1.0 PURPOSE

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

2.0 SPONSOR

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

William S. Stokes, DVM, DACLAM

Rear Admiral, U.S. Public Health Service

Chief Veterinary Officer, USPHS

Director, NICEATM

National Institute of Environmental Health Sciences, NIH, DHHS

Bldg. 4401, Room 3129, MD EC-14

79 T.W. Alexander Drive

Research Triangle Park, NC 27709

Phone: 919-541-7997

Fax: 919-541-0947

Email: stokes@niehs.nih.gov

Raymond Tice, Ph.D.

Deputy Director, NICEATM

National Institute of Environmental Health Sciences

MD EC-17, P.O. Box 12233

Research Triangle Park, NC 27709

Phone: 919-541-4482

FAX: 919-541-0947

Email: tice@niehs.nih.gov

David Allen, Ph.D.

Principal Investigator

ILS, Inc./Contractor supporting NICEATM

National Institute of Environmental Health Sciences

MD EC-17, P.O. Box 12233

Research Triangle Park, NC 27709

Phone: 919-316-4587

FAX: 919-541-0947

Email: allen7@niehs.nih.gov

Frank Deal, M.S.

Staff Toxicologist

ILS, Inc./Contractor supporting NICEATM

National Institute of Environmental Health Sciences

MD EC-17, P.O. Box 12233

Research Triangle Park, NC 27709

Phone: 919-316-4587

FAX: 919-541-0947

Email: dealf@niehs.nih.gov

Patricia Ceger, M.S.

Project Coordinator/Technical Writer

ILS, Inc./Contractor supporting NICEATM

National Institute of Environmental Health Sciences

MD EC-17, P.O. Box 12233

Research Triangle Park, NC 27709

Phone: 919-316-4556

Fax: 919-541-0947

E-Mail: cegerp@niehs.nih.gov

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

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Testing Facility

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

4.2 Key Personnel

- Study Director: John Gordon, Ph.D.
- Laboratory Technician(s): Cynthia Matherly
- Scientific Advisor: Mike Denison, Ph.D.
- Quality Assurance Director: Andrew Chu
- Safety Manager: Andrew Chu
- Facility Management: George Clark, Ph.D., Robert Clark, Tina Ginter

- Laboratory Director: Andrew Chu

5.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

5.1 Test Substances

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

5.2 Controls

Controls for the ER antagonist protocol are as follows:

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

Ral/E2 reference standard for range finder testing: Three concentrations (1.25×10^{-1}, 1.25×10^{3}, and 5.00×10^{-5} μg/mL) of raloxifene HCl (raloxifene, CASRN 84449-90-1) plus a fixed concentration (2.5×10^{-5} μg/mL) of 17β-estradiol (E2, CASRN: 50-28-2).

Ral/E2 reference standard for comprehensive testing: A serial dilution of raloxifene HCl (raloxifene, CASRN 84449-90-1) plus a fixed concentration (2.5×10^{-5} μg/mL) of 17β-estradiol (E2, CASRN: 50-28-2), consisting of nine concentrations of Ral/E2 in duplicate wells.

E2 control: 17β-estradiol, CASRN: 50-28-2, 2.5×10^{-5} μg/mL in tissue culture media used as a base line negative control.

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

6.0 OVERVIEW OF GENERAL PROCEDURES FOR ANTAGONIST TESTING

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

Antagonist range finder testing is conducted on 96-well plates using three concentrations of Ral/E2 (1.25×10^{-1}, 1.25×10^{-3}, and 5.00×10^{-5} μg/mL raloxifene with 2.50×10^{-5} μg/mL E2) in

duplicate as the reference standard, with three replicate wells for the E2 and DMSO controls. Range finder testing uses all wells of the 96-well plate.

Comprehensive testing is conducted on 96-well plates using nine concentrations of Ral/E2 in duplicate as the reference standard (**Table 6-1**). Three replicate wells for the DMSO control, Flavone/E2 and E2 controls are included on each plate. In order to avoid edging effects[1], wells on the perimeter of the plate are not used for experiments. These wells should contain tissue culture media only (i.e., no cells).

