103 enter the following information into the Compound Tracking worksheet:

1104 - Enter pass/fail results for plate reference standard and control parameters
1105 into the Plate Pass/Fail Table

1106 - Enter information from the testing of coded substances into the Testing
1107 Results Table

1108 - Reviewer Name – Enter the name of the person who Reviewed\QC'ed the
1109 data into cell A34

1110 - Date – Enter the date on which the data was reviewed into cell D32

1111 13.5.3 Determination of Outliers

1112 The Study Director will use good statistical judgment for determining "unusable" wells that will
1113 be excluded from the data analysis and will provide an explanation in the study notebook for any
1114 excluded data. This judgment for data acceptance will include Q-test analysis.

1115 The formula for the Q test is:

1116 $$\frac{Outlier - Nearest\ Neighbor}{Range\ (Highest - Lowest)}$$

1117 where the outlier is the value proposed for exclusion, the nearest neighbor is the value closest to
1118 the outlier, and the range is the range of the three values (Q values for samples sizes from 3 to 10
1119 are provided in Table 13-2). For example, if the value of this ratio is greater than 0.94 (the Q
1120 value for the 90% confidence interval for a sample size of three) or 0.76 (the Q value for the 90%
1121 confidence interval for a sample size of four), the outlier may be excluded from data analysis.

1122

1122 **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

1123

1124 For E2 reference standard replicates (sample size of two), any adjusted RLU value for a replicate
1125 at a given concentration of E2 is considered and outlier if its value is more than 20% above or
1126 below the adjusted RLU value for that concentration in the historical database.

1127 13.5.4 Acceptance Criteria

1128 13.5.4.1 *Range Finder Testing*

1129 Acceptance or rejection of a range finder test is based on reference standard and solvent control
1130 results from each experiment conducted on a 96-well plate.

1131 • Reduction: Plate reduction, as measured by dividing the averaged highest Ral/E2
1132 reference standard RLU value by the averaged DMSO control RLU value, must
1133 be greater than three-fold.

1134 • DMSO control results: DMSO control RLU values must be within 2.5 times the
1135 standard deviation of the historical solvent control mean RLU value (see **Section**
1136 **16.5**).

1137 An experiment that fails either acceptance criterion will be discarded and repeated.

1138 13.5.4.2 *Comprehensive Testing*

1139 Acceptance or rejection of a test is based on evaluation of reference standard and control results
1140 from each experiment conducted on a 96-well plate. Results are compared to quality controls
1141 (QC) for these parameters derived from the historical database (see **Section 16.5**), which are
1142 summarized below.

1143 • Reduction: Plate reduction, as measured by dividing the averaged highest Ral/E2
1144 reference standard RLU value by the averaged lowest Ral/E2 control RLU value,
1145 must be greater than three-fold.

1146 • DMSO control results: DMSO control RLU values must be within 2.5 times the
1147 standard deviation of the historical solvent control mean RLU value (see **Section**
1148 **16.5**).

1149 • Reference standard results: The Ral\E2 reference standard concentration-response
1150 curve should be sigmoidal in shape and have at least three values within the linear
1151 portion of the concentration-response curve.

1152 • E2 control results: E2 control RLU values must be within 2.5 times the standard
1153 deviation of the historical E2 control mean RLU value.

1154 • Positive control results: Flavone/E2 control RLU values must be less than the E2
1155 control mean minus three times the standard deviation from the E2 control mean.

