APPENDIX II
- H295R Cell Exposure Protocol -

University of Saskatchewan
Toxicology Centre
Environmental Toxicology Laboratory

STANDARD OPERATING PROCEDURE

Exposure of H295R human adrenocortical carcinoma cells to assess the effects of chemicals on testosterone and 17β-estradiol production
(ATCC CLR-2128)

Version 3 June 26, 2007

Markus Hecker, Ph.D.
John Laskey, Ph.D.
Angela Buckalew, B.S.
Paul D. Jones, Ph.D.
John L. Newsted, Ph.D.
John P. Giesy, Ph.D.

Supported through:
Supported through:
Toxicology Centre

Correspondence to:
Dr. Markus Hecker
137 Toxicology Centre
University of Saskatchewan
Saskatoon, SK S7N 5B3
T: (517)-432-6333
F: (517)-432-2310

Revisions to an existing SOP, addition of an SOP change form, or preparation of a new SOP must be reviewed, approved, and signed by the following:

Authored By: Markus Hecker, Ph.D.
John Laskey, Ph.D.
Angela Buckalew, B.S.
Paul D. Jones, Ph.D.
John L. Newsted, Ph.D.
John P. Giesy, Ph.D. Date: 26/06/06

Reviewed By: _____ Date:_____
(Principle Investigator)

Reviewed By: _____ Date:_____
(Co-Principle Investigator)

Reviewed By: _____ Date:_____
(Project Manager)

Reviewed By: _____ Date: _____
(QA Coordinator)

Reviewed By: _____ Date: _____
(US-EPA Project Officer)

DEFINITIONS AND ACRONYMS

DMEM w/ Hams F-12	Dulbecco's Modified Eagles Medium with Nutrient Mixture F-12 Hams
Passage	Identifier that describes the number of times cells were split after initiation of a culture from frozen stock. The initial passage that was started from the frozen cell batch is assigned the number one (1). Cells that were split 1x are labeled passage 2, etc.
PBS	Dulbecco's Phosphate Buffered Saline Solution
QC	Quality Control
QA	Quality Assurance
SOP	Standard Operating Procedure

TABLE OF CONTENTS

1 PURPOSE

The purpose of this document is to provide a consistent format for exposure studies with the H295R cell line and subsequent steps to measure hormone production of testosterone (T) and estradiol (E2) into medium after treatment with chemicals of interest or environmental samples.

2 SCOPE AND APPLICATION

The protocol gives a detailed description of the methodology for the plating of H295R cells (ATCC CLR-2128) in a 24-well plate setup and the conduct of exposure experiments with chemicals. It also lists all further steps and provides detailed information regarding the handling of samples including the extraction of hormones from medium, storage of samples and extracts, and refers to detailed SOPs or protocols for the subsequent analysis of T and E2. Finally, the protocol describes the procedure for the Live/Death® cytotoxicity test in the same plate to evaluate any possible effects of the test chemicals on cell viability.

The protocol provides a complete list of required reagents and solvents, and describes the preparation of all solutions and reagents used during the exposure experiments with the cells as well as during the subsequent extraction, hormone analyses and cell viability measurements. It also provides descriptions of QA/QC procedures including the analysis of positive controls with each experiment to verify proper performance of the cells.

Cell maintenance procedures including initiating cell cultures from frozen stock, cultivation and splitting of the growing cells, the freezing of cells for storage in liquid nitrogen and the maintenance of a proper cell culture diagram that allows for tracking the progress of a cell line are described in detail in a different protocol (H295R culture protocol).

Important:

- *Prior to the initiation of cell culture and any subsequent experiments each laboratory has to demonstrate the performance of their hormone detection system (e.g. ELISA, RIA, LC-MS) by analyzing supplemented medium spiked with an internal hormone control (APPENDIX I). The internal standard will be prepared by the lead laboratory (MSU/UofS) and send out to all participating laboratories. Non-conformance with these performance criteria results in the rejection of the selected hormone detection method. Performance criteria including allowable variation from target concentrations will be defined based on the repeated measurement of the internal standards by two of the laboratories that participated in the original inter-laboratory comparison studies (RTP and MSU/UofS).*
- *Due to changes in estradiol producing capacities of the cells with age (Hecker et al. 2006), cells have to be cultured following a specific pattern before they can be used for the conduct of experiments: After initiation of an H295R*

culture from an original ATCC batch following the procedures outlined in the H295R Culture Protocol, cells are to be grown for five (5) passages (cells need to be split 4-times). Passage five cells are to be frozen down in liquid nitrogen (H295R Culture Protocol; Chapter 5.3 Freezing H295R Cells). After starting cells from these frozen batches following the procedures described in the H295R Culture Protocol (H295R Culture Protocol; Chapter 5.1 Starting Cells from Frozen Stock), they need to be cultured for at least four (4) additional passages (passage # 5.4) until they can be used for exposure experiments. The maximum passage at which experiments are conducted shall not exceed 7 (passage # 5.7).

