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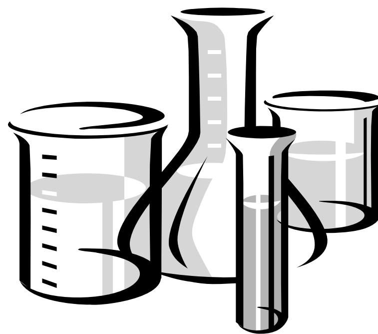
Endocrine Disruptor Screening Program Test Guidelines

OPPTS 890.1550: Steroidogenesis (Human Cell Line – H295R)



NOTICE

This guideline is one of a series of test guidelines established by the Office of Prevention, Pesticides and Toxic Substances (OPPTS), United States Environmental Protection Agency for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, *et seq.*), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*), and section 408 of the Federal Food, Drug and Cosmetic (FFDCA) (21 U.S.C. 346a).

The OPPTS test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting the test, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA and/or the FFDCA. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. The procedures contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the recommendations described in these guidelines, and the Agency will assess them for appropriateness on a case-by-case basis.

For additional information about OPPTS harmonized test guidelines and to access the guidelines electronically, please go to http://www.epa.gov/oppts and select “Test Methods & Guidelines” on the left side navigation menu. You may also access the guidelines in *http://www.regulations.gov* grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

OPPTS 890.1550: Steroidogenesis (Human Cell Line – H295R)

(a) **Scope.**

(1) **Applicability.** This guideline is intended to meet testing requirements of the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, *et seq.*), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*), and the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346a).

(2) **Background.** The Endocrine Disruptor Screening Program (EDSP) reflects a two-tiered approach to implement the statutory testing requirements of FFDCA section 408(p) (21 U.S.C. 346a). In general, EPA intends to use the data collected under the EDSP, along with other information, to determine if a pesticide chemical, or other substances, may pose a risk to human health or the environment due to disruption of the endocrine system.

This test guideline is intended to be used in conjunction with other guidelines in the OPPTS 890 series that make up the full screening battery under the EDSP to identify substances that have the potential to interact with the estrogen, androgen, or thyroid hormone (Tier 1 "screening"). The determination will be made on a weight-of-evidence basis taking into account data from the Tier 1 assays and other scientifically relevant information available. The fact that a substance may interact with a hormone system, however, does not mean that when the substance is used, it will cause adverse effects in humans or ecological systems.

Chemicals that go through Tier 1 screening and are found to have the potential to interact with the estrogen, androgen, or thyroid hormone systems will proceed to the next stage of the EDSP where EPA will determine which, if any, of the Tier 2 tests are necessary based on the available data. Tier 2 testing is designed to identify any adverse endocrine-related effects caused by the substance, and establish a quantitative relationship between the dose and that endocrine effect.

(3) **Source.** This protocol covers the plating of H295R cells (ATCC CLR-2128) in a 24-well plate setup and exposure of the cells to test chemicals. It provides detailed information regarding the handling of samples including the extraction of hormones from medium, storage of samples and extracts, and references detailed SOPs or protocols for the analysis of testosterone (T) and 17beta-estradiol (E2). The protocol also describes the procedure for the Live/Death® cytotoxicity test 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 that must be performed prior to initiating chemical testing and the analysis of positive controls with each run to verify proper performance of the cells.

Cell culturing 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 to track the progress of a cell line) are described in Section (f).

(b) **Purpose.** The H295R Steroidogenesis Assay is intended to identify xenobiotics that affect the steroidogenic pathway beginning with the sequence of reactions occurring after the gonatotropin hormone receptors (FSHR and LHR) through the production of testosterone and estradiol/estrone. The steroidogenic assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamus or pituitary gland.

(c) **Important Considerations.**

- ❑ Due to changes in the estradiol producing capacities of the cells with passage/age (Hecker et al. 2006), cells are cultured following a specific protocol before they can be used in experiments: After initiation of an H295R culture from an original ATCC batch following the procedures outlined below (Subsection (f)(3) Starting Cells from Frozen Stock), cells need to be grown for five (5.0) passages (*i.e.*, cells need to be split 4-times). Passage five cells are then frozen in liquid nitrogen (Subsection (f)(5) Freezing H295R Cells) for storage. For cells started from these frozen batches, the procedures as described in Subsection (f)(3) (Starting Cells from Frozen Stock) are followed. These cells are cultured for a minimum of four (4) additional passages (passage # 4.5) prior to their use in exposure experiments. The maximum number of passages that provide stable results is 10.
- ❑ Prior to initiation of cell cultures, it is recommended that Nu-serum be analyzed for background testosterone and estradiol concentrations.
- ❑ Prior to freezing cells for later use, a subset of passage five (5.0) cells is run in an QC plate (Subsection (g)(2)(iv) Quality Control Test for Cell Performance) to verify whether basal production of hormones and response to a positive control chemical meet the quality criteria in Table 5. Laboratories that have already frozen passage five (5.0) cells can thaw one of the frozen batches, grow it for three (3) passages (passage # 3.5), and use these cells for the QC plate run.
- ❑ All procedures involving cells are conducted using great care and the general considerations listed below should be followed.

- ALWAYS perform all operations with cells in a GENTLE manner.
- ALWAYS remove media/reagents from the plate well borders.
- ALWAYS add media/reagents by resting the pipette against the well wall.
- NEVER vortex or vigorously shake cells.

❑ To avoid clumping of cells in suspension that can result in potential seeding differences among wells of a plate, cells should be always thoroughly resuspended after trypsinization and centrifugation by gentle aspiration using a pipette.

❑ Prior to the initiation of cell culture and any subsequent experiments each laboratory is expected to

- demonstrate that the hormone measurement system to be used can detect hormone concentrations in supplemented medium with sufficient accuracy and precision to meet the QC criteria specified in Table 1, and
- conduct a qualifying experiment demonstrating that the laboratory is capable of maintaining and achieving appropriate cell culture and experimental conditions required for chemical testing (Subsection (g)(2)(iii)).

❑ Sufficient basal hormone production depends on a number of different factors including seeding density and cell passage. However, in some cases basal E2 production may be still less than desired (< 40 pg/mL) regardless of cell density and age/generation. In such cases 22-R Hydroxycholesterol can be added to the supplemented medium at concentrations between 20 and 40 μM to increase basal production of E2. In any case, performance of the assay must be demonstrated in the proficiency test (Subsection (g)(2)(iii)) prior to the conduct of any experiment.

❑ Prior to initiation of the actual exposure experiments, it is recommended that each chemical be tested for potential interference with the hormone measurement system to be utilized. This is of particular relevance for antibody-based assays such as ELISAs and RIAs because some chemicals can interfere with these tests (Shapiro and Page 1976).

❑ It is critical that exact volumes of solutions and samples are delivered into the wells during dosing because these volumes determine the concentrations used in the calculations of assay results. Therefore, only pipettes of the appropriate volume range are used (*e.g.*, use a 0.1 – 1 μL pipette for pipetting 1 μL of test solution stock into wells; use a 1 mL pipette for pipetting 0.99 mL of medium into wells) to avoid introduction of errors due to imprecise pipetting.

❑ Although the H295R cells are a human cell line and are considered non-infectious, it is recommended that proper biosafety measures be observed

such as the use of a biosafety cabinet, proper disposal/autoclaving of waste, and sterilization of all surfaces prior to and after work with the cells. Gloves and lab coat are considered to be the minimal amount of protection to be worn. Avoid wearing gloves that could trap liquid nitrogen next to the skin. Snug fitting non-permeable (waterproof) gloves are best. Eye protection is also recommended when exposure to splashing liquid nitrogen, solvents and biological material is possible. Special safety requirements need to be considered when working with ether, tritium and ethidium bromide.

(d) **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.

(e) **Preparation of Solutions and Reagents for Cell Culture.**

(1) **Dulbecco's Phosphate Buffered Saline (PBS).**

(i) **Equipment, Materials and Reagents.**

Equipment: Stir Plate; Analytical Balance; Pump for Filtration

Materials: Weigh Boats; Chemical Spatula; Pasteur pipettes; Stir Plate; 1000 ml Graduated Cylinder; 1000 ml Autoclaved Amber Bottle; 500 ml Bottle Top Filter (0.22 micron Cellulose Acetate; Low Protein Binding Membrane; Sterilizing; Corning Inc Cat# 430513); 10L Carboy or Storage Container.