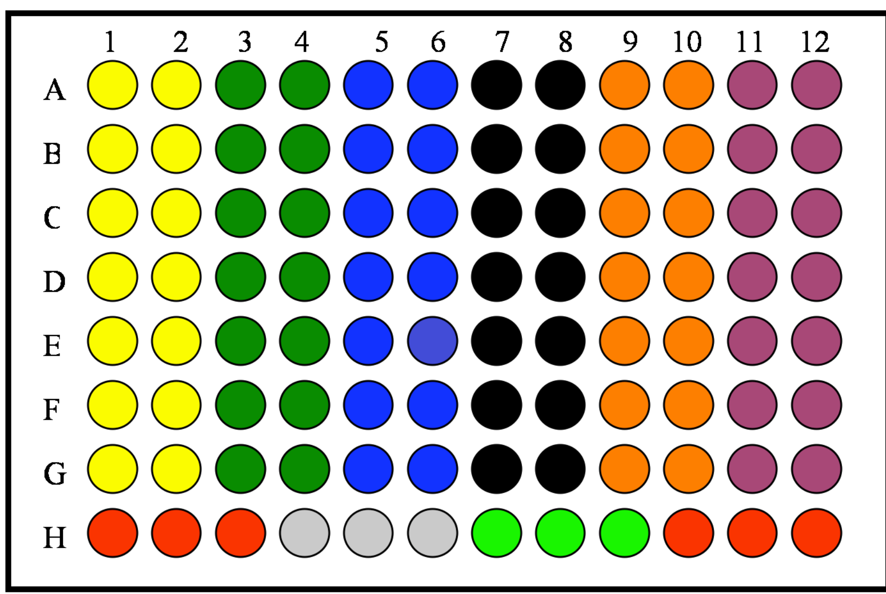
1156 An experiment that fails any single acceptance criterion will be discarded and repeated.

1157 14.0 RANGE FINDER TESTING

1158 Antagonist range finding for coded substances consists of seven point, 1:10 serial dilutions tested
1159 in duplicate wells of the 96-well plate. **Figure 14-1** contains a template for the plate layout used
1160 in antagonist range finder testing.

1161

1161 **Figure 14-1 Antagonist Range Finder Plate Layout**



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

1162

1163

1163 Evaluate whether range finder experiments have met acceptance criteria (see **Section 13.6.3**).

1164 To determine starting concentrations for comprehensive testing use the following criteria:

- 1165 If results in the range finder test suggest that the test substance is negative for 1166 antagonist activity (i.e., if there are no points on the test substance concentration 1167 curve that are less than the mean minus three times the standard deviation of the 1168 E2 control, see **Figure 14-2**), comprehensive testing will be conducted using an 1169 11-point 1:2 serial dilution using the maximum soluble concentration of test 1170 substance as the with the limit dose as the starting concentration.
- 1171 If results in the range finder test suggest that the test substance is negative for 1172 agonist activity (i.e., if there are no points on the test substance concentration 1173 curve that are greater than the mean plus three times the standard deviation of the 1174 DMSO control), and the higher concentrations in the range finder are cytotoxic, 1175 comprehensive testing will be conducted using an 11 point 1:2 serial dilution with 1176 the lowest cytotoxic concentration as the starting concentration (see **Figure 14-3**).
- 1177 If results in the range finder test suggest that the test substance is positive for 1178 antagonist activity (i.e., if there are points on the test substance concentration 1179 curve that are less than the mean minus three times the standard deviation of the 1180 E2 control), the top concentration to be used for the 11-point dilution scheme in 1181 comprehensive testing should be one of the following:
  - 1182 The concentration giving the lowest adjusted RLU value in the range 1183 finder
  - 1184 The maximum soluble concentration (See **Figure 14-2**)
  - 1185 The lowest cytotoxic concentration (See **Figure 14-3** for a related 1186 example).

1187 The 11-point dilution scheme will be based on either a 1:2 or 1:5 serial or dilution 1188 according to the following criteria:

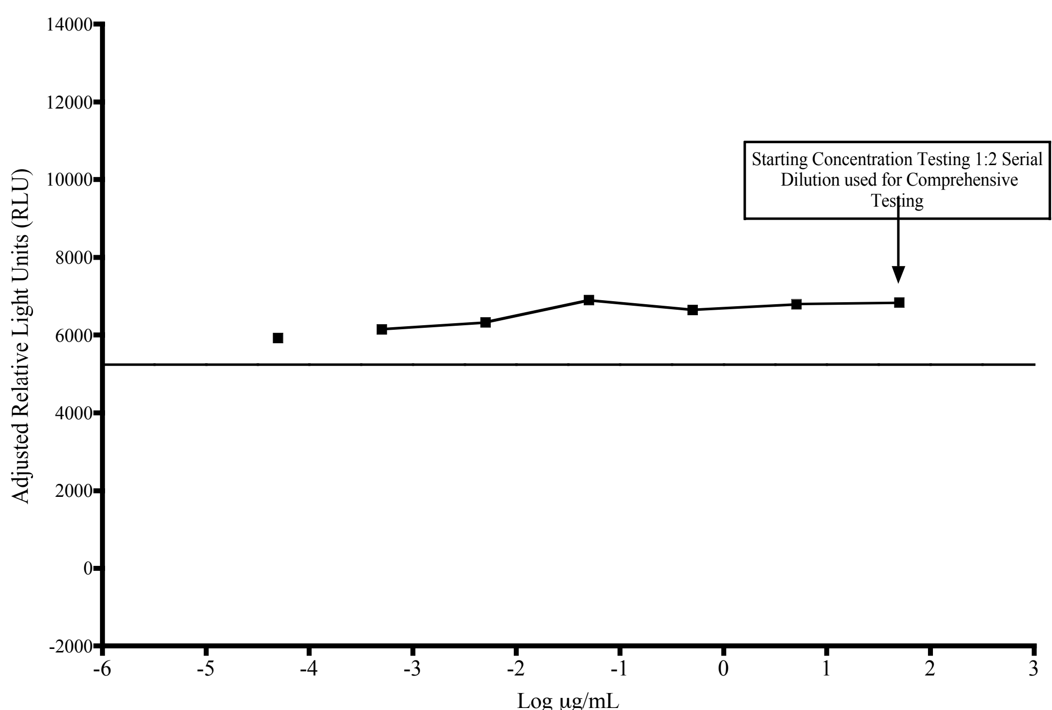
- 1189 An 11-point 1:2 serial dilution should be used if the resulting 1190 concentration range (note: an 11-point 1:2 serial dilution will cover a 1191 range of concentrations over approximately three orders of magnitude

1192 [three logs]) will encompass the full range of responses based on the
1193 concentration response curve generated in the range finder test (see **Figure**
1194 **14-4**).

1195 – If the concentration range that would be generated with the 1:2 serial
1196 dilution will not encompass the full range of responses based on the
1197 concentration response curve in the range finder test (see **Figure 14-5**), an
1198 11-point 1:5 serial dilution should be used instead.

1199 • If a substance exhibits a biphasic concentration response curve in the range finder
1200 test (see **Figure 14-6**), both phases should also be resolved in comprehensive
1201 testing. In this case, two peaks could potentially be used to identify the top
1202 concentration to be used for the 11-point dilution scheme in comprehensive
1203 testing. In order to resolve both curves, the top concentration should be based on
1204 the peak associated with the higher concentration and the top dose one log
1205 concentration higher than the concentration giving the lowest adjusted RLU value
1206 in the range finder. An 11-point 1:5 serial dilution should be used.