- *Prior to initiation of exposure and QC plate experiments, it must be demonstrated that the hormone detection system to be used can detect hormone concentrations in supplemented medium with sufficient accuracy and precision.*
- *Prior to freezing them down, a subset of passage five (5.0) cells is run in an QC plate (Chapter 5.1 Quality Control Experiment for cell performance) to verify whether basal production of hormones and response to a positive control chemical meet the quality criteria defined in the exposure protocol (Chapter 8 Records, Documentation and QC Requirements). For those laboratories that already frozen their passage five (5.0) cells down, they can thaw one of the frozen batches and grow it for three (3) passages (passage # 5.3) and use these cells for the QC plate experiment.*

3 SAFETY CONSIDERATIONS

The H295R cells are a human cell line, and though they are considered non-infectious, proper biosafety measures should be observed such as the use of a biosafety cabinet, proper disposal/autoclaving of waste, and sterilization of all surfaces prior and after work with the cells. Gloves and lab coat are the minimal amount of protection that should be worn. Avoid wearing gloves that could trap liquid nitrogen next to the skin for an extended period of time. Snug fitting non-permeable (waterproof) gloves are best. Eye protection is also recommended when exposure to splashing liquid nitrogen, solvents and biological active material is possible. Special safety requirements need to be considered when working with ether, tritium and ethidiumbromide.

4 SOLUTIONS AND REAGENTS

4.1 Exposure experiments

4.1.1 EQUIPMENT, MATERIALS AND REAGENTS

Equipment: Pipet-Aid, Stir Plate; Analytical Balance

Materials: Weigh Boats; Chemical Spatula; Pasteur pipettes; Waste Container, 2,10, 200 and 1,000 ul Pipettes, Sterile Pipette Tips, Sterile Amber Glass Vials (National Scientific Company; Cat# C4000-2W)

Reagents & Chemicals:

- Dulbecco's Phosphate Buffered Saline (PBS) *
- Stock Medium *
- Supplemented Medium *
- Sterile 1x Trypsin *
- DMSO (Aldrich, Cat. No. D2438)
- Forskolin (MW = 410.50); will be provided by Battelle)
- Prochloraz (MW = 376.67); will be provided by Battelle)
- Test Chemicals (will be provided by Battelle)

* The preparation and storage conditions for Dulbecco's Phosphate Buffered Saline (PBS), Stock and Supplemented Medium, and trypsin stock are described in detail in the in the H295R culture protocol.

4.1.2 METHOD, PROCEDURES AND REQUIRMENTS

1. Prepare stock concentrations of forskolin, prochloraz and test chemicals dosing solutions in DMSO. **Note:** All chemicals will be provided by Battelle and the necessary amounts are already pre-weighed into the shipping vials. For forskolin and prochloraz 100 mM stock solutions need to be prepared. This will be done by adding the amount of DMSO specified below. All descriptions for the preparation of Test Solutions are provided below.

2. If not specified differently in the data sheet provided with each shipment, add 100 uL of DMSO to the forskolin and prochloraz as well as all test chemical vials, cap and vortex to mix the chemical in the DMSO and to help to dissolve it. This results in 100 mM stock solutions for forskolin and prochloraz. For all test chemicals this results in the Stock 1 Test Solution.

3. Dilute these stock solutions as follows:

- *Forskolin:* Dilute 100 mM stock solution 1:10 (10 uL of 100 mM Stock 1 + 90 uL DMSO) to make 100 uL of a 10 mM solution. Dilute 10 uL of this 10 mM solution 1:10 to make 100 uL of a 1 mM solution. Store both, 10 and 1 mM stocks at 4°C until further use.
- *Prochloraz:* Dilute 100 mM stock solution 1:33 (3 uL of 100 mM Stock 1 + 97 uL DMSO) to make 100 uL of a 3 mM solution. Dilute 10 uL of this 3 mM solution 1:10 to make a 0.3 mM solution. Store both 3 and 0.3 mM stocks at 4°C until further use.
- *Test Chemicals:* Dilute Stock 1 1:10 (10 uL of 100 mM Stock 1 + 90 uL DMSO) to make 100 uL of Stock 2 solution. Continue diluting these stock solutions in similar manner with DMSO until a total of seven (7) dilutions have been made (Stock 1 – Stock 7). Dilutions should be made serially from the next greater concentration (e.g. a Stock 4 would be made by adding 10 μL of a Stock 3 to 90 μL of DMSO).

4. Label each vial as follows:
   - *Forskolin & prochloraz:* Chemical name, date the solution was made, concentration of chemical in mM, type of solvent (DMSO) and initial.
   - *Test chemicals:* Chemical ID number, date the solution was made, Stock identifier (e.g. Stock 1), type of solvent (DMSO) and initial.