Reagents:

- ❑ KCl (potassium chloride); J.T. Baker Cat# 3040-01
- ❑ KH_2PO_4 – monobasic, 99% (potassium phosphate); Aldrich Cat# 22130-9
- ❑ Na_2PO_4 – 99+% (sodium phosphate); Aldrich Cat# 21988-6

- ☐ NaCl – 100.2% (sodium chloride); J.T Baker Cat# 3624-05

(ii) **Method, Procedures and Requirements.**

1. Measure out 9L of ultra clean grade water (e.g., nanopure) into a carboy or storage container.

   Note: *As an alternative to the 10 L volume described here, smaller amounts of PBS buffer can be made with appropriate adjustments to the amounts of reagents used.*

2. Place the carboy on a stir plate and place the stir bar in the carboy. Turn on the stir plate.

3. Add 2.0 g of KCl, 2.0 g of KH_2PO_4, 11.5 g of Na_2PO_4, and 80 g of NaCl to the carboy.

   Note: *Add the chemicals slowly and wait for them to dissolve before proceeding.*
   Note: *Remember to rinse any traces of chemical out of the weigh boat and into the solution using nanopure water.*

4. Bring the total volume up to 10L with ultra clean grade water.

5. Adjust the pH to 7.4 using 10M HCl (if the pH needs to be decreased) or 10M NaOH (if the pH needs to be increased).

   Note: *Add the HCl or NaOH one drop at a time (using a Pasteur pipette) as the high concentration will change the pH considerably.*

6. Prior to use in the cell culture, sterilize the buffer by autoclaving or filtration using a sterile 0.22 micron pore bottle top filter in a 1L amber autoclaved bottle.

7. Label the bottle as follows:
   - PBS
   - H295R Cells
   - "Filter Sterilized" (only for the work solution to be used with cells)
   - pH = 7.4
   - Preparer's initials
   - Expiration date

8. Store the buffer at room temperature.

9. PBS can be used for up to 6 months, after that new PBS buffer should be made.

Note: Alternatively, Dulbecco's Phosphate Buffered Saline 10x, Modified, Without Calcium Chloride and Magnesium Chloride, Liquid, Sterile-Filtered, Cell Culture Tested can be Purchased from Sigma (Cat # D-1408).

(2) **Stock and Supplemented Medium.**

(i) **Equipment, Materials and Reagents.**

Equipment: Biosafety Cabinet; Stir Plate; Analytical Balance; Pump for Filtration.

Materials: Weigh Boats; Chemical Spatula; Pasteur pipettes; Stir Plate; 1000 ml Graduated Cylinder; 1000 ml Autoclaved Amber Bottle; 500 ml Bottle Top Filter (0.22 micron Cellulose Acetate; Low Protein Binding Membrane; Sterilizing; Corning Inc Cat# 430513).

Reagents:

- ❑ Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham; Sigma Cat # D-2906; stored at 2-8°C (equals a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DME) and Ham's F-12 Nutrient mixture in 15mM HEPES buffer without phenol red and sodium bicarbonate).
- ❑ Na_2CO_3 (sodium bicarbonate); Sigma Cat # S-5761.
- ❑ ITS+ Premix; BD Bioscience Cat # 354352; stored at 2-8°C; stable for at least 3 months (the premix contains insulin, transferrin, selenium, BSA and linoleic acid).
- ❑ BD Nu-Serum; BD Bioscience Cat # 355100 (=100 mL) or 355500 (=500 mL); stored at -20°C; stable for at least 3 months; upon arrival BD Nu-Serum should be aliquotted into 13 mL sterile storage vials under sterile conditions before freezing.

Final Concentrations of Components in Supplemented Medium:

- ❑ 15 mM HEPES
- ❑ 6.25 µg/ml insulin
- ❑ 6.25 µg/ml transferring
- ❑ 6.25 ng/ml selenium
- ❑ 1.25 mg/ml bovine serum albumin
- ❑ 5.35 µg/ml linoleic acid
- ❑ 2.5 % Nu Serum

(ii) **Method, Procedures and Requirements.**

Note: *Due to possible varying hormone concentrations in different batches of Nu-Serum, each new serum batch should be tested for background hormone concentrations prior to use. It has been shown that hormones present in medium are quickly metabolized by the cells, and do not affect the response of the cells to chemicals. However, for completeness reasons background concentrations of testosterone and estradiol should be documented. Furthermore, the same batch of Nu-Serum must be used for each set of experiments.*

Stock Medium (1L):

1. Place approximately 900 ml of ultra clean grade water (e.g., anopure) into the 1000 ml graduated cylinder.
2. Place the stir bar in the graduated cylinder and place the graduated cylinder on the stir plate. Turn the stir plate on.
3. As the water stirs, add one bottle of DME/F12 powder (bottle size for 1L).
4. Rinse the empty DME/F12 bottle with ultra clean grade water to remove all traces of chemical and add this to the solution once the initial powder added is totally dissolved.
5. Be sure to rinse the medium stuck on the sides of the graduated cylinder down into the solution using ultra clean grade water.
6. Add 1.2 g of sodium bicarbonate (Na_2CO_3) and rinse the weigh boat with water to remove all traces of chemical.
7. Adjust the pH of the solution to 0.1 to 0.3 below the desired final pH of 7.4 with 1N HCl or 1N NaOH (whichever appropriate).
8. Bring the final volume of the solution up to 1000 ml using ultra clean grade water.
9. Filter the medium using a sterile 0.22 micron pore bottle top filter into an amber autoclaved bottle. This should be done in the biosafety cabinet because to maintain the sterility of this solution.
10. Label the bottle as follows:
    - DME/F12 Stock Medium
    - H295R Cells
    - “Filter Sterilized”
    - pH = 7.4

- Preparer's initials
- Date
- Expiration date

11. Medium should be stored at 4°C.
12. Stock medium can be used for up to 3 months, after that new medium should be made.

Supplemented Medium (500 ml):

1. Thaw one vial (~13mL) BD Nu-Serum using either a 37°C water bath or incubator.
2. Allow the vial with 5 mL ITS+ premium mix to equilibrate to room temperature – swirl gently to mix content.
3. Place an autoclaved 500 mL graduate cylinder in the biosafety cabinet, and add 450 mL of stock medium.
4. Add 5 mL of the ITS+ premium mix and 12.5 mL of the BD Nu-Serum to the cylinder containing the 450 mL of stock medium, and bring the total volume up to 500 mL.
5. Filter the medium in the biosafety cabinet into an autoclaved 500 ml amber bottle using a 0.22 micron bottle top filter.
6. Label the bottle as follows:
    - DME/F12 Supplemented Medium
    - H295R Cells
    - "Filter Sterilized"
    - pH = 7.4
    - Preparer's initials
    - Date
    - Expiration date
7. Medium should be stored at 4°C.
8. Supplemented mediums can be used for up to 3 weeks, after that new supplemented medium should be made.

(3) **Freeze Medium.**

(i) **Equipment, Materials and Reagents.**

Equipment: Biosafety Cabinet.

Materials: 10 mL Strippettes; 15 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052); Autoclaved 100 mL Pyrex Bottle.

Reagents: Sterile Stock Medium (see chapter 4.2); Dimethyl Sulfoxide (DMSO) (A.C.S Reagent Baker Analyzed Cat# 9224-01); BD Nu-Serum (see chapter 4.2).

(ii) **Method, Procedures and Requirements.**

1. All work is conducted under sterile conditions in a biosafety cabinet.
2. The freeze media is made with H295R media supplemented with 7.5% Nu-Serum and 5% Dimethyl Sulfoxide (DMSO).
3. Add 7.5 mL Nu-Serum and 5.26 mL DMSO to 92.5 mL H295R stock medium in a sterile 100 mL Pyrex bottle.
4. Mix well and aliquot freeze media into 15ml centrifuge tubes (about 12 ml per tube).
5. Label the tubes as follows:
   - Freeze Medium
   - H295R Cells
   - “Filter Sterilized”
   - Preparer’s Initials
   - Expiration date
6. Store at –20ºC until needed.
7. Freeze medium can be stored for up to 6 months.

(4) **Trypsin 1X.**

(i) **Equipment, Materials and Reagents.**

Equipment: Biosafety Cabinet; Pipet-Aid.

Materials: 10 mL Strippettes; 15 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052); 500 ml Bottle Top Filter (0.22 micron Cellulose Acetate; Low Protein Binding Membrane; Sterilizing; Corning Inc Cat# 430513); 100 mL Autoclaved Pyrex bottle.

Reagents: Trypsin-EDTA 10X (Life Technologies Inc. Cat # 15400-054); Sterile PBS (see chapter 4.1).

(ii) **Method, Procedures and Requirements.**

1. All work is conducted under sterile conditions in a biosafety cabinet.

2. Filter 10X Trypsin-EDTA with a 0.22 micron filter and transfer 10 mL of the filtered solution into 15 mL polystyrene tubes.
3. Store unused filtered 10X trypsin at -20°C until needed.
4. Place 10ml of filtered 10X Trypsin-EDTA into a sterile 100 mL Pyrex bottle.
5. Bring the volume up to 100 mL using sterile PBS.
6. Aliquot the newly made 1X Trypsin into 15ml polypropylene tubes.
7. Label the tubes with:
   - Trypsin 1X
   - Date
   - Preparer's initials
   - Expiration date
8. Store in the freezer at -20ºC until needed.
9. Maximum storage time is 6 months.

