LUMI-CELL® ER ASSAY

AGONIST PROTOCOL

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

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TABLE OF CONTENTS

LIST OF ACRONYMS AND ABBREVIATIONS

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 reference standard	10 Point Serial Dilution of 17β-estradiol reference standard for the LUMI-CELL® ER agonist assay
EC_{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
G418	Gentamycin
Methoxychlor	*p,p'*-Methoxychlor
Methoxychlor control	3.13 μg/mL Methoxychlor Positive Control for the LUMI-CELL® ER Agonist Assay
RPMI	RPMI 1640 growth medium
T150	150 cm^2 tissue culture flask

1.0 PURPOSE

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

2.0 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 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.

3.0 CONTROLS AND REFERENCE STANDARDS

Controls for the ER agonist protocol are as follows:

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

Reference standard (17β-estradiol [E2]): Three concentrations of E2 (CASRN 50-28-2) in duplicate for range finder testing and a serial dilution consisting of 10 concentrations of E2 in duplicate for comprehensive testing

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

4.0 OVERVIEW OF GENERAL PROCEDURES FOR AGONIST 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.

Agonist range finder testing is conducted on 96-well plates using four concentrations of E2 (5.00 x 10^{-5}, 1.25 x 10^{-5}, 3.13 x 10^{-6} and 7.83 x 10^{-7} μg/mL) in duplicate as the reference standard and four replicate wells for the DMSO control. Range finder testing uses all wells of the 96-well plate to test six substances as seven point logarithmic serial dilutions in duplicate.

Comprehensive testing is conducted on 96-well plates using 11 concentrations of E2 in duplicate as the reference standard (**Table 4-1**). Four replicate wells for the DMSO control and four replicate wells for the methoxychlor control are included on each plate.

Table 4-1 Concentrations of E2 Reference Standard Used in Comprehensive Testing

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

[1]Concentrations are presented in μg/mL.

Visual observations for cell viability are conducted for all experimental plates just prior to LUMI-CELL® ER assay evaluation.

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 graphed, and evaluated as follows:

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

Where possible, the concentration that causes a half-maximal response (EC_{50}) is calculated using a Hill function analysis for substances that are posivitive. 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:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{logEC50} - X)\text{HillSlope}}}$$

where Y = response (i.e., relative light units); X = the logarithm of concentration; Bottom = the minimum response; Top = the maximum response; log EC_{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 EC_{50} parameters.

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.

4.1 Range Finder Testing

Agonist range finding for coded substances consists of a seven 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.

4.2 Comprehensive Testing

Comprehensive agonist testing for coded substances consists of 11 point, double serial dilutions, with each concentration tested in triplicate wells of the 96-well plate.

5.0 MATERIALS FOR LUMI-CELL® ER ASSAY AGONIST TESTING

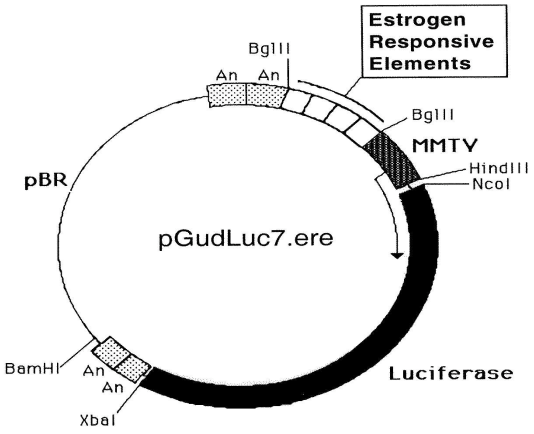
This section provides the materials needed to conduct LUMI-CELL® ER testing, with associated brand names/vendors[1] in brackets.

5.1 BG1Luc4E2 Cells:

Human ovarian cancer cell line stably transfected with a plasmid containing an estrogen response element pGudLuc7.0 (**Figure 5-1**) [XDS].

[1]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.

Figure 5-1 pGudLuc7.ERE Plasmid.



