Appendix B7

High-Throughput System for Screening Estrogen-Like Chemicals

(Provided by Dr. George C. Clark, Xenobiotic Detection Systems, Inc., Durham, NC, USA)

[This page intentionally left blank]

High-Throughput System for Screening Estrogen-Like Chemicals Submitted by Xenobiotic Detection Systems, Inc.

1.0 Detailed procedures for conducting the test:

Mass culture of BG1Luc4E2 cell line: The cell line BG1Luc4E2 has remained stably transfected with the reporter plasmid for over 2 years. Early clones of the cells are stored in liquid nitrogen in 1 ml ampules. Mass culture of the cells are initiated by quickly thawing an ampule and suspending in 5 ml of RPMI 1640 containing 5% fetal calf serum (Hyclone) and 1% pen/strep solution (RPMI growth media) in a T25 culture flask. The cells are then incubated in a 5% CO2 incubator at 37C and allowed to grow to confluence (approximately 24 hrs). The adherent BG1Luc4E2 cells are rinsed with Phosphate Buffered Saline (PBS) and a 1% solution of Trypsin (Gibco) is applied to the cells in the flask and they are incubated in this solution for approximately 1 minute. Hit the side of flask sharply against heel of your palm to dislodge cells from the bottom of the flask and confirm release of the cells by visual examination with a inverted microscope. If not, allow to incubate for another 30 seconds and try again. If cells have been mostly dislodged, then add 5-6 milliliters PBS to the flask and wash the cells out of the flask. The cells are washed out of the flask with PBS and are transferred to a 50 ml centrifuge tube. RPMI 1640 Media containing 5% fetal calf serum is immediately added to the tube to inhibit further cellular digestion by residual Trypsin. The cells are pelleted by centrifugation in a desk top clinical centrifuge at 1000 RPM and resuspended in2 ml of RPMI growth media and repeatedly drawn through a pipet to break up clumps of cells. One ml of the pelleted cells is transferred inoculated into two T75 flask containing 10 ml of RPMI growth media and allowed to grow in the incubator at 37 C with 5% CO2 atmosphere until confluent. The cells from one T75 flask can be used to inoculate four T75 flasks by repeating the trypsin procedure for passage of cells described above.

Conditioning of BG1Luc4E2 cells for measuring estrogen dependent luciferase activity:
The BG1Luc4E2 cells must be grown in estrogen free conditions to allow measurement of estrogen dependent induction of luciferase activity [Rogers, 2000 #197]. The following procedure has been developed to condition the cells in estrogen free conditions and then allow plating of the cells in a 96 well plate format for HTPS analysis of estrogen dependent induction of luciferase activity. Two T75 flasks of cells grown in RPMI growth media that have reached confluence are removed from the incubator and the media is poured off of the cells. The cells are washed with 10 ml of PBS and then 10 ml of estrogen free media is added to the flask. Estrogen free media consists of Dulbeco's Minimal Essential Media supplemented with 5% fetal calf serum that has been stripped of estrogen by treatment with activated carbon and is free of phenol red pH indicator. The flasks of cells are returned to the incubator for 24 hours. At this time the cells are ready to be plated into 96 well plates. The two T75 flasks are treated with trypsin and cells washed with PBS and the cells are resuspended in approximately 15 ml of Estrogen free media. The cells are counted with a hemocytometer and adjusted to a concentration of 300,000 cells per ml in Estrogen free media. Two hundred microliters of media are dispensed into each well of a 96 well plate (60,000 cells per well). The plate is returned to the incubator of 24 hours to allow them to adhere and grow in the plate.

Dosing 96 well plates of BG1Luc4E2 cells with Estrogen and test compounds:
Dilutions of beta-estradiol and test compounds are prepared in DMSO. A standard solution of 10 ng/ml of beta-estradiol in DMSO is used to prepare dilutions of this standard. Four microliters

of DMSO is added to ten 13 mm glass tubes. To the first tube 4 microliters of the 10 ng/ml standard solution of beta-estradiol is added to the 4 microliters of DMSO in the tube. The tube is vortexed and four microliters transferred to the next tube in the series. This is repeated for each of the 10 tubes creating a two fold dilution series. To each tube 400 micoliters of Estrogen free media is added to the DMSO solution and the tube vortexed vigorously. Similar dilution series are produced for test compounds or extracts being analyzed for estrogenic activity by the BG1Luc4E2 cells.

The 96 well plates of cells are removed from the incubator and media removed from the adherent cells by inversion onto absorbent plastic backed paper. The cells are rinsed with 50 microliters of PBS and this also removed by inversion on absorbent plastic backed paper. Two hundred microliters of beta-estradiol solutions or test compound is then applied to the 96 well plates. The outside rows of the plate are not used for determinations since we have found that these wells are very sensitive to environmental conditions and do not provide reproducible quantitative readings for induction of luciferase activity with the BG1Luc4E2 cells. The dosed plates of BG1Luc4E2 are returned to the incubator and incubated for 24 hours to allow maximal induction of luciferase activity in the cells.