(f) **Cell Maintenance and Culture Procedures.**

(1) **Purpose of H295R Cell Culture Protocol.** The purpose of this protocol is to define the appropriate culture of H295R cells obtained from ATCC (CLR-2128) in preparation for the conduct of exposure experiments utilizing the H295R Steroidogenesis Assay to identify chemicals with the potential to interfere with estrogen and/or androgen production. This protocol provides a consistent format for culturing H295R cell line. Such a consistent format has been shown to be essential for the successful conduct of the assay in terms of producing reproducible and accurate results (Hecker et al. 2006 & 2008).

(2) **Scope and Application of the H295R Cell Culture Protocol.** The protocol gives a detailed description of the methodology to culture and maintain the H295R human adrenocortical carcinoma cell line (ATCC CLR-2128). It provides a complete list of required reagents and solvents, and describes the preparation of all solutions and reagents used in the routine work with the cells. The protocol specifies cell maintenance procedures including initiating cell cultures from frozen stock, the cultivation and splitting of growing cells, and the freezing down of cells for storage in liquid nitrogen. Finally, the protocol provides a description of the proper maintenance of a cell culture diagram that allows for tracking the progress of a cell line and provides a platform for recording all of the work done with the cells.

Stock medium is used as the base for the supplemented and freezing mediums, which are used for a variety of different purposes in the cell culture laboratory. Supplemented medium is a necessary component for culturing cells. Freezing medium is specifically designed to allow for impact-free freezing down of cells for long-term storage.

The procedure for **starting the cells** is to be used when a new batch of cells is removed from liquid nitrogen storage for the purpose of culture and experimentation. **Splitting of the cells** is necessary to ensure the health and growth of the cells and to maintain cells for performing bioassays and other testing. H295R cells will need to be **frozen down** to make sure that there are always cells of the appropriate passage/age available for culture and conduct of experiments. This procedure should also be implemented when a cell line is not being actively used for research.

Cell Passage Nomenclature. In this protocol cell passages will be labeled using a simplified version of the tracking system utilized in the Cell Culture Diagram (Appendix 2) that is used in routine cell culture procedures. A two-number system will be used that provides information on the actual passage number as well as the passage number at which the cells were frozen down. The first number indicates the actual cell passage number and the second number describes the passage number at which the cells were frozen down. The numbers are separated by a dot (*e.g.,* cells that underwent 4 passages after they were brought up into culture again after being frozen down at passage 5 would be labeled passage 4.5).

(3) **Starting Cells from the Frozen Stock.**

(i) **Equipment, Materials and Reagents.**

Equipment: Biosafety Cabinet; Pipet-Aid; Centrifuge.

Materials: 10 ml Strippettes; Waste Container; 15 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052); 100 mm x 20 mm Cell Culture Dish (treated; polystyrene, non-pyrogenic; sterile; Corning Inc Cat# 430167) or Cell Culture Flask 75 cm^2 (treated; polystyrene, non-pyrogenic; sterile; Corning Inc Cat# 430641).

Reagents: Sterile Supplemented Medium (see chapter 4.2); H295R cells (ATCC Cat # CRL-2128).

(ii) **Method, Procedures and Requirements.**

1. Aliquot 10 ml of the supplemented medium to a 15 ml centrifuge tube. Use one centrifuge tube with medium for each H295R vial that is being thawed.

2. Remove a vial of H295R cells from liquid nitrogen storage.

3. Thaw the vial rapidly by agitation in a 37°C water bath or using the warmth of hands. Remember to be very careful, the vials are extremely cold and could cause damage to skin if held in one position for too long. Thawing should be rapid (within 40-60 seconds). As soon as the ice is melted, remove the ampoule/vial from water bath and wipe it down with 70% ethanol at room temperature and transfer to a biosafety cabinet. All following steps must be done under aseptic conditions.

4. In the biosafety cabinet, pipette the thawed cell solution into the medium that was aliquoted in step 1.

   Note: *The thawed cell solution should be placed into the aliquoted medium as quickly as possible. If the cells remain in the freezing medium for too long the viability will be poor.*

5. Centrifuge the cell suspension at 125 x g for 10 min, discard the supernatant and resuspend the cells with 12 mL of supplemented media by gentle swirling of the tube.

6. Transfer the cell suspension to a 100 mm x 20 mm culture plate (further referred to as "plate").

   Note: *Culture flasks can also be used as an alternative to culture plates. However, the amount of medium will need to be adjusted if using a different size plate/flask with a different surface area.*

7. Label the plate with:
   - Cell Type
   - Date
   - Initials of the person thawing the cells
   - The plates will be given a plate designation after their survival is assured.

8. Change the medium the next day (see following section on "Maintaining and Sub-culturing the Cells" for the description of H295R cell maintenance and medium change).

(4) **Maintaining and Sub-culturing the Cells.**

(i) **Equipment, Materials and Reagents.**

<u>Equipment</u>: Biosafety Cabinet; Pipet-Aid, Incubator (37°C, 5% CO_2).

<u>Materials</u>: 10 mL Strippettes; 100mm x 20mm Cell Culture Dish (treated; polystyrene, non-pyrogenic; sterile; Corning Inc Cat# 430167); Waste Container; 15 and 50 mL Centrifuge Tubes

(polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052 [15 mL] and 430290 [50 mL]).

Reagents: Sterile Supplemented Medium (Subsection (e)(2)); Sterile 1x Trypsin- EDTA (Subsection (e)(4)); Sterile PBS (Subsection (e)(1)).

(ii) **Method, Procedures and Requirements.** H295R cells are cultivated in an incubator at 37°C with 5% CO_2 in air atmosphere.

Medium Renewal - Renew medium 2-3 times weekly:

1. Pre-warm supplemented medium to 37°C in a water bath or incubator.
2. Wipe all tubes/containers off with 70% ethanol and transfer cell culture plates and supplemented medium into a biosafety cabinet.
3. Carefully pipette old medium off the culture plate without disturbing cells.
4. Add 12 mL of fresh supplemented medium to the plate.

Splitting of Cells - Split the cells when they are close to confluence (about 90% confluent):

1. Warm PBS and supplemented medium to 37°C in a water bath or incubator.
2. Thaw and warm 1x Trypsin-EDTA at 37°C in a water bath or incubator.
3. Wipe all tubes/containers off with 70% ethanol and transfer cell culture plates and solutions into a biosafety cabinet.
4. Measure 15 ml of PBS for each plate to be split into a 50 ml centrifuge tube (e.g., 45 mL for three plates; use 2nd tube for 4-6 plates, etc.).
5. Carefully pipette old medium off the culture plate without disturbing cells.
6. Rinse each 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.
7. 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).
8. 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.*

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 not take longer than 4-5 minutes).* ***Cells should not been exposed to trypsin for more than 10 minutes!***

9. Stop the trypsin action with 10.5 mL of supplemented medium (once again the volume will need to be adjusted for a different sized plate/flask).

10. Place the appropriate amount of cell solution in the new plate/flask. The amount of cell solution should be adjusted so that the cells are confluent within 5-7 days. The recommended sub-cultivation ratio is 1:3 to 1:4.

11. Label the plate with:
    - Cell Type
    - Date
    - Initials of the person splitting the cells
    - Unique identifier code containing ATCC Lot number, Freeze Down ID, Passage #, Total # of Passages since original ATCC cell batch was started in cell culture, Plate ID (Subsection (f)(6) and Appendix 1: Cell Culture Diagram)
    - Plate designation

(5) Freezing H295R Cells (Preparing Cells for Liquid Nitrogen Storage).

(i) Equipment, Materials and Reagents.

Equipment: Biosafety Cabinet; Pipet-Aid; Controlled Rate Freezing Container (1 Degree C Freezing Container; Nalgene Cat# 5100-0001); Centrifuge, Liquid Nitrogen Tank.

Materials: 10 ml Strippettes, Sterile Cryrogenic Vials (Polypropylene, Biohit Inc. Cat# 4503-1); Waste Container, 15 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052).

Reagents: Supplemented Medium (Subsection (e)(2)); Sterile 1x Trypsin-EDTA (Subsection (e)(4)); Freeze Medium (Subsection (e)(3)); Sterile PBS (Subsection(e)(1)).

(ii) Method, Procedures and Requirements.

1. Follow the procedure for splitting cell lines (Subsection (f)(4)) through step nine in the method (stopping of trypsin action with 10.5 mL supplemented medium).
2. Pipette all of the cell solution into a sterile 15 ml centrifuge tube that is labeled with the plate identifier code.
3. Centrifuge tube with cells for 5 minutes at 350 x g at room temperature.
4. Upon removal from the centrifuge there should be a pellet of cells in the bottom of the centrifuge tube. If not, spin again under the same conditions.
5. Pipette off the supernatant and place it into the waste container in the biosafety cabinet.