5.2 Equipment and Supplies:

General cell culture equipment, media and supplies suitable to a cell culture facility are needed. Equipment, media, and supplies specific to the LUMI-CELL® ER assay are specified below. Equivalent materials from other commercial sources can be used.

- Berthold Orion 1 Microplate Luminometer [Berthold Cat. No.: Orion 1 MPL3] or equivalent and dedicated computer
- Shaker for 96-well plates
- BackSeal-96/384, white adhesive bottom seal for 96-well and 384-well microplate [Perkin-Elmer, Cat. No. 6005199]
- 17 β-estradiol (CAS RN: 50-28-2)
- Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 10009186R38]
- DMSO, U.S.P. analytical grade
- Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L glucose, with sodium pyruvate, without phenol red or L-glutamine
- Fetal Bovine Serum
- Fetal Bovine Serum, charcoal/dextran treated, triple 0.1 μm sterile filtered
- Gentamycin Sulfate (G418), 50 mg/mL

- L-glutamine, 29.2 mg/mL
- Luciferase Assay System (10-Pack) [Promega Cat. No. E1501]
- Lysis Solution 5X [Promega, Cat. No. E1531]
- Methoxychlor (CAS RN: 72-43-5)
- Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 µg/mL streptomycin
- Phosphate buffered saline (PBS, 1X) without calcium and magnesium
- RPMI 1640 medium, containing L-glutamine
- Trypsin (10X), 2.5% in Hank's balanced salt solution (HBSS), without calcium and magnesium, without phenol red

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

6.0 PREPARATION OF TISSUE CULTURE MEDIA AND SOLUTIONS

6.1 RPMI 1640 Growth Medium (RPMI)

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

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.

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

6.2 Estrogen-free DMEM Medium

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

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.

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

6.3 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 10 mL 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 sterile centrifuge tubes.

1X Trypsin should be stored at -20°C.

6.4 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.

6.5 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 one year.

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 gently to mix; the Luciferase substrate should readily go into solution.
3. After solutions are mixed, aliquot to a 15mL centrifuge tube.
4. Store complete solution at –20°C.

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

7.0 OVERVIEW OF PROPAGATION AND EXPERIMENTAL PLATING OF BG1Luc4E2 CELLS

BG-1 cells are grown as a monolayer in tissue culture flasks in a dedicated tissue culture incubator at 37ºC ± 1ºC, 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/or 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.

7.1 Conditioning in Estrogen-free Medium, and Plating Cells for Experimentation

Cells must be conditioned to an estrogen-free environment 48 to 72 hours prior to plating the cells in 96-well plates for analysis of estrogen dependent induction of luciferase activity. Cells conditioned in estrogen-free medium are then plated (in estrogen-free medium) into 96-well plates at a plating density of 200,000 cells/mL.

8.0 PREPARATION OF TEST SUBSTANCES

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

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

8.1 Preparation of Reference Standards, Control and Test Substances for Range Finder and Comprehensive Testing

8.1.1 Preparation of Reference Standard and Control Stock Solutions

Stock solutions of E2 (1.0×10^{-2} μg/mL) and methoxychlor (313 μg/mL) are prepared in 100% DMSO and stored at room temperature for up to three years or until the expiration date listed in the certificate of analysis for that substance.

8.1.2 Preparation of Reference Standard, Control and Test Substance Dosing Solutions for Range Finder Testing

Range finder testing is conducted on 96-well plates using four concentrations of E2 (5.00×10^{-5}, 1.25×10^{-5}, 3.13×10^{-6} and 7.83×10^{-7} μg/mL) in duplicate as the reference standard. Four replicate wells are used for the DMSO control. Test substances are to be tested at 7 logarithmic dilutions starting at the highest soluble concentration of test substance.