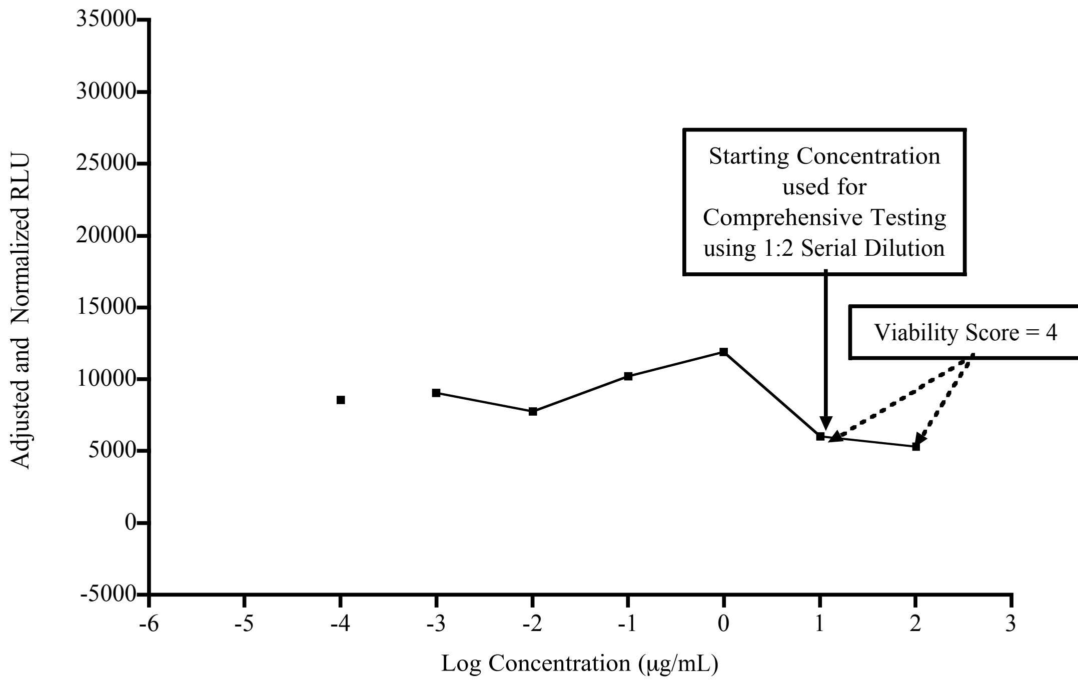
1207 **Figure 14-2 Antagonist Range Finder (example 1)**



1208

1209 The solid horizontal line represents the mean minus three times the standard deviation of the E2 control.

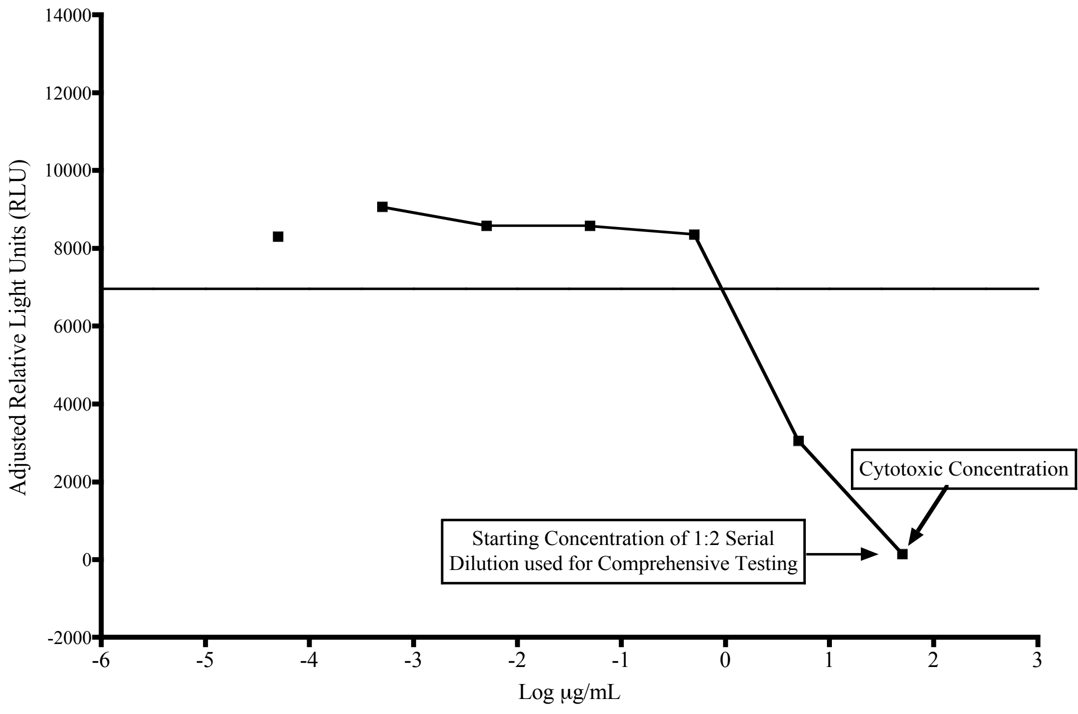
1210 **Figure 14-3 Antagonist Range Finder (example 2)**