   Note: *Be sure not to suck up the pellet. If this does happen, re-suspend the pellet in the medium and spin down in the centrifuge again.*

6. Re-suspend the pellet of cells in 1 ml of the appropriate cell freezing medium.
7. Transfer the solution to a sterile cryogenic vial and label with:
   - Cell Type.
   - Date.
   - Initials of the person freezing the cell line down.
   - Unique identifier code containing ATCC Lot number, Freeze Down ID, Passage #, Total # of Passages since original ATCC cell batch was started in cell culture, Plate ID (Subsection (f)(6) and Appendix 1: Cell Culture Diagram).
8. Place the vial(s) into the controlled rate freezing container.
9. Put the container into a -80°C freezer for 24 hours.
10. After 24 hours in the freezer, transfer to liquid nitrogen for storage.
11. The storage in liquid nitrogen vapor phase is recommended instead of having the cryogenic vials submerged in the liquid nitrogen fluid.

(6) **Cell Culture Diagram.** Cell culture diagrams are useful and necessary for the smooth operation of the cell culture laboratory. They are used for a variety of reasons including: (1) They allow for tracking the progress of a cell line and notice problems or incongruencies, which may arise over time (These are very important to note), and (2) They also provide a platform for recording all of the work done with the cells.

1. It is recommended that the following information from the saved cryovial is tracked:
   - Cell Type
   - Date the cells were frozen
   - ATCC Lot number
   - Freeze Down ID / Passage # / Total # of Passages since original ATCC cell batch was started in cell culture / Plate ID
2. Each time the cells are split the cell passage number and total number of passages are increased by one. Each time the cells are frozen the freeze down ID (Greek letter) is increased by one starting with Α and continuing with Β, Γ, Δ, Ε etc. (see Appendix 1 for key). When the cells that have been frozen are started in culture, the passage number is reset to 1, and the total number of passages is continued. Each different plate/flask is labeled with another letter with the first being A, the second B, and so on.
3. Each data sheet in the study will contain the information on the cells used (*i.e.*, Cell Type, Date the cells were frozen, ATCC Lot number, Freeze Down ID, Passage #, Total # of Passages since original ATCC cell batch was started in cell culture, and Plate ID). As the cells continue to grow, a record is made of each operation such as changing the media, freezing cells, splitting the cells etc. (See Appendix 2 for a sample diagram).
4. Continue with the cell culture diagram until the cells are no longer being used or are frozen.

(7) **Records, Documentation and QC Requirements.** When preparing media, remove label from the bottle or packet and place in notebook, with your initials and date that the medium was made. When starting new cells, start a “cell culture diagram” for the cell line (see the SOP for starting and maintaining a “cell culture diagram”). Record the culturing, splitting and freezing of the cells on the “cell culture diagram”. Be sure to include the date that the medium was changed, cells were split or frozen. It is recommended that the liquid nitrogen Dewar contents log book is updated if removing a vial to start new batch of cells or when transferring cryogenic vials with frozen cells to the liquid nitrogen. Any anomalies and/or deviation from the specified are documented.

(g) **Pre-test Procedures.**

(1) **Solutions and Reagents for Testing.**

(i) **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); 4.1050 mg
Prochloraz (MW = 376.67); 3.7667 mg
Test Chemicals

* 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 Section (e).

(ii) **Method, Procedures and Requirements.**

Step 1: Prepare 100 mM stock concentrations of forskolin, prochloraz and test chemicals dosing solutions in DMSO. Weigh out the appropriate amount of forskolin (4.1050 mg), prochloraz (3.7667 mg) and test chemicals (MW x 0.00001) in tared vials. Add 100 µL of DMSO to the forskolin, prochloraz and all test chemical vials, cap and vortex to dissolve each substance in the DMSO. This results in 100 mM stock solutions for forskolin and prochloraz. For all test chemicals this results in the Stock 1 Test Solution.

Step 2: Dilute these stock solutions as follows:

- *Forskolin:* Dilute 100 mM stock solution 1:10 (10 µL of 100 mM Stock 1 + 90 µL DMSO) to make 100 µL of a 10 mM solution. Dilute 10 µL of this 10 mM solution 1:10 to make 100 uL of a 1 mM solution.
- *Prochloraz:* Dilute 100 mM stock solution 1:10 (10 µL of 100 mM Stock 1 + 90 µL DMSO) to make 100 µL of a 10 mM solution. Dilute 10 µL of this 10 mM solution 1:10 to make a 1 mM solution. Dilute 10 µL of this 1 mM solution 1:10 to make a 0.1 mM solution.
- *Test Chemicals:* Dilute Stock 1 1:10 (10 µL of 100 mM Stock 1 + 90 uL DMSO) to make 100 µL 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).

Step 3: Label each vial as follows:

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

Step 4: Store the stock solutions at 4°C.

(2) **Requirements Before Initiating Testing.**

- ❑ Prior to the initiation of cell culture and any subsequent testing, each laboratory is expected to demonstrate the sensitivity of its hormone measurement system (Subsection (g)(2)(i)).
- ❑ If antibody-based hormone measurement assays are to be used, it is recommended that the chemicals to be tested be analyzed for their potential to interfere with the measurement system used to quantify testosterone and estradiol, as outlined in Subsection (g)(2)(ii) prior to initiating testing.
- ❑ Prior to conducting testing for the first time, the laboratory is expected to conduct a qualifying experiment demonstrating that the laboratory is capable of maintaining and achieving appropriate cell culture and experimental conditions required for chemical testing, as described in Subsection (g)(2)(iii).
- ❑ In addition to being run in parallel with each test run, it is recommended that a QC control plate be run before using a new batch of cells to evaluate the performance of the cells, as described in Subsection (g)(2)(iv).

(i) **Performance of Hormone Measurement System.** Each laboratory may use a hormone measurement 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 test runs, it is expected that each laboratory demonstrate the conformance of their hormone measurement system (e.g., ELISA, RIA, LC-MS) with the QC criteria defined in Table 1 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 is required prior to the measurement of hormones if an assay is used that employs antibodies.

Table 1. Performance Criteria for Hormone Measurement Systems.

Parameter	Criterion
Method Detection Limit	Testosterone: 100 pg/mL; Estradiol: 10 pg/mL [a]
Spike Sample Recovery	Supplemented medium are spiked with at least two concentrations of each hormone of interest (T: 500 and 2500 pg/mL; E2: 50 and 250 pg/mL). When analyzed with the hormone measurement assay, the average recovery rates (based on triplicate measures) for the spiked amounts of hormone are expected not to deviate more than 30% from nominal concentrations.
Hormone Cross reactivity (only antibody based systems)	No significant (≥30% of basal hormone production of the respective hormone) cross-reactivity with any of the hormones produced by the cells are expected to occur. These include: cholesterol, pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17alpha-pregnenolone,17alpha-progesterone, deoxycortisol, cortisol, DHEA, androstenedione, estrone [b]

[a] ***Note:*** *Method detection limits shown here are based on the basal hormone production values provided in Table 4, and are performance based. If greater basal hormone production can be achieved, a higher MDL is acceptable provided the criteria in Table 4 are met.*

[b] Some T and E2 antibodies may cross-react with androstendione and estrone, respectively, at a greater percentage In such cases it is not possible to accurately determine effects on 17β-HSD. However, the data can still provide useful information regarding the effects on estrogen or androgen production in general. In such cases data should be expressed as androgen/estrogen responses rather than E2 and T.

One additional criterion for the acceptance of data is the validity of the SC in terms of its position within the standard curve range. The SC is expected to be within the 75% range below the upper part (maximum optical density [OD] or similar response measured by hormone measurement system) and 75% above the lower part (minimum OD or similar response measured by hormone measurement system) of the linear range of standard curve for inducers, and inhibitors, respectively (Figure 1). Dilutions of medium (extracts) in the hormone measurement assay are to be selected accordingly.

