

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE 4XX

The H295R Steroidogenesis Assay

INTRODUCTION

1. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new, Test Guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals comprises five levels, each level corresponding to a different level of biological complexity (1). The *in vitro* H295R Steroidogenesis Assay (H295R) described in this Test Guideline utilises a human adreno-carcinoma cell line (NCI-H295R cells) and constitutes a level 2 “*in vitro* assay, providing mechanistic information”. Development and standardization of the assay as a screen for chemical effects on steroidogenesis, specifically the production of 17 β -estradiol (E2) and testosterone (T) was carried out in a multi-step process. The H295 assay has been optimized and validated (2)(3)(4).

2. The objective of the H295R Steroidogenesis Assay is to detect substances that affect androgen or estrogen steroid hormone production, specifically that of E2 and T. The H295R assay is intended to identify xenobiotics that have as their target site(s) the endogenous components that comprise the intracellular biochemical pathway beginning with the sequence of reactions from cholesterol to the production of the terminal steroid hormones. The H295R assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamic-pituitary-gonadal (HPG) axis. The goal of the assay is to provide a YES/NO answer with regard to the potential of a chemical to induce or inhibit the production of T and E2. The results of the assay are expressed as relative changes in hormone production compared with the solvent controls (SCs). The assay does not aim to provide mechanistic information concerning the interaction of the test substance with the endocrine system.

3. Definitions and abbreviations used in this Test Guideline are described in Annex 1. A detailed protocol including instructions on how to prepare solutions, cultivate cells and perform various aspects of the test is available as an annex to OECD monograph XXX: *Multi-Laboratory Validation of the H295R Steroidogenesis Assay to Identify Modulators of Testosterone and Estradiol Production* (4).

INITIAL CONSIDERATIONS AND LIMITATIONS

4. Five different enzymes catalyzing six different reactions are involved in sex steroid hormone biosynthesis. Enzymatic conversion of cholesterol to pregnenolone by the cytochrome P450 (CYP) cholesterol side-chain cleavage enzyme (CYP11A) constitutes the initial step in a series of biochemical reactions that culminate in synthesis of steroid end-products. Depending upon the order of the next two reactions, the steroidogenic pathway splits into two paths, the Δ^5 -

hydroxysteroid pathway and Δ^4 -ketosteroid pathway, which converge in the production of androstenedione (Figure 1).

5. Androstenedione is converted to testosterone (T) by 17β -hydroxysteroid dehydrogenase (17β -HSD). Testosterone is both an intermediate and end-hormone product. In the male, T can be converted to dihydrotestosterone (DHT) by 5α -reductase, which is found in the cellular membranes, nuclear envelope, and endoplasmic reticulum of target tissues of androgenic action such as prostate and seminal vesicles. DHT is significantly more potent as an androgen than T and is also considered an end-product hormone.

6. The last enzyme in the steroidogenic pathway is aromatase (CYP19) which converts androgenic substances into estrogenic substances. CYP19 converts T into 17β -estradiol (E2) in both sexes and, in the female, androstenedione into estrone. E2 and estrone are considered end-product hormones of the steroidogenic pathway.

7. The lyase activity of CYP17 differs for the intermediate substrates among species. In the human, CYP17 converts 17α -hydroxypregnenolone to DHEA (the Δ^5 -hydroxysteroid pathway) but not 17α -hydroxyprogesterone to androstenedione (Δ^4 -ketosteroid pathway). In contrast, in the rat, CYP17 converts the intermediates of both the Δ^5 -hydroxysteroid and Δ^4 -ketosteroid pathways equally. Such differences in the CYP17 lyase activity may explain species-dependent differences in response to substances that alter steroidogenesis (5).