8.1.3 Preparation of Reference Standard and Control Dosing Solutions for Comprehensive Testing

Comprehensive testing is conducted on 96-well plates using 11 concentrations of E2 (1.0×10^{-4}, 5.0×10^{-5}, 2.5×10^{-5}, 1.25×10^{-5}, 6.25×10^{-6}, 3.13×10^{-6}, 1.56×10^{-6}, 7.83×10^{-7}, 3.92×10^{-7}, 1.95×10^{-7}, 9.78×10^{-8} μg/mL) in duplicate as the reference standard. Four replicate wells for the DMSO and methoxychlor control are included on each plate.

8.1.4 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

Comprehensive testing experiments are used to determine whether a substance possesses ER agonist activity in the LUMI-CELL® ER assay. Agonist comprehensive testing for coded substances consists of 11 point, double serial dilutions, with each concentration tested in triplicate wells of the 96-well plate.

Start the 11-point serial dilution series at a single log dilution higher than the concentration giving the highest adjusted RLU value during the range finder (e.g., if the highest adjusted RLU value occurred at a concentration of 0.01 mg/mL, start the serial dilution at 0.1 mg/mL).

9.0 DATA ANALYSIS

Prior to measurement of luminescence, remove treated plates from the incubator. Remove media, then perform visual inspection of cell viability using the scoring in **Table 9-1**.

Table 9-1 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"

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

Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and with software that controls the injection volume and measurement interval. Light emission from each well is expressed as RLU per well.

9.1 Adjusting and Normalizing RLU Values

Subtract background luminescence (average DMSO solvent control RLU value) from test substance, reference standard and control RLU values. Plate induction is calculated using these corrected RLU values. Test substance, reference standard, and control RLU values are then adjusted relative to the highest E2 reference standard RLU value, which is set to 10,000.

9.1.1 Determination of Outliers

The Study Director will use good statistical judgment for determining "unusable" wells that will be excluded from the data analysis and will provide an explanation in the study notebook for any excluded data.

9.1.2 Acceptance Criteria

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 are compared to quality controls (QC) for these parameters derived from the historical database, which are summarized below.

- Induction: Plate induction, as measured by dividing the averaged highest E2 reference standard RLU value by the averaged DMSO control RLU value, must be greater than three-fold.
- Reference standard results: Calculated E2 reference standard EC_{50} values must be within 2.5 times the standard deviation of the historical database EC_{50} mean value.
- Solvent control results: Solvent control RLU values must be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- Positive control results: Methoxychlor control RLU values must be within 2.5 times the standard deviation of the historical methoxychlor control mean RLU value.

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

10.0 RANGE FINDER TESTING

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

- If there are no points on the test substance concentration curve that are above the line representing the mean plus three times the standard deviation of the DMSO control, the highest concentration used in comprehensive testing is the limit dose or the maximum soluble dose.
- If there are points on the test substance concentration curve that are above the line representing the mean plus three times the standard deviation of the DMSO

control, select a concentration that is a single log dilution higher than the concentration giving the highest adjusted RLU value in the range finder, and use that as the highest concentration for comprehensive testing.

- If a substance exhibits a biphasic concentration curve, the range finder experiment should be repeated unless the proposed concentration range for the comprehensive studies will include all concentrations of the biphasic region in the range finding study. If the range finder experiment is repeated and the substance still exhibits a biphasic concentration curve, comprehensive testing must be conducted on the peak of the biphasic curve at the lowest test substance concentration. If the substance is negative at this lowest concentration, then test at the higher concentration. For either peak of the concentration curve, select a concentration that is a single log dilution higher than the concentration giving the highest adjusted RLU value in the range finder and use that as the highest concentration for comprehensive testing.

11.0 COMPREHENSIVE TESTING

Evaluate whether comprehensive experiments have met acceptance criteria (see **Section 9.1.2**).

- If the substance has been tested up to the limit dose or the maximum soluble dose, without causing a significant decrease in cell viability, and there are no points on the concentration curve that are above the line indicating the mean plus three times the standard deviation of the DMSO control, the substance is considered negative for agonism
- If the substance has a positive response at any concentration, the substance is considered positive for agonism.