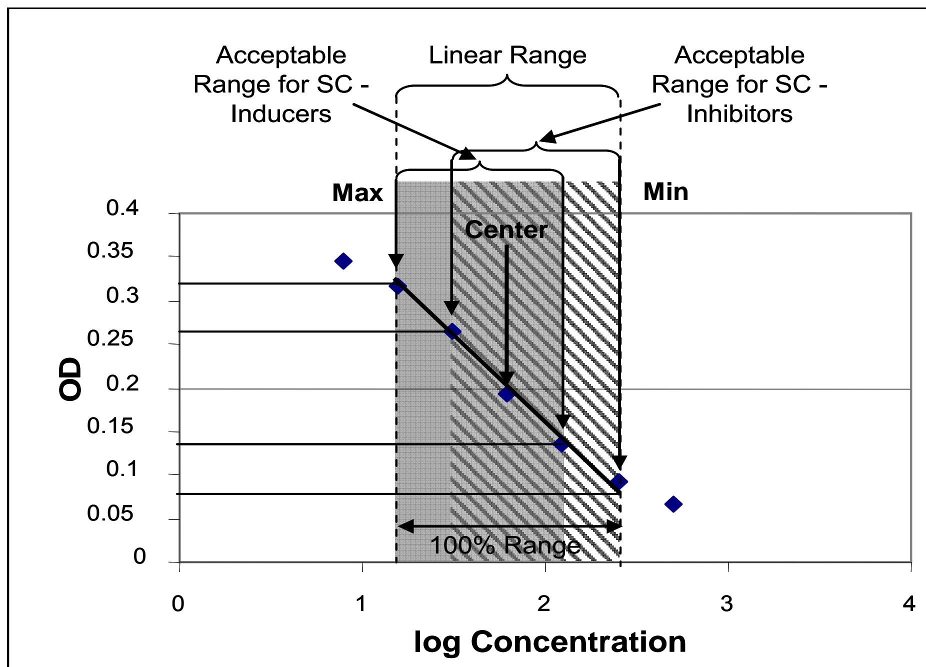


Figure 1. Example of hormone measurement system standard curve indicating expected range of hormone concentration of solvent control (SC) sample (not corrected for dilution in assay). Max = upper limit of linear range; Min = lower limit of linear range. Grey shaded area = 75% range below the maximum OD of the linear part of the standard curve. Diagonally striped area = 75% range above the minimum OD of the linear part of the standard curve.

(ii) **Chemical Hormone-Assay Interference Test.** If antibody-based hormone measurement assays are to be used, prior to initiation of testing, it is recommended that each chemical be tested for potential interference with the hormone measurement system being utilized. It has been previously shown that that some chemicals can interfere with antibody-based assays such as ELISAs and RIAs (Shapiro and Page 1976). This "chemical interference test" will be conducted as described for the analysis of medium samples (Subsection (h)(5)). Prior to extraction and analysis, chemical spiked medium is prepared as follows.

(A) **Equipment, Materials, and Reagents.**

Equipment: Biosafety Cabinet; Pipet-Aid, Vortex.

Materials: 10 mL or 25 mL Strippettes; Waste Container; 15 or 50 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052 [15 mL] or 430290

[50 mL]); 2 µL and 1000 µL Pipettes; Sterile Pipette Tips, Eppendorf Tubes (1.5 mL).

Reagents: Sterile Supplemented Medium (Subsection(e)(2); DMSO (Aldrich, Cat. No. D2438); Test chemical stock solutions (serial dilutions 1 – 6; see Subsection (g)(1)); Testosterone; 17β-Estradiol ; Ethanol (100%).

(B) **Method, Procedures and Requirements.**

Step 1: Prepare Stock Solutions:

- ❑ Dissolve 1 mg of T and E2 each in 1mL of 200 proof ethanol in Eppendorf tubes (***Primary Hormone Stocks***).
- ❑ Dilute 10 µl of ***Primary Hormone Stocks*** from previous step each in 990 µl of a supplemented medium ethanol solution (75 % EIA / 25 % ethanol) and vortex (***Seconda***ry Hormone Stocks)
- ❑ Dilute 10 µl of ***Secondary Hormone Stocks*** from previous step each in 990 µl supplemented medium and vortex (***Tertiary Hormone Stocks***).
- ❑ Dilute 100 µl of ***Tertiary Hormone Stocks*** from previous step each in 900 µl supplemented medium and vortex, resulting in a hormone stock concentration of 10 ng / mL (***Final Hormone Stock*** Solution).

Step 2: Prepare two Eppendorf Tubes per test chemical for initial interference test.

Step 3: Add 999 µL of supplemented medium to tube 1 and 779 µL of supplemented medium to tube 2.

Step 4: Add 200 µL of testosterone ***Final Hormone Stock*** Solution and 20 µL of estradiol Final Hormone Stock Solution to tube 2.

Step 5: Add 1 µL of Test Chemical Stock Solution 1 (greatest concentration) to tubes 1 and 2.

Step 6: Vortex thoroughly for >15 seconds.

Step 7: Extract medium supplemented with Test Chemical and Hormone Stock Solutions produced in previous steps and analyze for hormones as described in Subsection (h)(5).

Note: *If significant interference of chemical occurs as determined by hormone analysis (significant interference is defined as ≥ 30% of basal hormone production for testosterone and/or estradiol), it is recommended that the Chemical Hormone Assay Interference Test be repeated for all Test Chemical Stock Solution dilutions to identify the threshold dose at which significant interference occurs. If significant interference of a test chemical with a hormone measurement system occurs at more than one non-cytotoxic concentration, it is recommended that a different hormone measurement system be used.*

(iii) **Laboratory Proficiency Test.** Before testing unknown substances, a laboratory is expected to demonstrate that it is capable of achieving and maintaining appropriate cell culture and test conditions required for the successful conduct of the assay. As the performance of an assay is directly linked to the laboratory personnel conducting the assay, it is recommended that these procedures be repeated if a change in laboratory personnel occurs.

This qualification test will be conducted under the same conditions listed in Section (h) by exposing cells to six increasing concentrations of the model inducer forskolin and the model inhibitor prochloraz. These qualification tests will be done under a somewhat different exposure regime and plate layout compared to the standard test chemical exposure procedure in that the concentrations are more closely distributed about the expected values of the EC_{50} of each of the control chemicals than would be the case for an initial run with an unknown chemical.

Table 2. Dosing Schematic for the Laboratory Proficiency Study.

	1	2	3	4	5	6
A	DMSO 1µL	DMSO 1µL	DMSO 1µL	For 10 or Pro 3	For 10 or Pro 3	For 10 or Pro 3
B	For 0.03 or Pro 0.01	For 0.03 or Pro 0.01	For 0.03 or Pro 0.01	For 1 or Pro 0.3	For 1 or Pro 0.3	For 1 or Pro 0.3
C	For 0.3 or Pro 0.1	For 0.3 or Pro 0.1	For 0.3 or Pro 0.1	For 0.1 or Pro 0.03	For 0.1 or Pro 0.03	For 0.1 or Pro 0.03
D	For 3 or Pro 1	For 3 or Pro 1	For 3 or Pro 1	Blank	Blank	Blank

Exposure of H295R to either forskolin (For) or prochloraz (Pro) in a 24 well plate during the laboratory proficiency test. Dosing is in µM for all test chemical doses. Note: Doses will be administered in DMSO at 0.1% v/v per well. The DMSO solvent control will receive 1 µL of DMSO only. Nothing is added to the Blank wells**. Separate plates are to be run for forskolin and prochloraz.**

The effective concentrations expressed as 50% of the maximum response (EC_{50}s) are calculated using standard procedures (e.g., Probit analysis or moving average method) and compared with the values in Table 3. The data are expected to fall within the following ranges:

Table 3. EC_{50} Ranges for Control Substances.

	EC_{50} (µM)	
	T	**E2**
Prochloraz	0.01 – 0.1	0.03 – 0.3
Forskolin	0.2 – 2.0	0.3 – 3.0

(iv) **Quality Control Plate.** There are two uses of the quality control (QC) plate:

(A) It is recommended that H295R cell performance be assessed for possible changes in hormone production as a function of cell age prior to using a new ATCC batch or after using a previously frozen stock of cells for the first time, unless the laboratory proficiency test (Subsection (g)(2)(iii)) has been run with that batch of cells. To verify that the performance of H295R Cells under Standard Culture Conditions is meeting the QC requirements, a subset of passage five (5.0) cells is run in a QC plate. Laboratories that have already frozen passage five (5.0) cells can thaw one of the frozen batches, grow it for three (3) passages (passage # 3.5), and use these cells for the QC run.

(B) A QC plate provides the positive controls for the assay when testing chemicals and is included as part of each test run.

The QC plate is a 24 well plate shown in Table 4 and is incubated, dosed, and assessed (cell viability/cytotoxicity, hormones extraction and hormone analysis protocols) in the same manner as test plates described in Section (h). The QC plate will be dosed with a known inducer (forskolin) and inhibitor (prochloraz) of E2 and T synthesis each at two different doses of these compounds.

Table 4. 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.

	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 1 µL	DMSO 1 µL	DMSO 1 µL	DMSO 1µL + MeOH [b]	DMSO 1µL + MeOH [b]	DMSO 1 µL + MeOH [b]
C	FOR 1 µM	FOR 1 µM	FOR 1 µM	PRO 0.1 µM	PRO 0.1 µM	PRO 0.1 µM
D	FOR 10 µM	FOR 10 µM	FOR 10 µM	PRO 1 µM	PRO 1 µM	PRO 1 µM

[a]Blank wells receive medium only.
[b]A 70% methanol (MeOH) solution will be added to all MeOH wells after termination of the exposure experiment and removal of medium.