5. Store the stock solutions in DMSO in fridge.

5 CELL EXPOSURE PROCEDURES

Note: When initiating an experiment using a new batch or passage of cells, a control plate needs to be run in parallel with chemical testing to evaluate the performance of the cells. A description of the procedures for such a control experiment is given at the end of this section.

5.1 Quality Control Experiment for cell performance

A quality control (QC) H295R cell performance test needs to be conducted when using a new batch or new passage of cells to control for possible changes in hormone production as a function of cell age. This test will be conducted in a 24 well plate in parallel to the exposure experiment described below, following the same incubation, dosing, cell viability/cytotoxicity, hormones extraction and hormone analysis protocols described in this Standard Operating Procedure. The QC plate will be dosed with a known inducer (forskolin) and inhibitor (prochloraz) of E2 and T synthesis each at two different doses greater and less than the EC50s previously described for these compounds. A detailed description of the plate layout is provided in Table 1.

Table 1: Quality control plate layout for testing performance of unexposed H295R cells and cells exposed to known inhibitors (PRO = prochloraz) and stimulators (FOR =

forskolin) of E2 and T production. A 70% methanol solution will be added to all MeOH wells after termination of the exposure experiment and removal of medium (see Live/Dead® Product Insert).

	1	2	3	4	5	6
A	Blank [a]	Blank [a]	Blank [a]	Blank [a] + MeOH [b]	Blank [a] + MeOH [b]	Blank [a] + MeOH [b]
B	DMSO 1µL	DMSO 1µL	DMSO 1µL	DMSO 1µL + MeOH [b]	DMSO 1µL + MeOH [b]	DMSO 1µL + MeOH [b]
C	FOR 1µM	FOR 1µM	FOR 1µM	PRO 0.3µM	PRO 0.3µM	PRO 0.3µM
D	FOR 10µM	FOR 10µM	FOR 10µM	PRO 3µM	PRO 3µM	PRO 3µM

[a] Blank wells receive medium only.
[b] Methanol (MeOH) will be added **after** the exposure is terminated and the medium is removed from these wells.

Note: To verify that performance of H295R Cells under Standard Culture Conditions is meeting the QC requirements for this assay, prior to initiating the exposure experiments a subset of passage five (5.0) cells is run in a QC plate. For those laboratories that already froze their passage five (5.0) cells down, they can bring up one of the frozen batches and grow it for three (3) passages (passage # 5.3) and use these cells for the QC plate experiment. QC criteria for this verification experiment are provided in APPENDIX I.

5.2 Plating and Pre-Incubation of Cells

Note: Due to changes in estradiol producing capacities of the cells with age (Hecker et al. 2006), cells have to be cultured following a specific pattern before they can be used for the conduct of experiments: After initiation of an H295R culture from an original ATCC batch following the procedures outlined in the H295R Culture Protocol cells need to be grown for five (5.0) passages (cells need to be split 4-times). These cells are to be frozen down in liquid nitrogen (H295R Culture Protocol; Chapter 5.3 Freezing H295R Cells). After starting cells from these frozen batches following the procedures described in the H295R Culture Protocol (H295R Culture Protocol; Chapter 5.1 Starting Cells from Frozen Stock), they need to be cultured for at least four (4) additional passages (passage # 5.4) until they can be used for exposure experiments. The maximum passage at which experiments are conducted shall not exceed 7 (passage # 5.7).

5.2.1 EQUIPMENT, MATERIALS AND REAGENTS

Equipment: Biosafety Cabinet; Pipet-Aid; Centrifuge; Microscope

Materials: 10 mL Strippettes; Waste Container; 15 and 50 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052 [15 mL] and 430290 [50 mL]); 24-well Tissue Culture Plates (Corning Inc. Costar Cat# 524); Sterile 1.5 mL Eppendorf tubes; 10µL, 100 µL, 1mL Pipettes; Sterile Pipette Tips; Hemocytometer

Reagents: Sterile Supplemented Medium (see H295R culture protocol, chapter 4.2); NCI-H295R cells (ATCC Cat # CRL-2128) cultured under standard conditions as described in the H295R culture protocol for 6 and 11 generations; Sterile 1x Trypsin- EDTA (see H295R culture protocol, chapter 4.4); Sterile PBS (see H295R culture protocol, chapter 4.1)

5.2.2 METHODS, PROCEDURES AND REQUIREMENTS

1. Prewarm PBS and supplemented medium to 37°C in a water bath or incubator

2. Thaw and pre-warm 1x Trypsin-EDTA at 37°C in a water bath or incubator.

3. Wipe all tubes/containers off with 70% ethanol and transfer cells and solutions into a biosafety cabinet.

4. Remove a H295R cell culture plate cultured under standard conditions as outlined in the H295R culture protocol from incubator and place in biosafety cabinet.