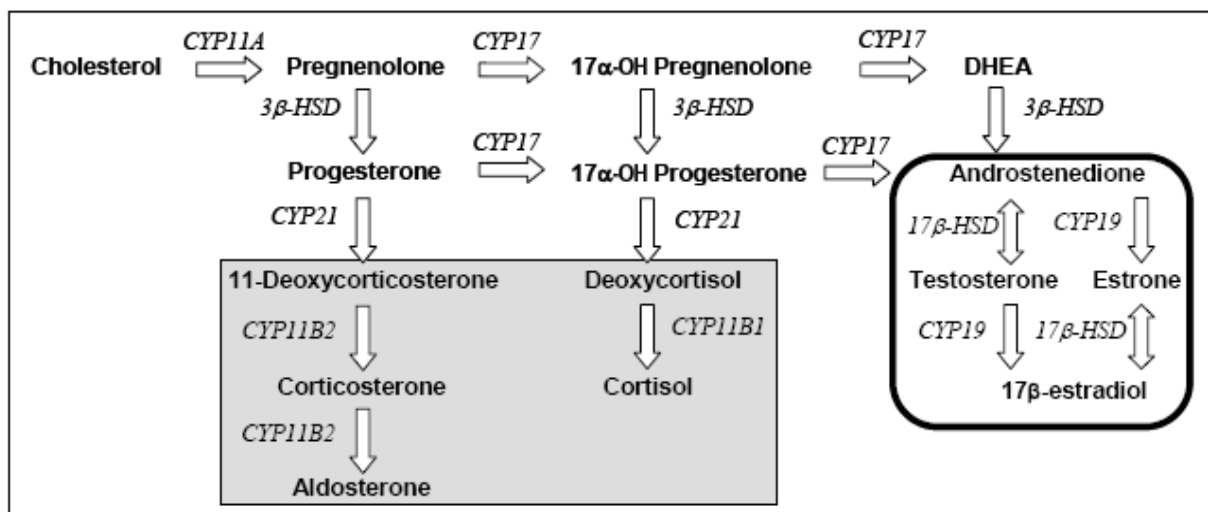


Figure 1: Steroidogenic pathway in H295R cells. Enzymes are in italics, hormones are bolded and arrows indicate the direction of synthesis. Gray background indicates corticosteroid pathways/products. Sex steroid pathways/products are circled. CYP = cytochrome P450; HSD = hydroxysteroid hydrogenase; DHEA = dehydroepiandrosterone.

8. The human H295R adreno-carcinoma cell line has been shown to be a useful *in vitro* model for steroidogenic pathways and processes (6)(7)(8). The H295R cell line expresses genes that encode for all the key enzymes for steroidogenesis noted above (9)(10) (Figure 1). This is a unique property because *in vivo* expression of these genes is tissue and developmental stage-specific with typically no one tissue or one developmental stage expressing all of the genes

involved in steroidogenesis. H295R cells have physiological characteristics of zonally undifferentiated human fetal adrenal cells (9). The cells represent a unique *in vitro* system in that they have the ability to produce all of the steroid hormones found in the adult adrenal cortex and the gonads, allowing testing for effects on both corticosteroid synthesis and the production of sex steroid hormones such as androgens and estrogens, although the assay was validated only to detect T and E2.

9. The H295R assay has several advantages over rodent tissue-based assays.
 - It allows for the detection of both increases and decreases in the production of both T and E2, and thus, permits identification of chemicals with the potential to induce or inhibit the enzymes of the steroidogenesis pathway.
 - It permits the direct assessment of the potential impact of a chemical on cell viability/cytotoxicity. This is an important feature as it allows for the discrimination between effects that are due to cytotoxicity from those due to the direct interaction of chemicals with steroidogenic pathways, which is not possible in tissue explants systems that consist of multiple cell types of varying sensitivities and functionalities.
 - It is of human origin and more appropriately reflects steroidogenesis in humans than in rodent tissue.
 - It does not require the use of animals.
 - The H295R cell line is commercially available.

10. The principle limitations of the assay are as follows:
 - Its metabolic capability is unknown but probably quite limited; therefore, substances that need to be metabolically activated will probably be missed in this assay.
 - Being derived from adrenal tissue, the H295R possesses the enzymes capable of producing the gluco- and mineral-corticoids as well as the sex hormones; therefore, effects on the production of gluco- and mineralcorticoids could influence the levels of T (CAS no. 58-22-0) and E2 (CAS no. 50-28-2) observed in the assay.
 - It does not measure dihydrotestosterone and, therefore, would not be expected to detect substances that inhibit 5 α -reductase in which case the Hershberger assay (11) can be used. The H295R assay will not detect substances that interfere with steroidogenesis by affecting the HPG-axis as this can only be studied in intact animals.

PRINCIPLE OF THE TEST

11. As the purpose of the assay is to detect substances that affect sex steroid hormone synthesis, the endpoints chosen for the assay are the production of T and E2, the two principal sex hormones. T is also an intermediate in the pathway to produce E2. The assay can detect chemicals that typically inhibit or induce the enzymes of the steroidogenesis pathway.

12. The assay is usually performed under standard cell culture conditions in 24-well culture plates. Alternatively, 48-well plates can be used for conducting the H295R Steroidogenesis Assay; however, seeding and experimental conditions should be adjusted accordingly. Tests with 48-well plates should be run with a final well volume of 0.5 mL and 100,000 – 150,000 cells per well. The adjusted assay must meet the quality and performance criteria listed in this guideline.

13. After an acclimation period of 24 h, cells are exposed for 48 h to six concentrations of the test chemical in triplicate. Solvent and a known inhibitor and inducer of hormone production are run at a fixed concentration as negative and positive controls. At the end of the exposure period, the medium is removed from each well. Cell viability in each well is analyzed immediately after removal of medium. Concentrations of hormones in the medium can be measured using a variety of methods including commercially available hormone measurement kits and/or instrumental techniques such as liquid chromatography-mass spectrometry (LC-MS). Data are expressed as fold change relative to the solvent control and the Lowest-Observed-Effect-Concentration (LOEC). If the assay is negative, the highest concentration tested is reported as the No-Observed-Effect-Concentration (NOEC). Conclusions regarding the ability of a chemical to affect steroidogenesis should be based on three independent test runs. The first test run may function as a range finding run with subsequent adjustment of concentrations for runs 2 and 3 if solubility or cytotoxicity problems are encountered or the activity of the chemical seems to be at the end of the range of concentrations tested.