Criteria for the QC plate are provided in Table 5.

Table 5. Criteria to be Met on Each QC Plate.

	Testosterone	Estradiol
Minimum Basal Production	500 pg/mL	40 pg/mL
Basal Production	≥ 5-times MDL	≥ 2.5-times MDL
Induction (10uM forskolin)	≥ 2-times SC	≥ 7.5-times SC
Inhibition (1uM prochloraz)	≤ 0.5-times SC	≤ 0.5-times SC

The minimum basal hormone production must be met in both solvent control and blank wells.

Note: *In some cases basal E2 production may be still less than desired (< 40 pg/mL) regardless of cell density and age/generation. In such cases 22-R Hydroxycholesterol can be added to the supplemented medium at concentrations between 20 and 40 µM to increase basal production of E2. In any case, the performance of the assay must be demonstrated in the proficiency test (Subsection (g)(2(iii)) prior to the conduct of any experiment.*

(h) **Test Procedures.**

(1) **Plating and Pre-Incubation of Cells.**

Note: *Due to changes in estradiol producing capacities of the cells with age (i.e., cell passage) (Hecker et al. 2006), cells are cultured following a specific protocol before they are 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 grown for five (5) passages (cells need to be split 4-times). These cells are frozen in liquid nitrogen (Subsection (f)(5)*

Freezing H295R Cells). Cells starting from these frozen batches, following the procedures described Subsection (f)(3) Starting Cells from Frozen Stock, need to be cultured for at least four (4) additional passages (passage # 4.5) before they can be used to conduct the assay. The maximum passage used in testing is 10 (passage # 5.10). Before using cells in a chemical exposure experiment, it is recommended that a QC plate be run to verify "readiness" of the cells for conducting the assay.

(i) **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 (Subsection (e)(2); Passage 4.5 to 10.5. NCI-H295R cells (ATCC Cat # CRL-2128) cultured under standard conditions as described in the H295R culture protocol; Sterile 1x Trypsin- EDTA (Subsection (e)(4)); Sterile PBS (Subsection (e)(1)).

(ii) **Methods, Procedures and Requirements.**

Step 1: Pre-warm PBS and supplemented medium to 37°C in a water bath or incubator.

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

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

Step 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: *The number of cell culture dishes that will be needed for an experiment depends on the number of plates needed for the exposure experiment and the confluency of the cells in the culture dishes. General rule: use 1 cell culture dish*

(100 mm) of 95-100% confluent cells to plate two 24-well plates at a target density of 200,000 to 300,000 cells per mL.

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

Step 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.).

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

Step 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.*

Step 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).

Step 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 from the plate 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).* ***The maximum exposure time to trypsin that cells can tolerate is 10 minutes!***

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

Step 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.*

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

Step 14: Clean the hemocytometer and the cover glass with 70% Ethanol. Add 10 µl 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

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

Step 16: Cells need to be seeded at 200,000 to 300,000 cells/mL medium resulting in approximately 50-60% confluency in the wells at 24 hours (Figure 2).

Note: *50 – 60% confluency in the wells at 24 hours is the preferred density of cells for optimal hormone production in the medium. At higher densities cells tend to be affected by hormonal feedback or other mechanisms and T as well as E2 production patterns are altered. Before conducting the assay the first time, different seeding densities between 200,000 and 300,000 cells per mL should be tested, and the density resulting in 50 to 60% confluency in the well at 24 hours should be selected for further experiments.*

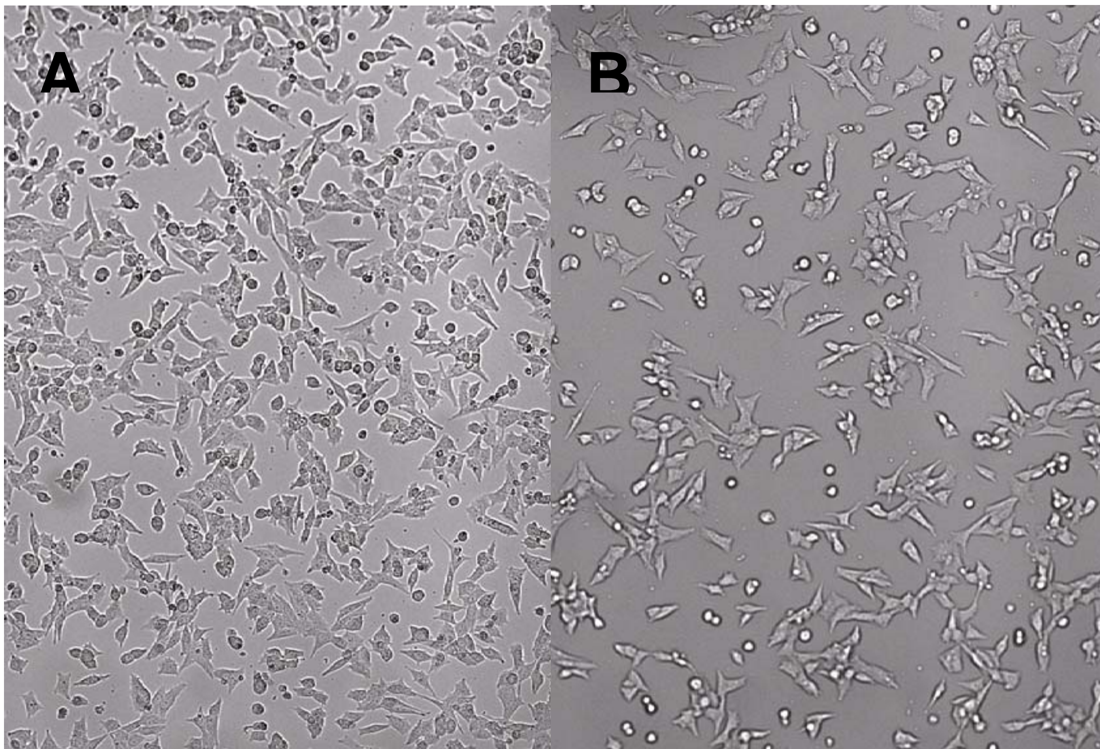


Figure 2. Photomicrograph of H295R Cells at a Seeding Density of 50 – 60% in a 24 Well Culture Plate at 24 hours.

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

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

Step 19: The new plates should be labeled with the cell type, preparer's 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.*

Step 20: Incubate seeded plate in incubator at 37°C under a 5% CO_2 in air atmosphere for 24 hours.

(2) **Exposure of Cells.**

(i) **Equipment, Materials and Reagents.**

Equipment: Biosafety Cabinet; Pipet-Aid, Incubator (37°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 200,000 to 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 (Subsection (e)(2)); DMSO; Test chemical stock solutions (serial dilutions 1 – 6; Subsection (g)(1)).

(ii) **Methods, Procedures and Requirements.**

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

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

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

Note: *Alternatively, a mastermix containing 3.94 mL of medium plus 4 µL of the respective chemical stock solution*

in DMSO can be prepared prior to dosing the cells. Then, 1 mL of the appropriate mastermix is to be dispensed per replicate well of each dose. If this approach is chosen, omit step 4.

Step 4: Dose cells by adding 1µl of the appropriate stock solution in DMSO per 1 mL medium (well volume). This results in a final concentration of DMSO of 0.1%.

Step 5: For solvent controls, add 1µl DMSO per 1 mL medium (well volume) directly into the well.

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

Table 6. Dosing Schematic for the Exposure of H295R to Test Chemicals in a 24 Well Plate.

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

Dosing is calculated based on a total volume of 1 mL per well.
Stock 1-7: 1 µL of appropriate stock solution needs to be added to each well.

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

(3) **Exposure Termination and Medium Storage.**

(i) **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 200,000 to 300,000 cells per well with H295R cells and exposed for 48 hours.

(ii) **Method, Procedures and Requirements.**

Step 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 cytotoxicity. Record your observations.

Step 2: Label Eppendorf tubes appropriately.

Step 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).

Step 4: Freeze media at -80ºC until further processing (Subsection (h)(5)).

Step 5: Immediately after removing medium conduct a cell viability test with each exposure plate (Subsection (h)(4)).

IMPORTANT: *Make sure that cells do not dry out, remove medium a row or column at a time and add 200 µL PBS with* Ca^{+} *and Mg+ to each well.*

(4) **Cell Viability Measurements.** A cell viability/cytotoxicity assay of choice can be used to determine to the potential impact of the test chemical on cell viability. The assay must provide a true measure of the percentage of viable cells present in a well, or it must be shown to be directly comparable to (a linear function of) the Live/Dead® Assay described below. An alternative assay that has been shown to work equally well as the Live/Dead® Assay is the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] test (Mosman et al. 1983).

