APPENDIX I
- H295R Cell Culture Protocol -

University of Saskatchewan
Toxicology Centre
Environmental Toxicology Laboratory

STANDARD OPERATING PROCEDURE

Culturing of the H295R human adrenocortical carcinoma cell line (ATCC CLR-2128)

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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

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1 PURPOSE

The purpose of this document is to provide a consistent format for culturing H295R cell line.

2 SCOPE AND APPLICATION

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, cultivation and splitting of the growing cells, and the freezing of cells down 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 used for a variety of different purposes in the cell culture laboratory. Supplemented medium is a necessary component for culturing cells. Freeze medium is specifically designed to allow for impact-free freezing down of cells for long-term storage.

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.

2.1 Passage Nomenclature

In this protocol passages will be labeled using a simplified version of the tracking system utilized in the Cell Culture Diagram (APPENDIX II) that 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.

2.2 Important Considerations

- *Do not freeze cells upon arrival from ATCC but immediately bring them into culture following the procedures listed in the H295R Culture Protocol (Chapter 5.1 Starting Cells from Frozen Stock).*

- *Due to changes in estradiol producing capacities of the cells with age (Hecker et al. 2006), cells have to be 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 (Chapter 5.1 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 to be frozen down in liquid nitrogen (Chapter 5.3 Freezing H295R Cells) for storage. For cells started from these frozen batches, the procedures as described in section 5.1 (Starting Cells from Frozen Stock) will need to be followed. These cells will need to be cultured for at least four (4) additional passages (passage # 5.4) prior to their use in exposure experiments. The maximum number of passages that is acceptable for experimentation shall not exceed 10.*
- *Prior to initiation of cell cultures, Nu-serum has to be analyzed for background testosterone and estradiol concentrations as well as for the presence of mycoplasma.*
- *Prior to freezing cells down, a subset of passage five (5.0) cells is run in an QC plate (H295R Exposure Protocol; Chapter 5.1 Quality Control Experiment for cell performance) to verify whether the basal production of hormones and the response to a positive control chemical meet the quality criteria as defined in the exposure protocol (H295R Exposure Protocol; Chapter 8 Records, Documentation and QC Requirements). For those laboratories that have 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 in the QC plate experiment.*

3 SAFETY CONSIDERATIONS

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.

4 PREPARATION OF SOLUTIONS AND REAGENTS

4.1 Dulbecco's Phosphate Buffered Saline (PBS)

4.1.1 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

4.1.2 METHOD, PROCEDURES AND REQUIRMENTS

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 amount of PBS buffer can be made with appropriate adjustments of 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 cell culture sterilize 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
    - Initials
    - Expiration date
8. Store 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).

4.2 Stock and Supplemented Medium

4.2.1 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^{o}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 ^{o}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 ug/ml insulin
6.25 ug/ml transferring
6.25 ng/ml selenium
1.25 mg/ml bovine serum albumin
5.35 ug/ml linoleic acid
2.5 % Nu Serum

4.2.2 METHOD, PROCEDURES AND REQUIRMENTS

Note:

1. 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. Final concentrations of E2 in supplemented medium should not exceed 40 pg/mL. If E2 concentrations should be greater than 40 pg/mL medium, a different batch of Nu-serum should be used. If a batch with appropriate E2 concentrations is not available, the medium can be charcoal striped prior to use in the exposure experiments to remove hormones. It has been shown that the use of charcoal striped medium during the 48h exposure period does not influence the performance of the assay. However, for the culture of cells as well as for the 24h pre-incubation time (see H295R Exposure Protocol) regular supplemented medium must be used because charcoal striping removes components from the medium that are essential for the routine culture of H295R cells. The same batch of Nu-Serum must be used for each set of experiments.
2. Each new batch of Nu serum needs to be tested for the possible contamination with mycoplasma because the presence of this can significantly alter cell characteristics. Mycoplasma detection kits are commercially available from different suppliers.

Stock Medium (1L):

1. Place approximately 900 ml of ultra clean grade water (e.g. nanopure) 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 in an amber autoclaved bottle. Should to be done in the biosafety cabinet because this solution needs to be sterile.
10. Label the bottle as follows:
    - DME/F12 Stock Medium
    - H295R Cells
    - "Filter Sterilized"
    - pH = 7.4
    - Initials
    - Date
    - Expiration date
11. Medium should be stored in the refrigerator 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
- Initials
- Date
- Expiration date

11. Medium should be stored in the refrigerator at 4°C.
13. Supplemented mediums can be used for up to 3 weeks, after that new supplemented medium should be made.

4.3 Freeze Medium

4.3.1 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).

4.3.2 METHOD, PROCEDURES AND REQUIRMENTS

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"
   - Initials
   - Expiration date

6. Store at –20 ºC until needed.
7. Freeze medium can be stored for up to 6 months.

4.4 Trypsin 1X

4.4.1 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)

4.4.2 METHOD, PROCEDURES AND REQUIRMENTS

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 in freezer until needed to make 1X Trypsin.
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
    - Initials
    - Expiration date
8. Store in the freezer at -20 ºC until needed.
9. Maximum storage time is 6 months.