Table 6-1 Concentrations of Ral/E2 Reference Standard Used in Range Finder and Comprehensive Testing

Raloxifene Concentrations[1]	E2 Concentrations
1.25×10^{-2}	2.5×10^{-5}
6.25×10^{-3}	2.5×10^{-5}
3.13×10^{-3}	2.5×10^{-5}
1.56×10^{-3}	2.5×10^{-5}
7.81×10^{-4}	2.5×10^{-5}
3.91×10^{-4}	2.5×10^{-5}
1.95×10^{-4}	2.5×10^{-5}
9.77×10^{-5}	2.5×10^{-5}
4.88×10^{-5}	2.5×10^{-5}

[1]Concentrations are presented in µg/mL.

Visual observations for cell viability are conducted for all experimental plates just prior to LUMI-CELL® ER evaluation. CellTiter-Glo® based cell viability testing (when used) is conducted concurrently in parallel plates on the same day, as outlined in **Section 11.4**.

Luminescence data, measured in relative light units (RLUs), is corrected for background luminescence by subtracting the mean RLU value of the vehicle control (DMSO) wells from the RLU measurements for each of the other wells of the 96-well plate. Data is then transferred into

[1]Edging effects are variations in response seen in the outermost wells in a cell culture plate. These variations are believed to be due to variations in temperature, evaporation, etc., that may occur in these wells that would ultimately affect cellular growth and health (ICCVAM, 2001).

Excel® data management spreadsheets and GraphPad PRISM® 4.0 statistical software, graphed, and evaluated for a positive or negative response as follows:

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

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

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log IC50 - X)\text{HillSlope}}}$$

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

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

6.1 Range Finder Testing

Antagonist range finding for coded substances consists of a six point, logarithmic serial dilution using 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, double 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**).

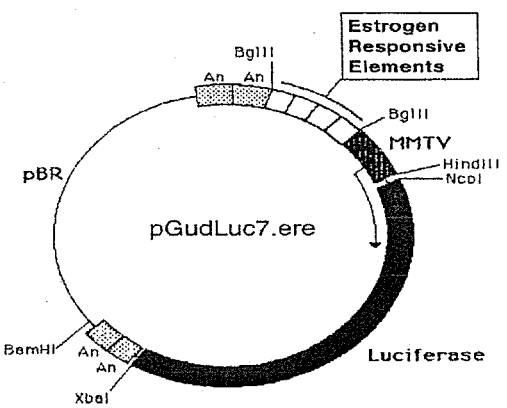
7.0 MATERIALS FOR LUMI-CELL® ER ANTAGONIST TESTING

This section provides the materials needed to conduct LUMI-CELL® 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.

7.2 Technical Equipment:

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

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

- Sonicating water bath (Cat. No. 15-335-30)
- Tissue culture incubator with CO_2 and temperature control (Cat. No. 11-689-4)
- Vacuum pump with liquid trap (side arm Erlenmeyer) (Cat. No. 01-092-29)
- Vortex mixer (Cat. No. 12-814)

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

7.3 Reference Standard, Controls, and Tissue Culture Supplies

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

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

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

- 17 β-estradiol (CAS RN: 50-28-2) [Sigma-Aldrich, Cat. No. E8875]
- CellTiter-Glo® Luminescent Cell Viability Assay [Promega Cat. No. G7572]
- Cryovial, 2 mL (Corning Costar) [Fisher Scientific Cat. No. 03-374-21]
- Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 10009186R38]
- Culture tube, 15 mL conical (Corning Costar) [Fisher Scientific Cat. No. 05-538-51]
- Culture tube, 50 mL conical (Corning Costar) [Fisher Scientific Cat. No. 05-526C]
- DMSO, U.S.P. analytical grade. [Sigma-Aldrich, Cat. No. 34869-100ML]
- Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L glucose, with sodium pyruvate, without phenol red or L-glutamine [Mediatech/Cellgro, Cat. No. 17-205-CV]