CULTURE PROCEDURE

Cell Line

14. The NCI-H295R cells are commercially available from the American Type Culture Collections (ATCC) upon signing a Material Transfer Agreement (MTA)¹.

Introduction

15. Due to changes in the E2 producing capacity of the cells with increasing age/passages (6), cells should be cultured following a specific protocol before they are used and the number of passages since the cells were defrosted as well as the passage number at which the cells were frozen and placed in liquid nitrogen storage should be noted. The first number indicates the actual cell passage number and the second number describes the passage number at which the cells were frozen and placed in storage. For example, cells that were frozen after passage five and defrosted and underwent 4 passages after they were cultured again would be labeled passage 4.5. An example of a numbering scheme is illustrated in Annex 1B to the validation report (4).

16. Stock medium is used as the base for the supplemented and freezing mediums, which are used for a variety of different purposes in culturing the cells and preparing them for use in testing. Supplemented medium is a necessary component for culturing cells. Freezing medium is specifically designed to allow for impact-free freezing of cells for long-term storage. Prior to use, Nu-serum (or a comparable serum of equal properties that has been demonstrated to produce data that meets the test performance and QC requirements), which is a constituent of supplemented media, should be analyzed for background T and E2 concentrations. The preparation of these solutions is described in Annex 1B to the validation report (4).

17 After initiation of an H295R cell culture from an original ATCC batch, cells should be grown for five (5.0) passages (*i.e.* the cells are split 4-times). Passage five cells are then frozen in liquid nitrogen for storage. Prior to freezing the cells, a sample of passage five (5.0) cells is run in a Quality Control (QC) plate (See paragraph 34 and 35) to verify whether the basal production of

¹ ATCC CRL-2128; ATCC, Manassas, VA, USA, [<http://www.lgcstandards-atcc.org/>].

hormones and the response to positive control chemicals meet the assay quality control criteria as defined in Table 5.

18. H295R cells need to be cultured, frozen and stored in liquid nitrogen to make sure that there are always cells of the appropriate passage/age available for culture and use. The maximum number of passages after taking a new or frozen batch of cells into culture that is acceptable for use in the H295R assay should not exceed 10. For cells started from these frozen batches, the procedure described in paragraph 18 should be followed. These cells should be cultured for at least four (4) additional passages (passage 4.5) prior to their use in testing.

Starting Cells from the Frozen Stock

19. The procedure for starting the cells from frozen stock is to be used when a new batch of cells is removed from liquid nitrogen storage for the purpose of culture and testing. Details for this procedure are set forth in Annex 1B of the validation report (4). All transfers of material should take place under sterile conditions in a bio-safety cabinet. H295R cells are removed from liquid nitrogen storage, thawed rapidly, placed in 10 ml of supplemented medium (37°C) in a 15 ml centrifuge tube, centrifuged at 125g for 10 min, re-suspended in 12 ml of supplemented medium, and transferred to a 100 x 20 mm culture plate. The medium should be changed the following day. The H295R cells are cultivated in an incubator at 37°C with 5% CO₂ in air atmosphere and the medium is renewed 2-3 times per week. When the cells are approximately 90% confluent, they should be split. Splitting of the cells is necessary to ensure the health and growth of the cells and to maintain cells for performing bioassays. The cells are rinsed three times with 5 ml of phosphate-buffered saline (PBS) and freed from the culture plate by the addition of 1.5 ml of sterile trypsin solution. After the cells detach from the plate, the trypsin action should be stopped with the addition of 10.5 ml of supplemented medium. The appropriate amount of cell solution is placed 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.

The plate should be labelled 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 (Annex4: Cell Culture Identification Scheme)
- Plate designation

The cells are now ready to be used in the assay (See paragraph 20). Excess cells should be frozen in liquid nitrogen as described in paragraph 20.

Freezing H295R Cells (preparing cells for liquid nitrogen storage)

20. The procedure described above for splitting cell lines through the step in which the action of trypsin is stopped by the addition of supplemented medium should be followed. Then, in the bio-safety cabinet the cell solution is pipetted into a sterile, labelled centrifuge tube and centrifuged for 5 minutes at 350g at room temperature. Upon removal from the centrifuge, there should be a pellet of cells in the bottom of the centrifuge tube. The supernatant is pipetted off and the pellet of cells is re-suspended in 1 ml of cell freezing medium. The solution is transferred to a

sterile cryogenic vial and labelled as above, and placed into the controlled rate freezing container and frozen at -80°C for 24 hours after which the cryogenic vial is transferred to liquid nitrogen for storage. The cells must be stored in an appropriate liquid nitrogen storage device. It is recommended to keep the vials in the vapour phase instead of having the cryogenic vials submerged in the liquid nitrogen fluid. Details for this procedure are set forth in Annex 1B of the validation report (4).