   Note: Depending on the number of plates needed for the exposure experiment and the confluency of the cells in the culture dishes a certain number of culture plates may be needed. General rule: use 1 petri dish of 95-100% confluent cells to plate two 24-well plates at a target density of 300,000 cells per mL.

5. Prepare centrifuge tube or small sterile glass bottle with about 11 mL media for every plate to be trypsinized.

6. Measure 15 mL of PBS for each plate to be split to a 50 mL centrifuge tube (e.g. 45 mL for three plates; use 2nd tube for 4-6 plates, etc.).

7. Carefully pipette old medium off the culture plate without disturbing cells.

8. Rinse plate with 5 mL of sterile PBS, and discard PBS. Rinse a total of 3 times. Make sure to change pipette tips between each rinsing. **Note:** Rinse gently to avoid detaching cells from the plate by adding PBS down the side of the well.

9. Add 1.5 mL of sterile 1x trypsin/plate and gently swirl plate to distribute trypsin evenly (volume should be adjusted in accordance with plate/flask size).

10. Wait for the cells to detach from the bottom of the plate.

    Note: Plate can be placed in the incubator if the cells do not separate easily. Also, need to be careful not to knock or shake plate to avoid clumping of cells.

    Note: Remember that the trypsin will kill the cells if left on for too long. Watch the cells closely and stop the trypsin action as soon as the cells have separated from the plate/flask (typically this should take not longer than 4-5 minutes). Cells should not been exposed to trypsin for more than 10 minutes!

11. Harvest the trypsinized cells and transfer them into the centrifuge tube or bottle with medium.

12. Thoroughly mix (aspirate using 10 mL stripette) the cell suspension of medium and trypsinized cells to make the content homogenous. Note: This is important for accurate cell counts because the cells tend to clump.

13. Take a small sub-sample (30-50 μL) of the well-mixed cell suspension and transfer to an Eppendorf tube.

14. Clean the hemocytometer and the cover glass with 70% Ethanol. Add 10 ul of the cell solution from the Eppendorf tube under the cover glass in the hemocytometer. Count the cells – at least 3 squares from each side of the hemocytometer. Calculate the mean of all the counts. The cell density is the mean cell count x 10^4. For example if the mean count is 110, there are 1,100,000 cells/mL = 1.1 x10^6 cells/mL

15. Calculate the volume of cell solution needed for the selected number of 24-well plates (calculate 1.2 mL media/well).

16. Cells need to be seeded at 300,000 cells/mL medium resulting in approximately 50-60% confluency in the wells.

    Note: This is the preferred density of cells for optimal hormone production in the medium. At higher densities cells tend to be affected by hormonal feedback mechanisms and hormone production per patterns were shown to be altered.

17. Dilute the cell solution to the desired plating density. Thoroughly mix the cell solution to assure homogenous cell density.

18. Plate the cells with 1 mL of the cell solution/well.

19. The new plates should be labeled with the cell type, initials and plating date. Individual wells (samples) should be labeled with sample name.

Note: All of the above steps with the exception of steps 14 & 15 (counting of cells) need to be conducted under sterile conditions in a biosafety cabinet.

20. Incubate seeded plate in incubator at 37^{o}C under a 5% CO_2 in air atmosphere for 24 hours.

5.3 Exposure of Cells

5.3.1 EQUIPMENT, MATERIALS AND REAGENTS

Equipment: Biosafety Cabinet; Pipet-Aid, Incubator (37^{o}C, 5% CO_2); Vortex, Microscope

Materials: 10 mL and 25 mL Strippettes; Waste Container; 15 and 50 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052 [15 mL] and 430290 [50 mL]); 24-well Tissue Culture Plates (Corning Inc. Costar Cat# 524) seeded at 300,000 cells per well with H295R cells and pre-incubated for 24 hours; 2 µL and 10µL Pipettes; Sterile Pipette Tips

Reagents:

- Sterile Supplemented Medium (see H295R culture protocol, chapter 4.2)
- DMSO (Aldrich, Cat. No. D2438)
- Test chemical stock solutions (serial dilutions 1 – 6; see chapter 4.1)

5.3.2 METHODS, PROCEDURES AND REQUIREMENTS

1. Remove cells from incubator that have been pre-incubated for 24 and check under microscope to assure good condition (attachment, morphology) prior to dosing.

2. Place cells in biosafety cabinet and remove old medium.

3. Add new medium (1mL/well) to all wells.

4. Dose cells in the well by adding 1ul of the appropriate stock solution in DMSO / 1 mL medium (well). This way the final concentration of DMSO is 0.1%.