5 CELL MAINTENANCE AND CULTURE PROCEDURES

5.1 Starting Cells from the Frozen Stock

5.1.1 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); NCI-H295R cells (ATCC Cat # CRL-2128)

5.1.2 METHOD, PROCEDURES AND REQUIRMENTS

1. Aliquot 10 ml of the supplemented medium to a 15 ml centrifuge tube. Use one centrifuge tube with medium for each NCI-H295R vial that is being thawed.
2. Remove a vial of NCI-H295R cells from liquid nitrogen storage.
3. Thaw the vial rapidly by agitation in a 37^{o}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 chapter on "Maintaining and Sub-culturing the Cells" for the description of H295R cell maintenance and medium change).

5.2 Maintaining and Sub-culturing the Cells

5.2.1 EQUIPMENT, MATERIALS AND REAGENTS

Equipment: Biosafety Cabinet; Pipet-Aid, Incubator (37°C, 5% CO_2)

Materials: 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 (see chapter 4.2); Sterile 1x Trypsin-EDTA (see chapter 4.4); Sterile PBS (see chapter 4.1)

5.2.2 METHOD, PROCEDURES AND REQUIRMENTS

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 cells 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 cells and solutions into a biosafety cabinet.
4. 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.).
5. Carefully pipette old medium off the culture plate without disturbing cells.
6. 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.
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 take not 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 ration 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 (see chapter 6 and APPENDIX I: Cell Culture Diagram)
    - Plate designation

5.3 Freezing H295R Cells (Preparing Cells for Liquid Nitrogen Storage)

5.3.1 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 (See chapter 4.2); Sterile 1x Trypsin- EDTA (see chapter 4.4); Freeze Medium (see chapter 4.3); Sterile PBS (see chapter 4.1)

5.3.2 METHOD, PROCEDURES AND REQUIRMENTS

1. Follow the procedure for splitting cell lines (chapter 5.2) 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.
6. 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.
7. Re-suspend the pellet of cells in 1 ml of the appropriate cell freezing medium
8. 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 (see chapter 6 and APPENDIX I: Cell Culture Diagram)
9. Place the vial(s) into the controlled rate freezing container.
10. Put the container into a -80°C freezer for 24 hours.
11. After the 24 hours in the freezer transfer to liquid nitrogen for storage.
12. 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: 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. They also provide us with a platform for recording all of the work done with the cells.

6.1.1 METHOD, PROCEDURES AND REQUIRMENT

1. Start with a clean page in your laboratory notebook. At the top of the page, record your initials, the date and "Cell Culture Diagram for H295R".
2. In a box at the top of the page record all of the information from the saved cryovial:
    - 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
3. Each time you split the cells you will increase the passage number and total number of passages by one. Each time you freeze down the cells you will increase the freeze down ID (Greek letter) by one starting with A and continuing with B, Γ, Δ, E etc. (see APPENDIX I for key). When you start cells from a freeze down you will restart the passage number to 1 and continue the total number of passages. Each different plate/flask will be labeled with another letter with the first being A, the second B, and so on.
4. 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 record everything that you do whether it is change the media, freeze cells down, split the cells etc. (See APPENDIX II for a sample diagram).
5. Continue with the cell culture diagram until the cells are no longer being actively used in research or they are all frozen down.

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. You should also update the liquid nitrogen Dewar contents log book if removing vial a to start new batch of cells or when transferring cryogenic vials with frozen cells to the liquid nitrogen.

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.

8 RESPONSIBILITIES

The person(s) working in the cell culture laboratory will be responsible for the preparation of stock medium and supplemented medium. They are also responsible for recognizing when more stock medium needs to be made, and when more medium needs to be ordered. See the "Ordering Sheet for the Cell Laboratory" to determine supplier and other information needed for ordering. The person(s) working in the cell culture laboratory will be responsible for thawing and culturing and splitting the cells. They will also need to be able to recognize when new cells need to be thawed. The person(s) working in the cell culture laboratory will be responsible for freezing down the cells. They will also need to be able to recognize when stocks are getting low and more cells need to be frozen.

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.

9 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., 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. ENTRIX, Inc., submitted to US-EPA, Dated September 2006.

APPENDIX I – FREEZE DOWN IDENTIFICATION

Symbol			Freeze Down Number
α	A	Alpha	0
β	B	Beta	1
γ	Γ	Gamma	2
δ	Δ	Delta	3
ε	E	Epsilon	4
ζ	Z	Zeta	5
η	H	Eta	6
θ	Θ	Theta	7
ι	I	Iota	8
κ	K	Kappa	9
λ	Λ	Lambda	10
μ	M	Mu	11
ν	N	Nu	12
ξ	Z	Xi	13
ο	O	Omicron	14
π	Π	Pi	15
ρ	P	Rho	16
σ	Σ	Sigma	17
T	τ	Tau	18
υ	Y	Upsilon	19
ϕ	Φ	Phi	20
χ	X	Chi	21
ψ	P	Psi	22
ω	Ω	Omega	23

APPENDIX II – 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 7.5.

Name:_____ Date:_____
Project:_____
ATCC Lot Number: _____