Plating and Pre-incubation of Cells for Testing

21. The H295R cell culture plates, prepared as outlined in Paragraph 18, are removed from the incubator and placed in a bio-safety cabinet. The number of cell culture plates that will be needed depends on the number of chemicals to be tested and the confluency of the cells in the culture dishes. As a general rule, one cell culture plate (cavity diameter 100 mm) of 95-100% confluent cells will supply sufficient cells for two 24-well plates at a target density of 200,000 to 300,000 cells per mL of medium resulting in approximately 50-60% confluency in the wells at 24 hours (Figure 2). This is the optimal cell density for hormone production in the assay. At higher densities cells tend to be affected by hormonal feedback mechanisms and T as well as E2 production patterns are altered. Before conducting the assay the first time, it is recommended that different seeding densities between 200,000 and 300,000 cells per mL be tested, and the density resulting in 50-60% confluency in the well at 24 hours be selected for further experiments.

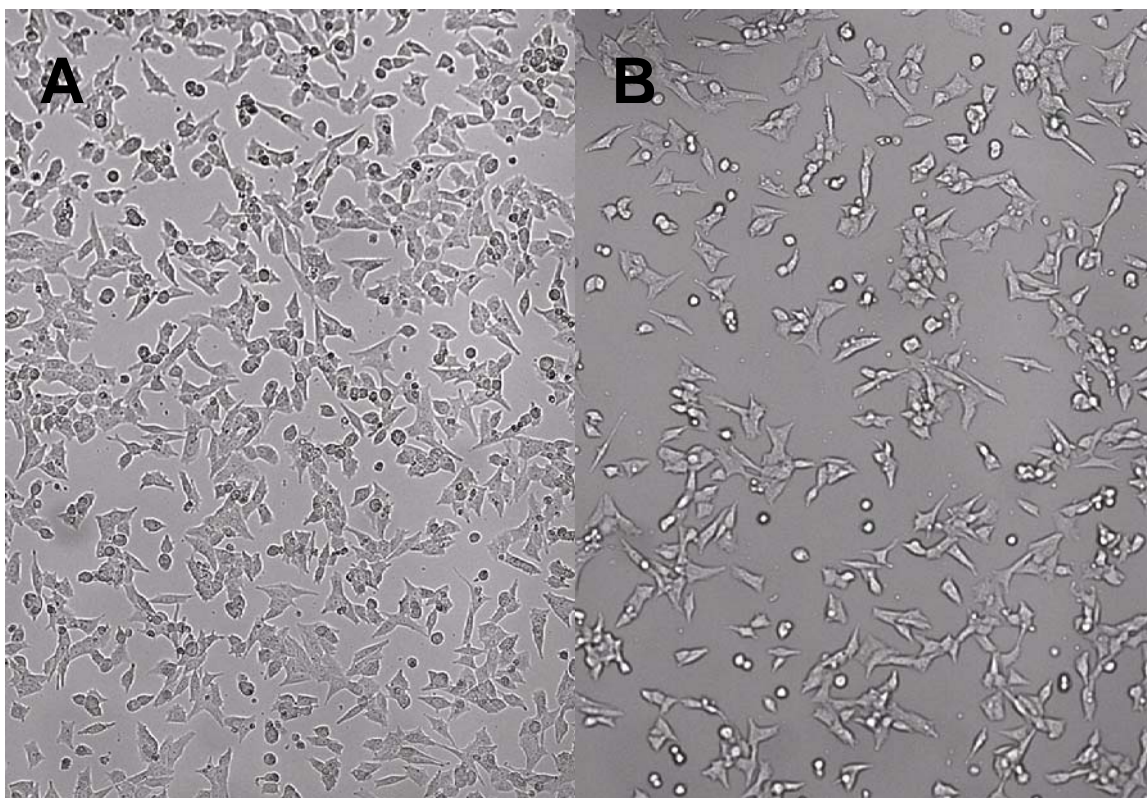


Figure 2: Photomicrograph of H295R cells at a seeding density of 50 – 60% in a 24 well culture plate at 24 hours

22. The medium is pipetted off the culture plate, and the cells are rinsed 3 times with 5 ml of

sterile PBS. Trypsin solution is added to detach the cells from the plate. The cells are placed into a centrifuge tube, suspended in medium and aspirated to make the content homogeneous. A small sub-sample (10µl) of the well mixed cell suspension is placed in a haemocytometer and the cells counted – at least 3 squares from each side of the haemocytometer. Alternatively, a cell counter can be used. The cell density is calculated as the mean cell count x 10⁴. The cell solution should be diluted to the desired plating density and thoroughly mixed to assure homogenous cell density. The cells should be plated with 1 mL of the cell solution/well and the plates and wells labelled. The seeded plates are incubated at 37°C under a 5% CO₂ in air atmosphere for 24 hours to allow the cells to attach to the wells.

QUALITY CONTROL REQUIREMENTS

23. 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 calibrated pipettes of the appropriate volume range should be used.

24. Prior to the initiation of cell culture and any subsequent testing, each laboratory should demonstrate the sensitivity of its hormone measurement system (paragraphs 28 - 30).

25. If antibody-based hormone measurement assays are to be used, the chemicals to be tested should be analyzed for their potential to interfere with the measurement system used to quantify T and E2 as outlined in paragraph 31 prior to initiating testing.

26. Prior to conducting testing for the first time, the laboratory should 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 paragraphs 32-34.