Measurement of estrogen induced luciferase activity in BG1Luc4E2 cells:
Luciferase that is produced in the BG1Luc4E2 cells in response to exposure to estrogen accumulates in the cytoplasm of the cells over the twenty-four hour incubation. To measure luciferase the cells must be lysed and substrates for measurement of luciferase enzyme activity added and results, light emission by the enzymatic activity measured in a luminometer. To accomplish this, the cells are removed from the incubator and media removed by inversion of the plate on absorbent plastic backed paper, and the plates tapped on the paper to remove residual media. The cells in the 96 well plates are washed with PBS and the cells examined with an inverted microscope to observe whether any observable toxicity or displacement of the lawn of cells grown on the bottom of the plates has occurred. The PBS is then removed by inversion of the plate on absorbent plastic backed paper and a reflective white plate tape (Packard) applied to the clear bottoms of the 96 well plates to increase the efficiency in measuring emitted light from the wells of the plate. A dilute detergent lysis reagent (Promega) is then added to the cells and the cells shaken in a vibrating mixer to aid in lysis of the cells. The cells are then placed in a Lucy 2 Luminometer (Anthos Analytical) which is a robotic instrument that delivers 50 microliters of luciferase enzyme reagent (Promega) to each well and then measures the resulting light omitted (integrating light emission from the wells for a 15 second period). The light emission is expressed as Relative Light Units (RLU) for each well. The measured RLU by the instrument is then exported to a Compaq computer and analyzed with software designed to provide analysis of the RLU of the beta-estradiol standard, subtraction of blank responses and interpolation of unknown responses to the standard curve.

A sample template for the 96 well plate analysis includes, the B-estradiol standard, test chemicals for analysis of potential estrogenic activity, and measurements of extracts of environmental chemicals for luciferase activity as well as control samples of background or solvent blank in the system. As described earlier, we have determined that the responsiveness of the BG1Luc4E2 cells is extremely sensitive to an edge effect in which determinations made in the outer wells of the plate are extremely variable and result in reduced confidence of analysis of luciferase activity in these wells. Therefore, on a 96 well plate 56 wells of the plate are useful for determination of estrogen dependent induction of luciferase activity. The ten standard dilutions of beta-estradiol are therefore applied to wells B2 through E3. This provides a standard curve of beta-estradiol in a two fold dilution series from 50 pg/ml down to 0.097 pg/ml. Solvent or blank controls are applied to a number of wells of the plate to provide replicate of estimates of the background response expected for luciferase expression by the BG1Luc4E2 cells (wells F3, G3, B4, F10, G10).

We have determined that the output of receptor mediated gene expression systems is best estimated by a 4 parameter Hill equation. Input of the RLU for samples is entered into this equation and the pg of estrogenic like activity for the sample is estimated from the model. The output is expressed as pg of estrogenic activity derived from the model.

The output of the analysis corrected for the amount of sample extracted for the determination. The estimated estrogenic activity of each sample is corrected for the dilution of sample extract that was used in the analysis. This analysis also displays a non-modeled graphic display of the data. The figure provides a graphic display of the output from the B-estradiol standard and a test chemical.

2.0 Dose-selection procedures, including the need for any dose range-finding studies or acute toxicity data prior to conducting the test:

The dose selection for Beta-estradiol standard is based upon the responsiveness of our genetically engineered BG1Luc4E2 cells to estrogen. The cells are extremely sensitive to estrogen and estrogen-like chemicals demonstrating a significant response to as little as 0.39 pg/ml solution of beta-estradiol. The BG1Luc4E2 cells respond with a dose dependent induction of luciferase activity up to a maximal concentration of 50 pg/ml.

A screening testing for estrogenic activity of a chemical is performed by initially performing a dose range finding experiment with the chemical. Ten milligrams of a pure chemical for testing of estrogenic activity is weighed out into glass vial and dissolved in one-milliliter of DMSO. A 10 fold dilution series is of the chemical is then produced by adding 10 microliters of the test compound to 90 microliters of DMSO in a 13 mm glass tube and repeating this procedure for six dilutions creating a dilution series of 1 mg/ml down to 1 ng/ ml. Four micoliters of these solutions is then added to 400 microliters of media (final concentrations of 10 micrograms/ml down to 10 picrograms/ml) and applied to the BG1Luc4E2 cells to evaluate induction of luciferase activity. Using this screening format 8 compounds can be evaluated per plate of BG1Luc4E2 cells. If a test chemical is positive for induction of luciferase activity a second experiment using a two fold dilution series at the concentrations that are active is performed. An example two-fold analysis of the activity of diethlstilbesterol was included in the example analysis provided from 50 pg/ml down to a concentration of 1.56 pg/ml.

3.0 Endpoint(s) measured:

The endpoint measured is the induction of luciferase activity in a human ovarian carcinoma, BG-1 that has been genetically engineered with a reporter gene construct that expresses the enzyme luciferase in response to exposure of the BG1Luc4E2 cell line to estrogen or estrogen-like chemicals.