1211

1212 The solid horizontal line represents the mean minus three times the standard deviation of the E2 control.

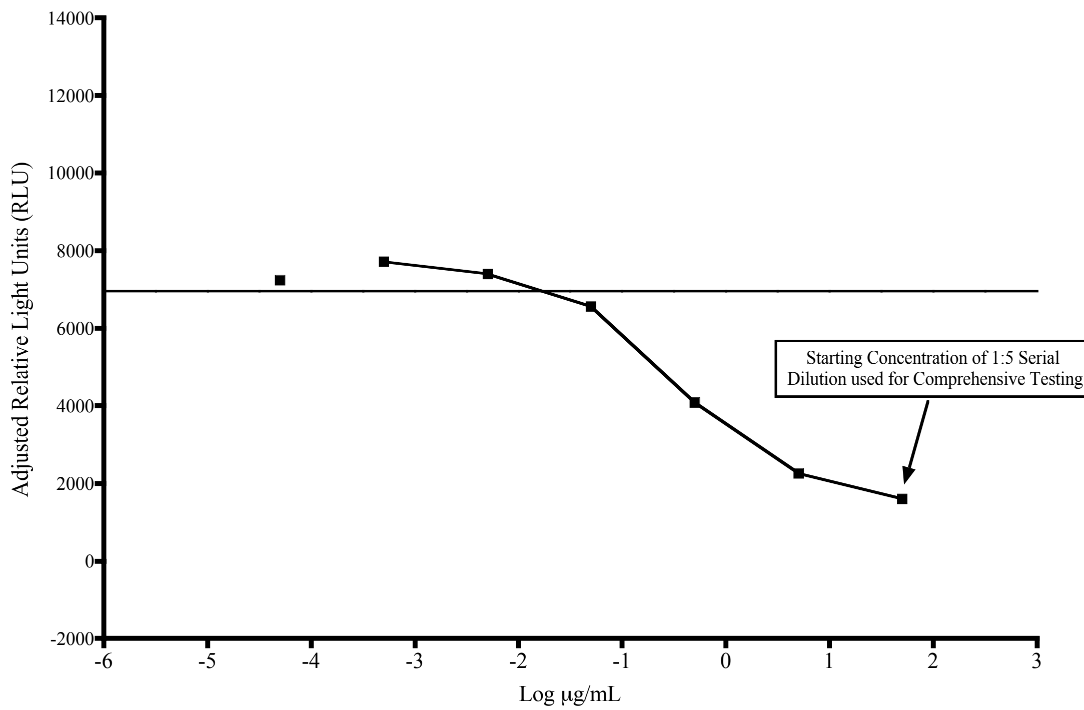
1213 **Figure 14-4 Antagonist Range Finder (example 3)**