- Fetal Bovine Serum [Mediatech/Cellgro Cat. No. MT 35-010-CV]
- Fetal Bovine Serum, charcoal/dextran treated, triple 0.1 μm sterile filtered [Hyclone, Cat. No. SH30068.03]
- Flavone (CASRN: 525-82-6) [Sigma-Aldrich, Cat. No. F2003]
- Gentamycin Sulfate (G418), 50 mg/mL [Mediatech/Cellgro Cat. No. 30-234-CR]
- L-glutamine, 29.2 mg/mL [Cellgro, Cat. No. 25005-CI]
- Luciferase Assay System (10-Pack) [Promega Cat. No. E1501]
- Lysis Solution 5X [Promega, Cat. No. E1531]
- Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 μg/mL streptomycin [Cellgro, Cat. No. 30-001-CI].
- Phosphate buffered saline (PBS, 1X) without calcium and magnesium [Cellgro, Cat. No. 21-040-CV]
- Pipettes, serological: 2.0 mL [Sigma-Aldrich, Cat. No. P1736], 5.0 mL [Sigma-Aldrich, Cat. No. P1986], 25 mL [Sigma-Aldrich, Cat. No. P2486]
- Raloxifene (CASRN 84449-90-1) [Sigma-Aldrich Cat. No. R1402]
- RPMI 1640 medium, containing L-glutamine [Mediatech, Cat. No. 10-040-CV]
- Tissue culture flasks (Corning-Costar): 25 cm^2 (T25) [Fisher Cat. No. 10-126-28]; 75 cm^2 (T75) [Fisher Cat. No. 10-126-37]; and 150 cm^2 (T150) [Fisher Cat. No. 10-126-34]
- Tissue culture plates (Corning-Costar): 96-well [Thomas Scientific Cat. No. 6916A05]
- Trypsin (10X), 2.5% in Hank's balanced salt solution (HBSS), without calcium and magnesium, without phenol red [Cellgro, Cat. No. 25-054-CI].

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

8.0 PREPARATION OF TISSUE CULTURE MEDIA AND SOLUTIONS

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

8.1 RPMI 1640 Growth Medium (RPMI)

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

Procedure for one 549 mL bottle:

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

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

8.2 Estrogen-Free DMEM Medium

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

Procedure for one 539 mL bottle:

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

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

8.3 1X Trypsin Solution

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

Procedure for making 100 mL of 1X trypsin:

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

1X Trypsin should be stored at -20°C.

8.4 1X Lysis Solution

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

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

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

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

8.5 Reconstituted Luciferase Reagent

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

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

To reconstitute luciferase reagent:

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

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

8.6 Reconstituted CellTiter-Glo® Reagent

CellTiter-Glo® reagent consists of two components, CellTiter-Glo® buffer and lyophilized CellTiter-Glo® substrate.

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

To reconstitute CellTiter-Glo® reagent:

1. Remove CellTiter-Glo® buffer and CellTiter-Glo® substrate from -70°C freezer and allow them to equilibrate to room temperature.
2. Add CellTiter-Glo® buffer solution to CellTiter-Glo® substrate container and swirl or vortex gently to mix; the CellTiter-Glo® substrate should readily go into solution.
3. CellTiter-Glo® substrate should readily go into solution.
4. After solutions are mixed aliquot to a 15mL centrifuge tube.
5. Store complete solution at –20°C.

Reconstituted CellTiter-Glo® reagent is stable for up to 3 months at –20°C.

9.0 OVERVIEW OF PROPAGATION AND EXPERIMENTAL PLATING OF BG1Luc4E2 CELLS

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

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

9.1 Procedures for Thawing Cells and Establishing Tissue Cultures

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

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

9.1.1 Thawing Cells

1. Remove a cryovial of frozen BG-1 cells from the liquid nitrogen flask.
2. Facilitate rapid thawing by loosening the top slightly (do not remove top) to release trapped gasses and retightening it. Roll vial between palms.
3. Use a micropipette to transfer cells to a 50 mL conical centrifuge tube.
4. Rinse cryovial twice with 1X PBS and add PBS rinse material to the conical tube.
5. Add 20 mL of RPMI to the conical tube.
6. Centrifuge at 1000 x *g* for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
7. Aspirate media from pellet and re-suspend it in 5 mL RPMI, drawing the pellet repeatedly through a 2.0 mL serological pipette to break up any clumps of cells.
8. Transfer cells to a T25 flask, place them in an incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