27. When initiating testing using a new batch or passage of cells, a control plate should be run before using a new batch of cells to evaluate the performance of the cells as described in paragraph 35 and 36.

Performance of the Hormone Measurement System

Standard curve

28. The solvent control (SC) values should fall within the linear portion of the standard curve, i.e. the SC should 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 3). Dilutions of medium (extracts) in the hormone measurement assay are to be selected accordingly.

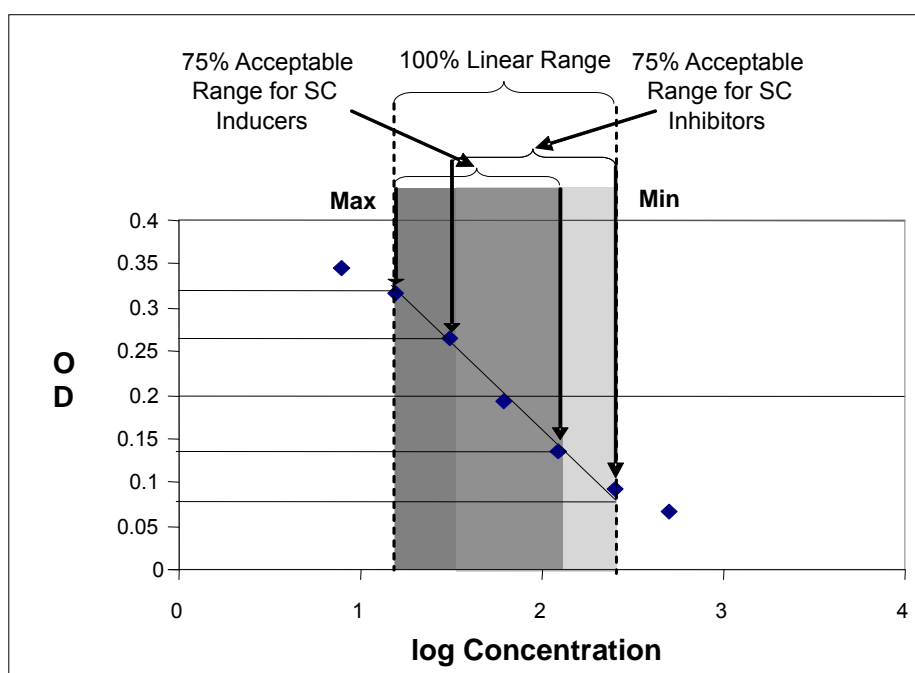


Figure 3: Example of hormone measurement system standard curve indicating acceptable range of hormone concentration of solvent control (SC) sample (not corrected for dilution in assay). Max = upper limit of linear range; Min = lower limit of linear range. Grey shaded area = 75% range below the maximum optical density (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.

Method sensitivity, accuracy, precision and cross-reactivity with sample matrix

29. Each laboratory may use a hormone measurement system of its choice for the analysis of the production of T and E2 by H295R cells so long as it meets performance criteria, including the minimum detection limits (MDL). Nominally these are 100 pg/mL for T and 10 pg/mL for E2, which are based on the basal hormone levels observed in the validation studies. However, greater or lower levels may be appropriate depending upon the basal hormone levels achieved in the performing laboratory. Prior to initiation of QC plate and test runs, the laboratory should demonstrate that the hormone assay to be used can measure hormone concentrations in supplemented medium with sufficient accuracy and precision to meet the QC criteria specified in Tables 1 and 7 by analyzing supplemented medium spiked with an internal hormone control. Supplemented medium should be spiked with at least three concentrations of each hormone (100, 500 and 2500 pg/mL of T and 10, 50 and 250 pg/mL of E2) and analyzed. Measured hormone concentrations of non-extracted samples should be within 30% of nominal concentrations, and variation between replicate measurements of the same sample should not exceed 25% (see also table 7 for additional QC criteria). If these QC criteria are fulfilled it is assumed that the selected hormone measurement assay is sufficiently accurate, precise and does not cross-react with components in the medium (sample matrix) such that a significant influence on the outcome of the assay would be expected. In this case, no extraction of samples prior to measurement of hormones is required.

30. In the case that the QC criteria in tables 1 and 7 are not fulfilled, it should be assumed that

significant matrix effects occur, and an experiment with extracted spiked medium described in Annex 2B of the validation report (4) should be conducted. Measurements of the hormone concentrations should be made in triplicate. If it can be shown that after extraction the components of the medium do not interfere with the hormone detection method as defined by the QC criteria, all further experiments should be conducted using extracted samples. If the QC criteria cannot be met after extraction, the utilized hormone measurement assay is not suitable for the purpose of the H295R Steroidogenesis Assay, and an alternative hormone detection method should be used.