1214

1215 The solid horizontal line represents the mean minus three times the standard deviation of the E2 control.

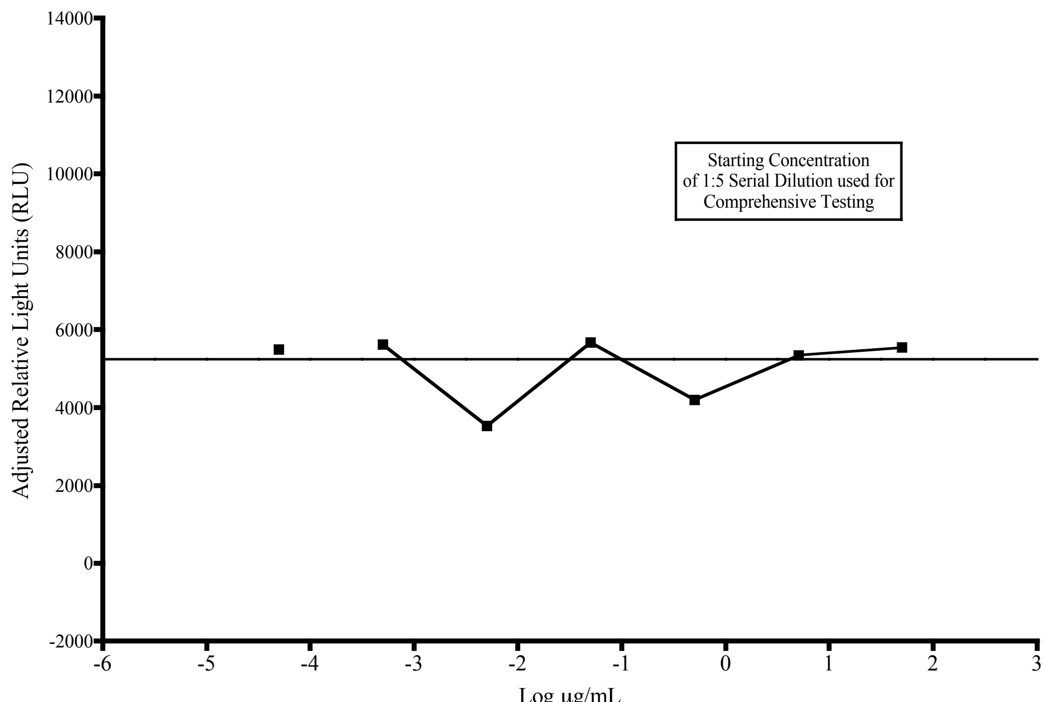
1216 **Figure 14-5 Antagonist Range Finder (example 4)**



1217

1218 The solid horizontal line represents the mean minus three times the standard deviation of the E2 control.

1219 **Figure 14-6 Antagonist Range Finder (example 5)**



1220

1221 The solid horizontal line represents the mean minus three times the standard deviation of the E2 control.

1222

1223 **15.0 COMPREHENSIVE TESTING**

1224 Antagonist comprehensive testing for coded substances consists of 11 point, 1:2 serial dilutions,
1225 with each concentration tested in triplicate wells of the 96-well plate. **Figure 15-1** contains a
1226 template for the plate layout to be used in antagonist comprehensive testing.
1227
1228

1228 **Figure 15-1 Antagonist Comprehensive Test Plate Layout**

1229
1230 1 2 3 4 5 6 7 8 9 10 11 12
1231 A
1232 B
1233 C
1234 D
1235 E
1236 F
1237 G
1238 H
1239
1240
1241 **9 Point Duplicate Ral/E2 Reference Standard**
1242 **DMSO (Solvent Control)**
1243 **Test Substance #1**
1244
1245 **Test Substance #2**
1246 **E2 Control**
1247 **Flavone Control**
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258

1258 Evaluate whether comprehensive experiments have met acceptance criteria (see **Section 13.6.3**)
1259 and graph the data as described in the NICEATM Prism® users guide.

1260 • If the substance has been tested up to the limit dose or the maximum soluble dose
1261 without causing a significant decrease in cell viability, and there are no points on
1262 the concentration curve that are less than the mean minus three times the standard
1263 deviation of the E2 control, the substance is considered negative for antagonism.

1264 • If the substance has been tested up to the limit dose and there are points on the
1265 concentration curve that are less than the mean minus three times the standard
1266 deviation of the E2 control, but cell viability has a visual inspection score of 2 or
1267 greater, at all points falling below the E2 line, the substance is considered
1268 negative for antagonism.