Note: *The assessment of cell viability using the above methods is a relative measurement that does not necessarily exhibit linear relationships with the absolute number of cells in a well. Therefore, a subjective parallel visual assessment of each well by the analyst should be conducted, and digital pictures of the SCs and the two greatest non-cytotoxic concentrations are to be taken and archived to enable later assessment of true cell density if this should be required.*

(i) **Live/Dead® Cell Viability/Cytotoxicity Assay.** The *LIVE/DEAD* Viability/Cytotoxicity Kit (Molecular probes, Eugene OR, USA, Cat # 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 as determined by the enzymatic conversion of the virtually non-fluorescent cell-permeate 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. The cytotoxicity testing will be conducted in the chemical exposure plate 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.

(A) **Equipment, Materials and Reagents.**

Equipment: Vortex; Microscope; Eppendorf Multipipette; Plate reading fluorometer *(**Note:** this protocol has been tested with the 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 to 300,000 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; Subsection (e)(1)).

(B) **Method, Procedures and Requirements.**

Preparation Steps (prior to assay):

Step 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.

Step 2: Prepare viability assay reagent (refer to Supplies and Biochemicals Section): Each plate will need 16 mL (24 wells * 600 µL) plus a little extra (1.6 mL).

Step 3: Dilute the appropriate amounts of calcein and Ethidium with the appropriate volume of of PBS with Ca^{2+} and Mg^{2+}:

- Calcein stock (µL): 0.5 µL/mL
- Ethidium bromide stock (µL) 2.0 µL/mL

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

Step 4: Set up fluorometric plate reader following the manufacturer's specifications.

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

Step 1: Remove plate from incubator and remove media for subsequent hormone analysis or extraction as described in Subsection (h)(5), and add 300 µL of PBS with Ca^{2+} and Mg^{2+}. Then rinse 1 time with 300 µL of PBS with Ca^{2+} and Mg^{2+}. **Note:** Remove medium from not more than 4 wells at a time and fill with PBS with Ca^{2+} and Mg^{2+} before removing medium from second set of wells to prevent cells from drying.

Step 2: **QC plate only:** Remove the PBS from wells A4-6 and B4-6 and add 300 µL Methanol to these wells, and allow standing at room temperature for 30 minutes.

Step 3: Remove the methanol and rinse those wells three times with 300 µL PBS with Ca^{2+} and Mg^{2+}. Rinse the balance of the wells once with 300 µL PBS with Ca^{2+} and Mg^{2+}. Leave the last 300 µL rinse on the cells.

Step 4: Add 600 µL of viability assay reagent to all wells.

IMPORTANT CONSIDERATION: *Since the staining is progressive up to at least 3 to 4 hours, it is important to*

space the staining of each plate to the time it takes to read the fluorescence.

Step 5: Incubate at room temperature for 1 hour (cover the plate with lid to prevent evaporation).

Step 6: Scan the plate in the fluorometer using the above described settings.

Step 7: Export/print data (check that values are appropriate, otherwise adjust sensitivity and rescan).

(C) **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 is adversely affected in the least dose and continues to decrease 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.

(5) **Hormone Extraction and Analysis.**

(i) **Hormone Extraction from Medium.** This procedure is followed if the hormone measurement system may be affected by the presence of culture medium. If it can be demonstrated that hormone measurements can be made in the presence of medium, this procedure may be omitted.

(A) **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).

(B) **Method, Procedures and Requirements.**

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

Step 2: Pipet 450 μL medium into the test tube.

Step 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).

Step 4: Briefly vortex medium samples after spiking.

Step 5: Add 10 μL of the 3H-labeled hormone to a liquid scintillation vial containing 4 mL of scintillation cocktail (this tube is used as a reference for the calculation of CPM of the "CPM spike tube" used later for the derivation of extraction efficiencies;.

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

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

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

Step 9: Allow the ether and water fractions to separate or centrifuge for 10 min at 2,000 rpm. Carefully collect the ether fraction (supernatant) into a new test tube using a glass pipet without disturbing water fraction.

***Alternative method**: Prepare a dry ice-acetone bath in a fume hood by placing several large chunks of dry ice in a large glass beaker and filling with acetone. Hold plasma samples in the dry ice bath for approximately 30 sec or until water fraction is completely frozen. Then collect non-frozen ether fraction as described above.*

Step 10: Re-extract original water fraction by adding 2.5 mL ether to the tube, cap, vortex, and centrifuge as above.

Step 11: Collect ether fraction into the same tube containing first ether fraction.

Step 12: Wash ether with 1 mL nanopure water to remove hydrophilic contaminants that may be present. Cap, vortex, and centrifuge as above, and transfer ether to new glass vial.

Note: *May not be necessary. Can be omitted if laboratory can demonstrate that there is no difference in analytical results between washed and non water treated samples.*

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

Step 14: If samples are to analyzed immediately, reconstitute in 250μL assay buffer that is provided with each ELISA kit and vortex. If analyses are to be conducted at a later date, cap vial and store dry at -80°C for up to 8 weeks.

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

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

Step 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 of reconstituted samples should not exceed 4 weeks).

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

Equation:

$$\%\ \text{recovery} = \frac{(CPM_{Sample} - CPM_{Blank}) \times 25}{(CPM_{Spike} - CPM_{Blank})} \times 100$$

(ii) **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 (Subection (g)(2)(i) and Figure 1). 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:

Equation:

Hormone concentration (per mL) / recovery * dilution factor

Example:

Extracted:	450 µL medium
Reconstituted in:	250 µL 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/mL (already adjusted for final concentration per mL)
Recovery:	89 %

Final Hormone Conc. = (150pg/mL) ÷ (0.89) x (250 µL/450 µL) x10 = 936.3 pg/mL

(6) **QC Parameters During Testing.** In addition to meeting the criteria for the QC plate, other quality criteria that pertain to variation between replicate wells, replicate experiments, linearity and sensitivity of hormone measurement systems, variability between replicate hormone measures of the same sample, and % recovery of hormone spikes after extraction of medium are provided in Table 7.

Table 7. Recommended Ranges and/or Variation (%) for H295R Assay Test Plate Parameters.

	Comparison Between	T	E2
Basal hormone production in SCs	Fold-greater than MDL	≥ 5-fold	≥ 2.5-fold
Exposure Experiments - Within Plate CV for SCs (Replicate Wells)	Absolute Concentrations	≤ 30%	≤ 30%
Exposure Experiments - Between Plate CV for	Fold-Change	≤ 30%	≤ 30%

	Comparison Between	T	E2
SCs (Replicate Experiments)			
Hormone Measurement System –Sensitivity	Detectable fold-decrease relative to SC	≥ 5-fold	≥ 2.5-fold
Hormone Measurement System – Replicate Measure CV for SCs	Absolute Concentrations	≤ 25%	≤ 25%
Medium Extraction – Recovery of Internal 3H Standard (If Applicable)	CPM	≥ 65% Nominal	

(7) **Data Reporting and Analysis.**

(i) **Data Processing and Statistics.** To evaluate the relative increase/decrease in chemically altered hormone production, results need to be normalized to the mean solvent control (SC) value for each assay (*i.e.*, each 24-well plate of cells used to test a given chemical), and results are expressed as changes relative to the SC in each exposure plate. ll data are expressed as mean +/- standard deviation (SD). All doses that exhibit cytotoxicity greater 20% are omitted for further evaluation. Relative changes are calculated as follows:

Equation:

Relative Change = (Hormone conc. in each well)÷(Mean solvent hormone conc.)

If by visual inspection of the well and the digital photographs described in Section 7 there appears to be an increase in cell number, the apparent increase needs to be verisfied. If an increase in cell numbers is verified, this should be stated in the data evaluation report, and the hormone data normalized by dividing hormone concentration by the relative change in the number of viable cells.

Prior to conducting statistical analyses, the assumptions of normality and variance homogeneity are evaluated. Normality are evaluated using standard probability plots or any other appropriate statistical method (e.g., Shapiro-Wilk's test). If the data are not normally distributed, it is recommended that the data be transformed to approximate a normal distribution. If the data are normally distributed or approximate normal distribution, differences between chemical treatments and solvent controls (SCs) are analyzed using parametric test (e.g., Dunnett's Test). If data are not normally distributed, an appropriate non-parametric test is used

(e.g., Kruskal Wallis, Steel's Many-one rank test). Differences are considered significant at $p \leq 0.05$.

A summary of criteria for the evaluation of data has been provided below (Table 8).