5. For solvent controls dose 1ul DMSO / 1 mL medium (well) directly into the well.

6. Dose cells according to the exposure layout as indicated in Table 3.

Table 3: Dosing schematic for the exposure of H295R to test chemicals in a 24 well plate. Dosing is calculated based on a total volume of 1 mL per well

	1	2	3	4	5	6
A	DMSO 1µL	DMSO 1µL	DMSO 1µL	Stock 4 1µL	Stock 4 1µL	Stock 4 1µL
B	Stock 1 1µL	Stock1 1µL	Stock 1 1µL	Stock 5 1µL	Stock 5 1µL	Stock 5 1µL
C	Stock 2 1µL	Stock 2 1µL	Stock 2 1µL	Stock 6 1µL	Stock 6 1µL	Stock 6 1µL
D	Stock 3 1µL	Stock 3 1µL	Stock 3 1µL	Stock 7 1µL	Stock 7 1µL	Stock 7 1µL

Stock 1-7: 1 µL of appropriate stock solution needs to be added to each well.

7. Incubate dosed plate in incubator at 37°C under a 5% CO_2 in air atmosphere for 48 hours.

5.4 Exposure Termination and Medium Storage

5.4.1 EQUIPMENT, MATERIALS AND REAGENTS

Equipment: Microscope; -80ºC freezer

Materials: 1.5 mL Eppendorf tubes; 1 mL Pipette, Pipette Tips; 24-well Tissue Culture Plates (Corning Inc. Costar Cat# 524) seeded at 300,000 cells per well with H295R cells and exposed for 48 hours

5.4.2 METHOD, PROCEDURES AND REQUIRMENTS

1. Remove exposure plate from incubator and check every well under the microscope for cell condition (attachment, morphology, degree of confluence), look for signs of distress or cytotoxicity. Record your observations.

2. Label Eppendorf tubes appropriately.

3. Split medium from each well into two equal amounts (approx. 490 µL each) and transfer to two separate Eppendorf tubes appropriately labeled (e.g. a & b).

4. Freeze media at -80ºC until further processing (see 7.1; Hormone Extraction).

5. Subject cells immediately after removing medium to cell viability test (see Chapter 6). Remove medium a row or column at a time and add 200 µL PBS with Ca^{+} and Mg+ to each well. **IMPORTANT:** Make sure that cells do not dry out.

6 CELL VIABILITY MEASUREMENTS USING THE LIVE/DEAD® VIABILITY/CYTOTOXICITY KIT

The *LIVE/DEAD* Viability/Cytotoxicity Kit (L-3224) gives a simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability – intracellular esterase activity and plasma membrane integrity. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (Ex/Em ~495 nm/~515 nm). Ethidium homodimer 1 (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (Ex/Em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually non-fluorescent before interacting with cells.

The purpose of this section is to provide a consistent format for cytotoxicity testing with H295R cell line using a *LIVE/DEAD* Viability/Cytotoxicity Kit (L-3224). The cytotoxicity testing will be conducted in the same plate the chemical exposure took place, and must be conducted immediately after termination of the exposure experiments.

The methods described here are have been optimized based on the use of 24 well plates and the Fluoroskan Ascent plate reader.

6.1.1 EQUIPMENT, MATERIALS AND REAGENTS

Equipment: Vortexer; Microscope; Eppendorf Multipipette; Fluoroskan Ascent Fluorometric Microtiter Plate Reader (Thermo Electron Corporation)

Materials: 100-1000, 20-200, 2-20 and 0.5-2 µl Pipettes, Pipette Tips; 15 and 50 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052 [15 mL] and 430290 [50 mL]); 24-well Tissue Culture Plates (Corning Inc. Costar Cat# 524) seeded at 200,000 cells per well with H295R cells and exposed for 48 hours

Reagents:

- Cell viability assay reagents; sold either as a kit from Molecular Probes (#L-3224; Eugene, OR) or as individual components:
  - Calcein AM (Molecular Probes #C-3100); MW = 994.87; made up as 4000x (2 mM) stock (50 µg/12.56 µL DMSO)
  - Ethidium homodimer I (Molecular Probes #E 1169); MW = 857; made up as 2000x (1 mM) stock in DMSO
- Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBS; see chapter 4.1.1)

6.1.2 METHOD, PROCEDURES AND REQUIRMENT

Preparation steps (prior to assay)

1. Inspect plates visually with and without microscope - check degree of confluence, homogeneity from well-to-well, and any signs of cytotoxicity or altered morphology. Note all observations in laboratory notebook.