1269 • If there are points on the test substance concentration curve that are less than the
1270 mean minus three times the standard deviation of the E2 control that do not cause
1271 a visual inspection score of 2 or greater, the substance is positive for antagonism.

1272 – Points in the test substance concentration curve that cause a visual
1273 inspection score of 2 or greater, are not included in data analyses.

1274 **16.0 COMPILATION OF THE HISTORICAL QUALITY CONTROL DATABASE**

1275 Historical databases are maintained in order to ensure that the assay is functioning properly.
1276 Historical databases are compiled using Excel® spreadsheets and are separate from the
1277 spreadsheets used to collect the data for individual test plates. Reference standard and control
1278 data is used to develop and maintain the historical database and are used as quality controls to
1279 determine acceptance of individual test plates.

1280 The sources of data needed to compile the historical database for the E2 control and flavone/E2
1281 control values are the experiment specific Excel® data collection and analysis spreadsheets (**see**
1282 **Section 13.5.2)** used for LUMI-CELL® ER antagonist testing. The sources of the data needed to
1283 compile the historical database for the DMSO control are the experiment specific Excel® data
1284 collection and analysis spreadsheets used for LUMI-CELL® ER antagonist and agonist testing

1285 (see **Section 13.5.2** of the LUMI-CELL® ER antagonist protocol and **Section 11.5.2** in the
1286 LUMI-CELL® ER agonist protocol).

1287 16.1 E2 Control

1288 Open the LUMI-CELL® ER antagonist specific historical database Excel® spreadsheet
1289 (LUMI_AgandAntQC.xls) and save under a new name using the Excel® "Save As" function,
1290 adding the laboratory designator to the file name (e.g., for Laboratory H, the new name would be
1291 HLUMI_AgandAntQC.xls). Open the E2 Control worksheet and enter the date and experiment
1292 name into worksheet columns A and B respectively. Enter the experimental mean adjusted E2
1293 control value (from cell D37 in the ER Antagonist Report worksheet of the Excel® data
1294 collection and analysis spreadsheet) into the Antagonist E2 control worksheet, column C.
1295 Acceptance or rejection of plate E2 control data for comprehensive testing is based on whether
1296 the mean plate E2 RLU value falls within 2.5 times the standard deviation of the E2 value in the
1297 historical database (columns G and H in theE2 Control worksheet).

1298 16.2 DMSO

1299 Open the combined agonist and antagonist LUMI-CELL® ER historical database Excel®
1300 spreadsheet (LUMI_AgandAntQC.xls) and save under a new name using the Excel® "Save As"
1301 function, adding the laboratory designator to the file name (e.g., for Laboratory H, the new name
1302 would be HLUMI_AgandAntQC.xls). Enter the date and experiment name into worksheet
1303 columns A and B respectively. Enter the experimental mean DMSO control value (from cell H37
1304 in the RAW DATA worksheet of the agonist and antagonist Excel® data collection and analysis
1305 spreadsheet) into worksheet column C. Acceptance or rejection of the plate DMSO control data
1306 for range finding and comprehensive testing is based on whether the mean plate DMSO RLU
1307 value falls within 2.5 times the standard deviation of the DMSO value in the historical database
1308 (columns G and H in the DMSO worksheet).

1309

1310 17.0 QUALITY TESTING OF MATERIALS

1311 All information pertaining to the preparation and testing of media, media supplements, and other
1312 materials should be recorded in the Study Notebook.

1313 **17.1 Tissue Culture Media**

1314 Each lot of tissue culture medium must be tested in a single growth flask of cells before use in
1315 ongoing tissue culture or experimentation (**note:** each bottle within a given lot of
1316 Charcoal/Dextran treated FBS must be tested separately).

1317 1. Every new lot of media (RPMI and DMEM) and media components (FBS,
1318 Charcoal/Dextran treated FBS, and L-glutamine) must first be tested on the
1319 LUMI-CELL® ER assay prior to being used in any GLP acceptable assays.