Chemical interference test

31. If antibody-based assays such as Enzyme-Linked Immunosorbent Assays (ELISAs) and Radio-Immuno Assays (RIAs) are going to be used to measure hormones, each chemical should be tested for potential interference with the hormone measurement system to be utilized prior to initiation of the actual testing of chemicals (Annex 2B of the validation report (4)) because some chemicals can interfere with these tests (12). If interference occurs that is $\geq 20\%$ of basal hormone production for T and/or E2 as determined by hormone analysis, the Chemical Hormone Assay Interference Test (see Annex 4, section 5.0) should be run on all test chemical stock solution dilutions to identify the threshold dose at which significant ($\geq 20\%$) interference occurs. If interference is less than 30%, results may be corrected for the interference. If interference exceeds 30%, the data are invalid and the data at these concentrations should be discarded. If significant interference of a test chemical with a hormone measurement system occurs at more than one non-cytotoxic concentration, a different hormone measurement system should be used.

Table 1: Performance criteria for hormone measurement systems.

<i>Parameter</i>	<i>Criterion</i>
Measurement Method Sensitivity	Method Detection Limit T: 100 pg/mL; E2: 10 pg/mL ^a
Hormone Extraction Efficiency	When analyzed with the hormone measurement assay, the average recovery rates (based on triplicate measures) for the spiked amounts of hormone should not deviate more than 30% from nominal concentrations.
Chemical Interference (only antibody based systems)	No substantial ($\geq 30\%$ of basal hormone production of the respective hormone) cross-reactivity with any of the hormones produced by the cells should occur. ^{b, c}

^a Note: Method measurement limits are based on the basal hormone production values provided in Table 5, and are performance based. If greater basal hormone production can be achieved the limit can be greater.

^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 17 β -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.

^cThese include: cholesterol, pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17 α -pregnenolone, 17 α -progesterone, deoxycortisol, cortisol, DHEA, androstenedione,

estrone.

Laboratory Proficiency Test

32. Before testing unknown substances, a laboratory should 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, these procedures should be repeated if a change in laboratory personnel occurs.

33. This qualification test will be conducted under the same conditions listed in paragraphs 37 through 39 by exposing cells to six increasing concentrations of the model inducer forskolin (CAS no. 66575-29-9) and the model inhibitor prochloraz (CAS no. 67747-09-5) as shown in Table 2. Separate plates are run for forskolin and 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 EC₅₀ of each of the control chemicals than would be the case for an initial run with an unknown chemical (Table 2).

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 run for forskolin and prochloraz.

34. Cell viability and hormone analyses should be conducted as provided in paragraphs 43 through 45. The hormone effective concentrations, expressed as 50% of the maximum response (EC₅₀s), should be calculated using standard procedures (e.g. Probit analysis or moving average method) and compared with the values in Table 3. The data are considered acceptable if they fall within the following ranges:

Table 3: EC₅₀ Ranges for Control Substances

	CAS no.	EC₅₀ (µM)
--	----------------	-----------------------------

		<i>T</i>	<i>E2</i>
<i>Prochloraz</i>	67747-09-5	0.01 – 0.1	0.03 – 0.3
<i>Forskolin</i>	66575-29-9	0.2 – 2.0	0.3 – 3.0

Quality Control Plate

35. There are two uses of the quality control (QC) plate:

1. H295R cell performance should 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 (paragraphs 32-34) 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.
2. A QC plate provides the positive controls for the assay when testing chemicals and should be part of each test run.

36. The QC test is conducted in a 24 well plate and follows the same incubation, dosing, cell viability/cytotoxicity, hormone extraction and hormone analysis procedures described in paragraphs 37 through 46 for testing chemicals. The QC plate contains two concentrations of a known inducer (forskolin) and inhibitor (prochloraz) of E2 and T synthesis. A detailed description of the plate layout is provided in Table 4. The criteria to be met on the QC plate are listed in Table 5. The minimum basal hormone production should be met in both the solvent control and blank wells.

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. A 70% methanol solution will be added to all MeOH wells after termination of the exposure experiment and removal of medium (see Live/Dead® Assay procedure in Annex 3).

	1	2	3	4	5	6
A	Blank ^a	Blank ^a	Blank ^a	Blank ^a + MeOH ^b	Blank ^a + MeOH ^b	Blank ^a + MeOH ^b
B	DMSO 1µL	DMSO 1µL	DMSO 1µL	DMSO 1µL + MeOH ^b	DMSO 1µL + MeOH ^b	DMSO 1µL + MeOH ^b
C	FOR 1µM	FOR 1µM	FOR 1µM	PRO 0.1µM	PRO 0.1µM	PRO 0.1µM
D	FOR 10µM	FOR 10µM	FOR 10µM	PRO 1µM	PRO 1µM	PRO 1µM

^a Blank wells receive medium only.

^b Methanol (MeOH) will be added **after** the exposure is terminated and the medium is removed from these wells.

Table 5: Performance criteria for the Quality Control Plate

	T	E2
Minimum Basal Production	500 pg/mL	40 pg/mL
Basal Production of hormone in the solvent control (SC)	\geq 5-times the minimum detection limit (MDL)	\geq 2.5-times the MDL
Induction (10 μ M forskolin)	\geq 2 times the SC	\geq 7.5 times the SC
Inhibition (3 μ M prochloraz)	\leq 0.5 times the SC	\leq 0.5 times the SC

CHEMICAL EXPOSURE PROCEDURE

37. The pre-incubated cells are removed from the incubator (paragraph 21) and checked under microscope to assure that they are in good condition (attachment, morphology) prior to dosing.