2. Prepare viability assay reagent (refer to Supplies and Biochemicals Section): Each plate will need 9.6 mL (24 wells * 400 µL) plus a little extra (1.4 mL). Dilute the appropriate amounts of calcein and Ethidium with the appropriate volume of media without FBS as shown below:

   For 11 mL of viability assay reagent, add
   Calcein stock (µL): 5.5 µL
   Ethidium bromide stock (µL) 22 µL

   CAUTION: Ethidium bromide homodimer is a powerful mutagen - handle with care and throw contaminated tips, etc., into biohazard bags

3. Set up fluorometric plate reader following the manufacturers specifications.

Cell Viability Assay Procedure (process one plate at a time)

1. Remove plate from incubator and remove media for subsequent hormone extraction as described in chapter 5.3. Then rinse 1 time with PBS. **Note:** Remove medium from one row or column at a time and fill with PBS before removing medium from second row/column to prevent cells from drying.

2. Add 200 µL of PBS with Ca^{2+} and Mg^{2+} to all wells.

3. Add 400 µL of viability assay reagent to all wells using an Eppendorf Multipette.

4. Incubate at room temperature for 45 minutes (seal plate to prevent evaporation).

5. Scan plate in the Fluoroskan Ascent reader using the above described settings.

6. Export/print data (check that values are appropriate, otherwise adjust sensitivity and rescan). Data analysis is discussed in Section 6.1.3 along with an example raw data file and a Microsoft Excel spreadsheet version of a sample data analysis.

6.1.3 Data analysis

Average the three measurements for calcein AM and ethidium bromide. Divide the average calcein AM fluorescence for each sample by its ethidium bromide homodimer fluorescence to obtain a live to dead ratio. Graph the average calcein AM fluorescence and standard deviation for the negative control, solvent control, and each concentration tested. Examine the calcein AM data visually. If the blank has greater viability than the other treatments, the solvent may be toxic to the cells. If viability decreases with increasing concentration of the test substance, the test substance may be toxic to the cells. In either of these cases, the hormone data must be regarded with great suspicion. If the solvent is toxic, try a different solvent or a lower concentration of solvent. If the test substance is toxic conclude that cytotoxicity is likely to preclude any other effects at this test concentration.

7 HORMONE EXTRACTION AND ANALYSIS

Each laboratory can use a hormone detection system of its choice for the analysis of the production of T and E2 by H295R cells. Prior to the initiation of cell culture and any subsequent experiments each laboratory has to demonstrate the conformance of their hormone detection system (e.g. ELISA, RIA, LC-MS) with the QC criteria defined in APPENDIX I by analyzing supplemented medium spiked with an internal hormone control. Due to the cross-reactivity of some of the antibody based hormone ELISA's and RIA's with hormone metabolites/conjugates produced by the H295R cells an extraction of the medium may be required prior to the measurement of hormones (Chapter 7.1). If a laboratory wishes to omit this extraction it has to demonstrate the hormone detection system to be used does not exhibit any significant cross-reactivities with any of the metabolites/conjugates of the hormone of interest. One key criterion for the acceptance of data generated during an experiment is the validity of the SC in terms of its position within the standard curve range. The SC should in the center of the linear range of the standard curve of the applied hormone detection assay to maximize the ability to detect increases and decreases in hormone production (APPENDIX I). Dilutions of medium (extracts) in the hormone detection assay have to be selected accordingly.

7.1 Hormone Extraction from Medium

7.1.1 EQUIPMENT, MATERIALS, AND REAGENTS

Equipment: Vortex; Scintillation Counter (Beckman Coulter- LS6500 Multi-purpose SC); Nitrogen Evaporator

Materials: 100-1000, and 2-20 µL Pipettes, Pipette Tips; Glass Scintillation Vials (Research Products International Corp. Cat#-211000); Glass Test Tubes (> 5 mL) with caps

Reagents:

- Scintillation cocktail Bio-Safe II (Research Products International Corp Cat# 111195)
- Ether, Anhydrous (J.T. Baker Cat# 9244-22)
- Testosterone(1,2,6,7-3H(N))-1mCi (PerkinElmer Cat# 370001)

7.1.2 METHOD, PROCEDURES AND REQUIRMENT

1. Label two glass test tubes for each medium sample to be extracted.

2. Pipet 450 µL medium to the test tube.

3. Spike medium samples with 10 µL of ^{3}H-testosterone (concentration = 0.0002 µCi/µL) to test for extraction recoveries. (At this low concentration the radio-labeled hormone spike will be detectable in a liquid scintillation counter, but will not affect the end result of the hormone concentration in the ELISA test).

4. Briefly vortex medium samples after spiking.

5. Add 10 µL of the 3H-labeled hormone to a liquid scintillation vial containing 4 mL of scintillation cocktail.

6. Bring all medium samples to 1 mL with nanopure water.

7. Add 2.5-mL ether to each test tube, and cap. Be careful not to remove test tube label in the process.

8. Vortex each tube for a minimum of 1 minute to allow the water and ether layers to mix.

9. Allow the ether and water fractions to separate or centrifuge for 10 min at 2,000 rpm.

10. Carefully collect the ether fraction (supernatant) into a new test tube using a glass pipet without disturbing water fraction.