1320 2. Add 4 µL of DMSO (previously tested) into four separate 13 mm tubes.

1321 3. Add 400 µL media (to be tested) to 13 mm tube.

1322 4. Dose an experimental plate as in **Section 12.0,** treating the media being tested as a
1323 test substance.

1324 5. Analyze 96-well plate as described in **Section 12.0,** comparing the data from the
1325 DMSO controls made using previously tested tissue culture media to the new
1326 media being tested.

1327 6. Use the agonist historical database to determine if the new media with DMSO lies
1328 within 2.5 standard deviations of the mean for the media. If the RLU values for
1329 the new media with DMSO lie within 2.5 standard deviations of the DMSO mean
1330 from the historical database, the new lot of media is acceptable. If the RLU values
1331 for the new media with DMSO do not lie within 2.5 standard deviations of the
1332 DMSO mean from the historical database, the new lot may not be used in the
1333 assay.

1334 7. Note date and lot number in study notebook.

1335 8. If the new bottle passes quality testing as described in **Section 15.1 step 6**, apply
1336 the media to a single flask cells and observe the cells growth and morphology
1337 over the following 2 to 3 days. If there is no change in growth or morphology, the
1338 new media is acceptable for use.

1339

1339 **17.2 G418**

1340 1. New lots of G418 must first be tested on the LUMI-CELL® ER assay prior to
1341 being used in any GLP acceptable assays.

1342 2. Add 220 µL of G418 (previously tested) to a single flask containing cells growing
1343 in RPMI.

1344 3. Add 220 µL of G418 (to be tested) to a different flask containing cells growing in
1345 RPMI.

1346 4. Observe cellular growth and morphology in both tissue culture flasks over a 48 to
1347 72 hour period. If there are no differences in observed growth rate and
1348 morphology between the two flasks, the new G418 lot is acceptable.

1349 5. If cellular growth is decreased, or the cells exhibit abnormal morphology, the new
1350 lot of G418 is not acceptable.

1351 6. Note date and lot number in study book.

1352 **17.3 DMSO**

1353 1. Every new bottle of DMSO must be tested on the LUMI-CELL® ER assay prior
1354 to use in any GLP acceptable assays.

1355 2. Add 4 µL of DMSO (to be tested) into four separate 13 mm tubes.

1356 3. Add 400 µL media (previously tested) the same tubes.

1357 4. Dose an experimental plate as in **Section 15.0,** treating the media being tested as a
1358 test substance.

1359 5. Analyze 96-well plate as described in **Section 15.0,** comparing the data from the
1360 DMSO controls made using previously tested tissue culture media to the new
1361 media being tested.

1362 6. Use the agonist historical database to determine if media with new DMSO lies
1363 within 2.5 standard deviations of the DMSO mean from historical database. If the
1364 RLU values for the media with new DMSO lie within 2.5 standard deviations of
1365 the DMSO mean from the historical database, the new lot of DMSO is acceptable.

1366 If the RLU values for media with new DMSO do not lie within 2.5 standard
1367 deviations of the DMSO mean from historical database, the new lot may not be
1368 used in the assay.

1369 7. Note the date, lot number, and bottle number in study book.

1370 8. If no DMSO has been previously tested, test several bottles as described in
1371 **Section 15.3**, and determine whether any of the bottles of DMSO have a higher
1372 average RLU than the other bottle(s) tested. Use the DMSO with the lowest
1373 average RLU for official experiments.

1374 **17.4 Plastic Tissue Culture Materials**

1375 1. Grow one set of cells, plate them for experiments on plastic ware from the new lot
1376 and one set of cells in the plastic ware from a previous lot, and dose them with E2
1377 reference standard and controls.

1378 2. Perform the LUMI-CELL® ER experiment with both sets of cells.

1379 3. If all of the analysis falls within acceptable QC criteria, then the new
1380 manufacturer's products may be used.

1381

1381 ## 18.0 REFERENCES

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