Table 8. Data Categorization Parameters for the Analysis of Results Obtained with the H295R Steroidogenesis Assay

Parameter	Criterion
Statistical Significance	Response is considered to be statistically significant if the difference from the Solvent Control has p ≤ 0.05
Dose response	Data is expected to follow a dose response type profile at non-cytotoxic doses, or doses that do not interfere with the hormone measurement assay (note: response can be bi-phasic such as an increase at lower and a decrease at higher doses, but changes randomly observed at only a few concentration within the dose range are to excluded).
Interference with hormone measurement assay	When marked interference of the chemical of interest with the hormone measurement system utilized occurs (≥ 30% of hormone concentration measured at the same dose at which interference occurred), this data is omitted. In the case of weak to moderate interference (< 30% of hormone concentration measured at the same dose at which interference occurred), results may be corrected for the % interference.
Solubility	The results at concentrations for which cloudiness or a precipitate is observed is not included.
Cell viability	Only non-cytotoxic concentration (> 80% cell viability) are included

(ii) **Data Interpretation and Graphical Representation.** Results are provided both in graphical (bar graphs representing mean +/- 1*SD) and tabular (LOEC, direction of effect, and strength of maximum response that is part of the dose-response portion of the data) formats (for example see Figure 1). The bars that correspond to statistically significant results are designated with an asterisk. Data assessment is only considered valid if it has been based on three independently conducted experiments[1]. Furthermore, the coefficient of variation for the LOECs (or NOECs if appropriate) among the three experiments is expected not to exceed 30%. The concentration range used in runs 2 and 3 may be tailored on the basis of the results of run 1 to better define the dose response range containing the LOEC.

[1] An experiment or run is considered independent if it has been conducted at a different date and with cells from a different culture plate.

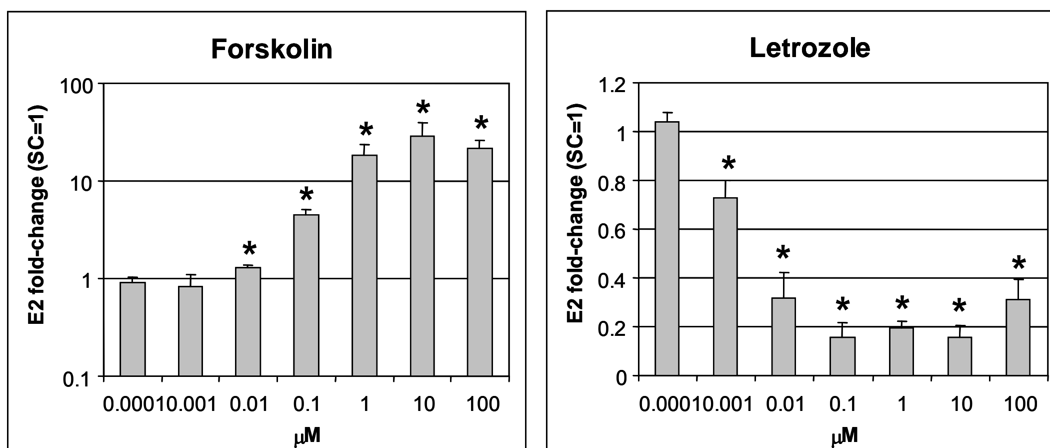


Figure 3. Example of the graphical presentation and evaluation of data obtained during the conduct of the H295R Steroidogenesis Assay. Asteriks indicate statistically significant differences from the solvent control ($p<0.05$).

A chemical is judged to be positive if the fold induction is statistically different from the solvent control following the procedures described in Subsection (h)(5) at doses that fall within the increasing or decreasing portion of the dose-response curve. Statistically significant increases in fold induction indicate the chemical is an inducer of one or more enzymes in the steroid synthesis pathway. Statistically significant decreases in fold induction indicate the chemical is an inhibitor of one or more enzymes in the steroid synthesis pathway. Statistically significant differences at concentrations that do not follow a dose-response curve may be due to random effects; such results are considered to be equivocal. As noted in Subsection (h)(5) results exceeding the limits of solubility or at cytotoxic concentrations are not included in interpreting results.

(iii) **Data Reporting.** The test report should include the following information:

(A) **Testing Facility:**

- ❑ Name of facility and location.
- ❑ Name of study director, other personnel, and their study responsibilities.
- ❑ Dates the study began and ended.

(B) **Test Substance, Reagents and Controls:**

- ❑ Identity (name/CAS No as appropriate), source, lot/batch number, purity, supplier, and characterization of test substance, reagents, and controls.
- ❑ Physical nature and relevant physicochemical properties of test substance.
- ❑ Storage conditions and the method and frequency of preparation of test substances, reagents and controls.
- ❑ Stability of test substance.

(C) **Cells:**

- ❑ Source and type of cells.
- ❑ Number of cell passages (cell passage identifier) of cells used in test.
- ❑ Description of procedures for maintenance of cell cultures.

(D) **Pre-test Requirements (if applicable):**

- ❑ Description and results of chemical hormone-assay interference test.
- ❑ Description and results of hormone extraction efficiency measurements.
- ❑ Standard and calibration curves for all analytical assays to be conducted.

(E) **Test Conditions:**

- ❑ Composition of media.
- ❑ Concentration of test chemical.
- ❑ Cell density (estimated or measured cell concentrations at 24 hours and 48 hours).
- ❑ Solubility of test chemical.
- ❑ Incubation time and conditions.

(F) **Test Results:**

- ❑ Raw hormone concentration data for each well for controls and test substances--each replicate measure in form of the original data provided by the instrument utilized to measure hormone production (e.g., OD, fluorescence units, CPM, etc.).
- ❑ Validation of normality or explanation of data transformation.
- ❑ Mean responses +/- 1 SD for each well measured.

- ❑ Viability (cytotoxicity) data (If using the Live/Dead® stain, include values for both live and dead fluorescence).
- ❑ Check that QC requirements were met.
- ❑ A bar graph showing relative (fold) change at each concentration, SD and statistical significance as stated in Subsection (h)(7)(ii).

(G) **Data Interpretation, Discussion and Conclusions:**

- ❑ Apply the data interpretation procedure to the results and discuss findings.

(8) **Records, Documentation and QC Requirements.** All QC requirements and criteria are listed in Appendix 1. Data are expected to fall within the acceptable ranges defined for each parameter. If these criteria are not met it is recommended that the sample be re-analyzed or be dropped from the data set (A notation is made on the spreadsheets that QC criteria were not met).

Any anomalies and/or deviation from the specified method are documented.

It is expected that all testing be conducted according to Good Laboratory Practice standards, but if a lab is not GLP certified, GLPs should be followed to the extent possible.

(i) **References.**

1. Hecker, M., Giesy, J.P., Jones, P.D., Higley, E.B., Newsted, J.L., Mehrle, P (2006). Influence of cell passage and freeze/thaw events on basal production of 17β-estradiol and testosterone by H295R cells. Interim draft report by ENTRIX, Inc., submitted to US-EPA, Contract Code # GS-10F-0041L. September 2006.

2. Hecker, M., Giesy, J.P., Timm, G (2008). Multi-Laboratory Validation of the H295R Steroidogenesis Assay to Identify Modulators of Testosterone and Estradiol Production. Report by ENTRIX, Inc., submitted to the U.S.-Environmental Protection Agency, Contract Code # GS-10F-0041L. February 2008.

3. Mosman T (1983). Rapid colorimetric assay for growth and survival: application to proliferation and cytotoxicity. J. Immunol. Methods. 100, 45-50.

4. Shapiro R, and Page LB (1976). Interference by 2,3-dimercapto-1-propanol (BAL) in agiotensim I radioimmunotassay. J Lab Clin Med. 1976 Aug (2): 22-31.

Appendix 1 – Freeze Down Identification

Symbol			Freeze Down Number
α	Α	Alpha	0
β	Β	Beta	1
γ	Γ	Gamma	2
δ	Δ	Delta	3
ε	Ε	Epsilon	4
ζ	Ζ	Zeta	5
η	Η	Eta	6
θ	Θ	Theta	7
ι	Ι	Iota	8
κ	Κ	Kappa	9
λ	Λ	Lambda	10
μ	Μ	Mu	11
ν	Ν	Nu	12
ξ	Z	Xi	13
ο	Ο	Omicron	14
π	Π	Pi	15
ρ	Ρ	Rho	16
σ	Σ	Sigma	17
τ	Τ	Tau	18
υ	Υ	Upsilon	19
φ	Φ	Phi	20
χ	Χ	Chi	21
ψ	P	Psi	22
ω	Ω	Omega	23

Appendix 2 – Cell Culture Diagram (Example)

Note: *For the specific purpose of the validation studies cells will be grown from an original ATCC batch for five (5.0) passages, and then frozen down resulting (given that cells from one culture plate were always split into three new plates as shown below) in a maximum of 162 batches. One of these batches will then be started up in culture again and grown for at least four (4) passages (passage # 4.5 = plate ID# B/4.5/9/X) prior to initiation of the exposure experiments. Only cells that have been frozen down one (1) time (B generation) will be used for these experiments. The passage number is not to exceed 10.*

Name:____

Date:______________________

Project:____

ATCC Lot Number: __________________________