11. Add an additional 2.5 mL ether to the original water fraction, cap, vortex, and centrifuge as above.

12. Collect ether fraction into the same tube containing previous ether fraction.

13. Evaporate the ether fractions to dryness under a stream of nitrogen.

14. Either reconstitute in 250µL assay buffer that is provided with each ELISA kit by vortexing or cap vial and store dry at -80°C for up to 8 weeks.

15. Remove 10 µL of the assay buffer extract from each sample and place in a liquid scintillation vial containing 4 mL of cocktail.

16. Test for extraction efficiency by running 3H-labled spike, 3H-labled plasma and a blank sample (10 µL of assay buffer) extracts in the liquid scintillation counter.

17. After reconstitution of the extract either use sample within 24 hrs in the ELISA (see next section) or freeze at –80 °C until further processing (maximum storage time should not exceed 4 weeks).

18. Calculate recoveries from scintillation counter readings (CPM) as follows:

% recovery = ((CPM sample – CPM blank)*25) / (CPM spike – CPM blank) * 100

7.2 Hormone Measurements using Commercial Test Kits

Conduct hormone analysis as specified in the manuals provided by the test kit manufacturer. Most manufacturers have a unique procedure by which the hormone analyses are run. Each sample should be run at two dilutions each in triplicate to ensure that at least one reading falls within the linear range of the standard curve of the assay.

Dilutions in the plates need to be adjusted such that expected **hormone concentrations for the solvent controls fall within the center of the linear range of the standard curve** of the individual assay (APPENDIX I). Non conformance with this increases the risk of an over- or underestimation of the true changes due to chemical exposure, and therefore, results in the rejection of the data.

Final hormone concentrations are calculated as follows:

Hormone concentration (well) / recovery * dilution factor

Example:

Extracted: 450 µL medium
Reconstituted in: 250 mL assay buffer
Dilution in Assay: 1:10 (to bring the sample within the line range of the standard curve)
Hormone Concentration in Assay: 150 pg
Recovery: 89 %

Final hormone concentration = 150 pg * 0.89 / (450mL / 250 mL) * 10 = 741.7 pg / mL

8 RECORDS, DOCUMENTATION AND QC REQUIREMENTS

All QC requirements and criteria are listed in APPENDIX I. Data needs to fall within the acceptable ranges defined for each parameter to be considered for further evaluation. If these criteria are not met the sample either has to be re-analyzed or should be dropped from the data set (need to indicate in spreadsheets that QC criteria were not met).

The primary analyst shall document any anomalies and/or deviation from the specified method in a bound, serially numbered, laboratory notebook with tear-out carbon copies. All electronic files and hardcopies will be kept at the Laboratory and duplicate copies will be provided to the lead laboratory (Aquatic Toxicology Laboratory, Michigan State University) and ENTRIX. This information will also be recorded in the data package to EPA. The primary analyst will sign and date any forms as the analyst.

The technical reviewer will record any problems noted during the technical review. The technical reviewer will return the items to the analyst for corrections prior to inclusion into the data package. The technical reviewer will sign and date all forms as the reviewer.

9 RESPONSIBILITIES

The person(s) working in the cell culture laboratory will be responsible for the preparation of supplemented medium and PBS. They are also responsible for recognizing when more solutions need to be made, and when more medium supplement needs to be ordered. See the "Ordering Sheet for the Cell Laboratory" to determine supplier and other information needed for ordering.

The primary analyst will complete the analysis as specified in this SOP and provide documentation of raw data and any anomalies and provide data to the data analyst.

The technical reviewer will determine if data quality objectives were met, notify the analyst if any problems were found.

10 REFERENCES

Aquatic Toxicology Laboratory/Michigan State University, Laboratory Quality Control Plan (LQCP), September 1998.

Aquatic Toxicology Laboratory/Michigan State University, Safety Manual, September 1998.

Aquatic Toxicology Laboratory/Michigan State University, Requirements for the Preparation, Review, Approval, and Implementation of Standard Operating Procedures (SOPs), September 1998.

Hecker M., Hilscherova K., Laskey J., Buckalew A., Jones P.D., Newsted J.L., Giesy J.P. 2006. Culturing of the H295R human adrenocortical carcinoma cell line (ATCC CLR-2128). Michigan State University, Aquatic Toxicology Laboratory Standard Operating Procedure.

APPENDIX I – QC REQUIREMENTS

Quality Criteria for the Conduct of the H295R Steroidogenesis Assay

Laboratories must demonstrate competence in performing any of the procedures that are part of the H295R Steroidogenesis Assay before they can start testing chemicals. Furthermore, there will be a series of quality controls that are part of the actual conduct of the assay that allow for the evaluation of the assay performance during each experiment.