4.0 Duration of exposure:

The duration of exposure to B-estradiol to induce maximal expression of the luciferase reporter gene in our BG1Luc4E2 bioassay is 24 hours. A significant induction of estrogen dependent expression of luciferase activity can be measured as early as two hours after exposure of the cells with half maximal induction occurring at eight hours following exposure of the BG1Luc4E2 cells [Rogers, 2000 #197].

5.0 Known limits of use:

The only known limits of use of the BG1Luc4E2 bioassay for measuring estrogen dependent induction of luciferase activity is if the chemical or environmental extract is toxic to the cellular

system. Toxicity could potentially inhibit induction of estrogen-dependent induction of luciferase activity. However, overt toxicity is assessed in the system by visual observation of the cells before measurement of luciferase induction. The sensitivity and large dynamic range of the BG1Luc4E2 bioassay system allows for dilution of the sample test compound to limit toxicity and yet estimate potential induction of estrogen-dependent luciferase expression.

6.0 Nature of the response assessed:

The response that is measured is the enzymatic activity of luciferase that is induced in our genetically engineered cells BG1Luc4E2 that express this enzyme in response to exposure to estrogen and estrogen-like chemicals. The enzyme activity is assessed by the production of light in a luminometer following addition of enzyme reagents.

7.0 Appropriate vehicle, positive, and negative controls and the basis for their selection:

The vehicle used for application of chemicals is DMSO. The response from the vehicle is the negative control for chemicals and solvent for extraction of environmental samples is the vehicle in testing environmental extracts. The positive control is B-estradiol which is the hormone ligand for the estrogen receptor.

8.0 Acceptable range of vehicle, positive and negative control responses:

Criteria have not been established for the range of vehicle, positive and negative control responses as yet since this system is in development. However, control charts are being established for responses. Generally, the vehicle response should be less than 20% of the maximal induction of the positive control at this time in development.

9.0 Nature of the data to be collected and the methods used for data collection:

The data collected are measurements of the light induction produced by the luciferase enzyme and are measured as relative light units detected by a luminometer. The data are stored as electronic files in a computer system that is backed up daily. They are secured in the laboratory and follow methods described in EPA method 2185: Good Automated Laboratory Practices.

10.0 Type of media in which data are stored:

The data are stored electronically in a Windows NT network. The network hard disk is backed up every 24 hours on a Compaq workstation.

11.0 Measures of variability:

In the screening mode of the assay replicate analysis are not performed, however the use of a varying doses of compound allows an estimate if the response demonstrates a trend. In confirmation assays, triplicate analysis is performed and statistical model testing can be performed on this data.

12.0 Statistical or non-statistical method(s) used to analyze the resulting data (including methods to analyze for a dose-response relationship). The method(s) employed should be justified and described:

The data that is generated from the B-estradiol standard is modeled using a four parameter Hill equation. The Hill equation is a mathematical model that generates the best fit for receptor mediated induction of gene expression (Kohn, Lucier et al. 1993; Kohn, Sewall et al. 1996; Kohn, Walker et al. 2001).

13.0 Decision criteria or the prediction model used to classify a test chemical (e.g., positive, negative, or equivocal), as appropriate:

There have been three initial criteria adopted for assigning a positive designation for a chemical in the BG1Luc4E2 estrogen screen. The first criteria is that the chemical induces luciferase activity that is greater than 3 times the standard deviation of the DMSO blank at an applied concentration of 10 micrograms/ml (designated +). The second more restrictive criteria are that the chemical induces BG1Luc4E2 bioassay system at both 10 and 1 microgram/ml (designated as ++). The third criteria is that the chemical induces luciferase activity at a number of concentrations in a two-fold dilution re-analysis demonstrating dose-dependent induction of luciferase and a relative response to B-estradiol can be assigned (designated +++). A negative designation for activity in the BG1Luc4E2 bioassay estrogen screen is assigned when no induction of luciferase activity is detected at any concentration over 3 times the standard deviation of the DMSO blank.

14.0 Information that is included in a test report.

Information in test reports include the standard curve generated by a two-fold dilution series of the positive control chemical B-estradiol, background determinations of solvent carrier (DMSO), modeling of the B-estradiol response using a four parameter Hill equation, and response of at six different 10 fold dilutions from 10 micrograms/ml down 10 picrograms/ml in our BG1Luc4E2 bioassay.

15.0 References

Kohn, M. C., G. W. Lucier, et al. (1993). "A mechanistic model of effects of dioxin on gene expression in the rat liver." Toxicol Appl Pharmacol **120**(1): 138-54.

Kohn, M. C., C. H. Sewall, et al. (1996). "A mechanistic model of effects of dioxin on thyroid hormones in the rat." Toxicol Appl Pharmacol **136**(1): 29-48.

Kohn, M. C., N. J. Walker, et al. (2001). "Physiological modeling of a proposed mechanism of enzyme induction by TCDD." Toxicology **162**(3): 193-208.

Rogers, J. M. and M. S. Denison (2000). "Recombinant cell bioassays for endocrine disruptors: development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals." In Vitr Mol Toxicol **13**(1): 67-82.

[This page intentionally left blank]