9.1.2 Establishing Tissue Cultures

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

1. Remove the T25 flask from the incubator.
2. Aspirate the RPMI, then add 5 mL 1X PBS.
3. Aspirate 1X PBS, then add 1 to 2 mL 1X trypsin to the T25 flask, gently swirling the flask to coat all cells with the trypsin.
4. Place the flask in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
5. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.
6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, then hit the flask again.
7. After cells have detached, add 5 mL PBS, and transfer the suspended cells to a 50 mL centrifuge tube. Wash the flask one additional time with 5 mL PBS.
8. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular digestion by residual trypsin.
9. Pellet the cells by centrifugation, as described in **Section 9.1.1**, and re-suspend the cells in 10 mL RPMI medium.
10. Draw the pellet repeatedly through a 25 mL serological pipette to break up clumps of cells
11. Transfer cells to a T75 flask, then place the flask in an incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

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

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

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

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

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

16. Remove the T150 flask from the incubator.

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

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

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

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

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

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

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

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

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

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

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

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

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

1. Remove both T150 flasks from the incubator.
2. Aspirate the medium and rinse the cells with 5 mL 1X PBS.
3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask to coat all cells with the trypsin.
4. Incubate cells in the incubator (see conditions in **Section 9.0**) for 5 to 10 min.
5. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.
6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, then hit the flask again.
7. After cells have detached, add 5 mL 1X PBS to the first T150 flask and transfer the suspended cells to the second T150 flask.
8. Transfer the contents of both flasks to a 50 mL conical tube. Repeat step 7 with an additional 5 mL 1X PBS and transfer to the 50 mL conical tube.

9. Immediately add 20 mL estrogen-free DMEM to the 50 mL conical tube to inhibit further cellular digestion by residual trypsin.
10. Centrifuge at 1000 x *g* for eight minutes. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
11. Aspirate media from pellet and re-suspend it in 4 mL estrogen-free DMEM, drawing the pellet repeatedly through a 25 mL serological pipette to break up clumps of cells.

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

9.2.1 Ongoing Tissue Culture Maintenance

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

9.2.2 Conditioning in Estrogen-free Medium

1. Add 20 mL estrogen-free DMEM to two T150 flasks.
2. Add 150 µL G418 to the estrogen-free DMEM in the T150 flasks.
3. Add 1 mL of cell suspension from **Section 9.2 step 11** to each flask.
4. Tissue culture medium may need to be changed 24 hours after addition of G418 to remove cells that have died because they do not express reporter plasmid.
5. G418 does not need to be added to the flasks a second time.

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

9.2.3 Plating Cells Grown in Estrogen-free DMEM for Experimentation

1. Remove the T150 flasks that have been conditioned in estrogen-free DMEM for 48 to 72 hours from the incubator.
2. Aspirate the medium, then rinse the cells with 5 mL 1X PBS.
3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask to coat all cells with the trypsin.
4. Place the flasks in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
5. Detach cells by hitting the side of the flask sharply against the palm or the heel of the hand.
6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for 2 additional minutes, then hit the flask again.
7. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the flask, then transfer to the 50 mL conical tube.
8. Immediately add 20 mL estrogen-free DMEM to each conical tube to inhibit further cellular digestion by residual trypsin.
9. Centrifuge at 1000 x *g* for eight minutes. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
10. Aspirate off the media from the pellet and re-suspend it in 20 mL DMEM, drawing the pellet repeatedly through a 25 mL serological pipette to break up any clumps of cells.
11. Pipette 15 µL of the cell suspension into the "v" shaped slot on the hemocytometer. Ensure that the solution covers the entire surface area of the hemocytometer grid, and allow cells to settle before counting.