1. Performance of Hormone Detection System

Prior to the initiation of cell culture and any subsequent experiments each laboratory has to demonstrate the performance of their hormone detection system (e.g. ELISA, RIA, LC-MS) by analyzing supplemented medium spiked with an internal hormone control. The internal standard will be prepared by the lead laboratory (MSU/UofS) and send out to the participating laboratories. Non-conformance with these performance criteria results in the rejection of the selected hormone detection method. Performance criteria including allowable variation from target concentrations will be defined based on the repeated measurement of the internal standards by two of the laboratories that participated in the original inter-laboratory comparison studies (RTP and MSU/UofS).

2. Performance of H295R Cells under Standard Culture Conditions

The qualifying experiment requires growing cells for 5 passages, seeding and exposing them as described for the QC plates in the H295R exposure protocol, and the measuring E2 and T in the cell media using pre-validated hormone detection methods. Threshold concentrations for basal production of testosterone (T) and estradiol (E2) are provided (Table 1). As production of E2 in passage 5 cells may not be sufficient to detect decreases greater than 1.5- to 2-fold in response to exposure to an inhibitor, during the qualifying experiments it is only expected that the laboratory shows conformance with the performance criteria for E2 induction after exposure to the stimulator forskolin (Table I.1).

Table I.1: *Performance criteria to me met by each laboratory during the qualifying experiments.*

	Testosterone	***Estradiol***
Basal Production	*≥ 2000 pg/mL*	*≥ 50 pg/mL*
Induction (10uM forskolin)	*2-fold*	*15-fold*
Inhibition (3uM prochloraz)	*2-fold*	*n/a*

3. Performance of H295R Cells during Exposure Experiments

The first step in the QC validation studies should be related to measuring the alterations in hormone production in cells exposed to the model compounds forskolin and prochloraz in the QC plates. The target performance criteria to be achieved for the exposure to 10 μM forskolin and 3 μM prochloraz are as follows:

Forskolin (10 μM): ≥ 15-fold induction of E2 production, and ≥ 2-fold induction of T production after 48 h.

Prochloraz (3 μM): ≥ 2-fold reduction of E2 production, and ≥ 2-fold reduction of T production after 48 h.

Furthermore, in both solvent control and blank wells in the QC-plate basal hormone production of T and E2 need to be at least 2000 and 80 pg/mL, respectively (Figure I.1). Other quality criteria that pertain to acceptable variation between replicate wells, replicate experiments, linearity and sensitivity of hormone detection systems, variability between replicate hormone measures of the same sample, and % recovery of hormone spikes after extraction of medium are provided in Table I.2. One key criterion for the acceptance of data generated during an experiment is the validity of the SC in terms of its position within the standard curve range. Allowable variation of the SC from the center of the linear range of the standard curve of the applied hormone detection assay is within the 50% range around the center (Figure I.1). Dilutions of medium (extracts) in the hormone detection assay have to be selected accordingly.

Table I.2: *Acceptable ranges and/or variation (%) for H295R assay QC parameters.*

	Comparison Between	***T***	***E2***
Basal hormone production in blanks and SCs	*Absolute Concentrations*	*≥ 2000 pg/mL*	*≥ 80 pg/mL*
Exposure Experiments - Within Plate CV (Replicate Wells)	*Absolute Concentrations*	*≤ 25%*	*≤ 25%*
Exposure Experiments - Between Plate CV (Replicate Experiments)	*Fold-Change*	*≤ 30%*	*≤ 30%*
Hormone Detection System - Sensitivity	*Absolute Concentrations*	*≤ 100 pg/mL*	*≤ 30 pg/mL*
Hormone Detection System – Replicate Measure CV	*Absolute Concentrations*	*≤ 25%*	*≤ 25%*
Hormone Standard Recoveries (Spiked Medium Samples)	*Absolute Concentrations*	*Nominal ± 20%*	*Nominal ± 20%*
Medium Extraction – Recovery of Internal ^{3}H Standard (If Applicable)	*CPM*	*≥ 75% Nominal*	

The ability to meet these criteria will be used as the qualifier to accept data generated during an experiment. In those instances where the data does not meet these criteria, the experiment will have to be repeated. If multiple laboratories repeatedly fail meeting this criterion, the QC criteria may need to be re-defined. A second level of QC will be

required for the acceptance of the test chemical data generated during the validation studies. These QC criteria will be defined on the basis of the variation observed during the validation experiments, and will include measures of effective concentrations (EC5, EC10 and EC50) and fold changes relative to the SCs.

Figure I.1: *Example of hormone detection system standard curve indicating acceptable range of hormone concentration of solvent control (SC) sample (not corrected for dilution in assay). Max = upper limit of linear range; Min = lower limit of linear range. Grey shaded area = 50% range around the center of the linear part of the standard curve.*