38. The cells are placed in a bio-safety cabinet and the medium removed and replaced with new medium (1 mL/well). Cells are exposed to the test chemical by adding 1 μ L of the appropriate stock solution in DMSO (see Annex 2B of the validation report (4)) per 1 mL medium (well volume). This results in a final concentration of DMSO of 0.1%. Cells can be exposed either indirectly by adding the solvent/ stock solution to the medium or by direct dosing of each well. With indirect dosing the old medium is pipetted off and the dosed medium added. If cells are dosed directly, DMSO or the test chemical solution should be added by slowly pipetting the solvent or stock solution into the medium with a swirling motion. DMSO sinks to the bottom, and if not distributed during dosing, may concentrate at one spot possibly impacting cells at this location. The plate is returned to the incubator at 37°C under a 5% CO₂ in air atmosphere for 48 hours. The test chemical plate layout is shown in Table 6. Dosing is calculated based on a total volume of 1 mL per well. Stock 1-7: 1 μ L of the appropriate stock solution is added to each well.

Table 6: Dosing schematic for the exposure of H295R cells to test chemicals in a 24 well plate

	1	2	3	4	5	6
A	DMSO 1 μ L	DMSO 1 μ L	DMSO 1 μ L	Stock 4 1 μ L	Stock 4 1 μ L	Stock 4 1 μ L
B	Stock 1 1 μ L	Stock 1 1 μ L	Stock 1 1 μ L	Stock 5 1 μ L	Stock 5 1 μ L	Stock 5 1 μ L
C	Stock 2 1 μ L	Stock 2 1 μ L	Stock 2 1 μ L	Stock 6 1 μ L	Stock 6 1 μ L	Stock 6 1 μ L
D	Stock 3 1 μ L	Stock 3 1 μ L	Stock 3 1 μ L	Stock 7 1 μ L	Stock 7 1 μ L	Stock 7 1 μ L

39. After 48 hours the exposure plates are removed from the incubator and every well is checked under the microscope for cell condition (attachment, morphology, degree of confluence) and signs of cytotoxicity. The medium from each well is split into two equal amounts (approximately 490 μ L each) and transferred to two separate Eppendorf tubes appropriately labeled. To make sure that cells do not dry out, medium is removed a row or column at a time and 200 μ L PBS with Ca^+ and Mg^+ is added to each well. The media are frozen at -80°C until further processing to analyze hormone concentrations (see paragraphs 43-45).

40. Immediately after removing the medium, cell viability is determined for each exposure plate.

Cell Viability Determination

41. A cell viability/cytotoxicity assay of choice can be used to determine the potential impact of the test chemical on cell viability. The assay should be able to provide a true measure of the percentage of viable cells present in a well, or it should be demonstrated that it is directly comparable to (a linear function of) the Live/Dead® Assay (Molecular probes, Eugene OR, USA, Cat # L-3224)(see Annex 2B of the validation report (4)). An alternative assay that has been shown to work equally well is the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] test (13). 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.

42. The minimum cell viability required per well is 80%, and wells with lower viability should not be included in the final data analysis.

Hormone Analysis

43. Each laboratory can use a hormone measurement system of its choice for the analysis of T and E2. As noted in paragraph 29, each laboratory should demonstrate the conformance of their hormone measurement system (*e.g.* ELISA, RIA, LC-MS) with the QC criteria by analyzing supplemented medium spiked with an internal hormone control prior to conducting QC runs or testing of chemicals. In order to ensure that the components of the test system do not interfere with measurement of hormones, the hormones are extracted from the media following the procedures in Annex 2B of the validation report (4) prior to their measurement.

44. If a commercial test kit is being used to measure the hormone production, the hormone analysis should be conducted 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 (paragraph 28). Dilutions in the plates need to be adjusted such that expected hormone concentrations for the solvent controls fall within

the centre of the linear range of the standard curve of the individual assay (Annex 2B of the validation report (4)). Values outside of the linear portion of the standard curve should be rejected.

45. Final hormone concentrations are calculated as follows:

Example:

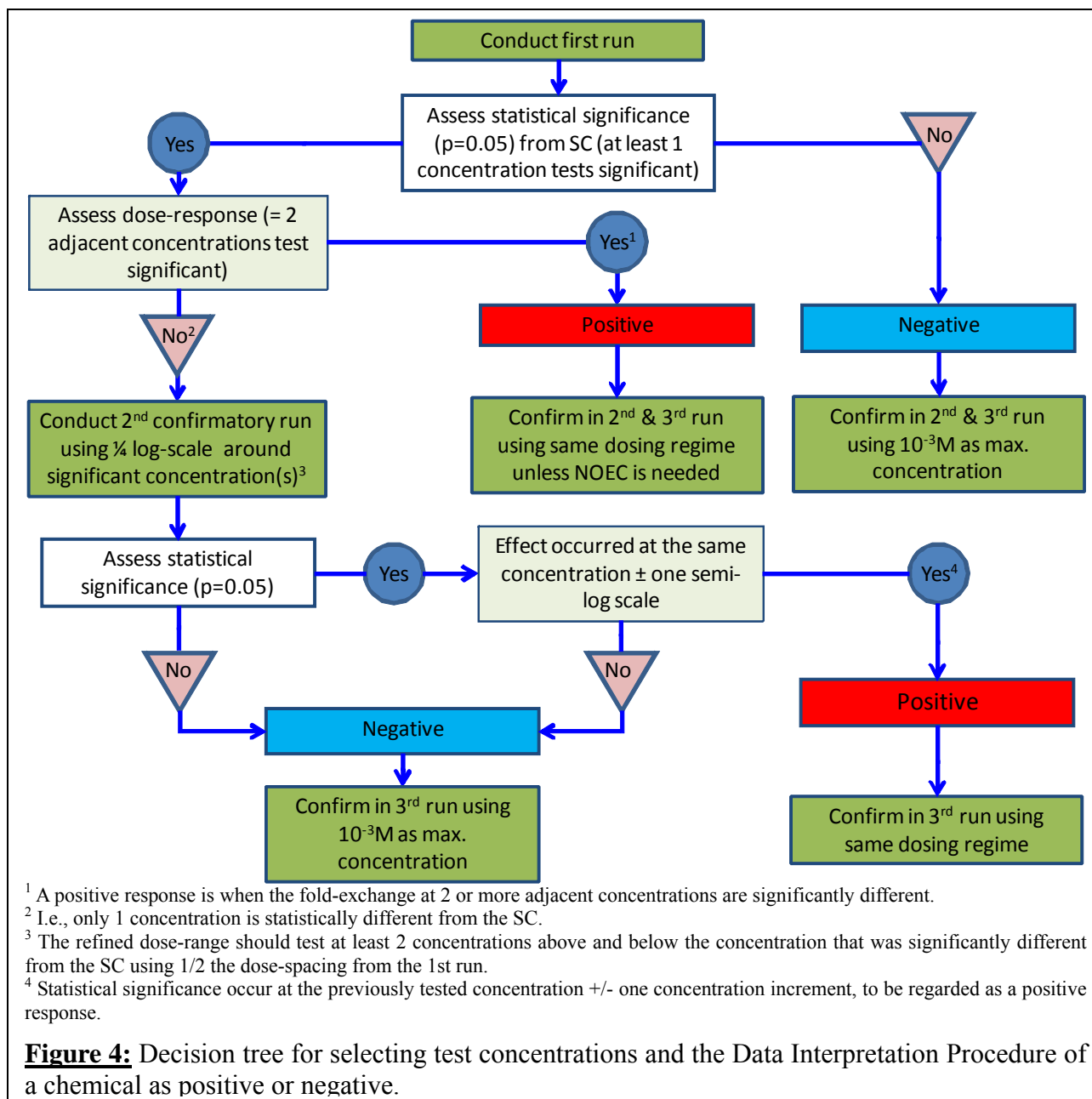
Extracted:	450 µL medium
Reconstituted in:	250 µL assay buffer
Dilution in Assay:	1:10 (to bring the sample within the line range of the standard curve)
Hormone Concentration in Assay:	150 pg/mL (already adjusted for final concentration per mL)
Recovery:	89 %

Final hormone concentration = (Hormone concentration (per mL) ÷ recovery) (dilution factor)

Final hormone concentration = (150 pg/mL) ÷ (0.89) × (250 µL/450 µL) × 10 = 936.3 pg/mL

Selection of test concentrations and Data Interpretation Procedure (DIP)

46. Three independent runs of the assay should be conducted. Unless prior information such as information on solubility limits or cytotoxicity provide a basis for selecting test concentrations, it is recommended that the test concentrations for the initial run be spaced at approximately half-log intervals with 10^{-4} M being the maximum concentration. Test concentrations in runs two and three



should be adjusted on the basis of the results of the initial run as indicated in Figure 4. If the results of the first run are positive (see paragraph 53), i.e. the fold change at two or more adjacent concentrations is statistically significant, the test should be repeated two additional times using the same conditions under which the first run was conducted, unless lower concentrations need to be employed to achieve a LOEC. If only one concentration is statistically different from the SC, the second test should be conducted with closer spaced concentrations. The refined dose range will test at least two concentrations above and below the concentrations that tested significant using $1/2$

the dose spacing than the first two experiments (quarter log vs. semi-log scale). Should the second experiment not confirm the first test (statistical significance must occur at the previously positively tested concentration plus/minus one concentration-increment), a third experiment is to be conducted using the original dosing regime. If no positive results are obtained during the first run, the maximum test concentrations is to be adjusted to 10^{-3} M using a semi-log scale during the second and third experiment.

DATA ANALYSIS AND REPORTING

Quality Control of the Test Plate

47. In addition to meeting the criteria for the QC plate, other quality criteria that pertain to acceptable variation between replicate wells, replicate experiments, linearity and sensitivity of hormone measurement systems, variability between replicate hormone measures of the same sample, and percentage recovery of hormone spikes after extraction of medium should be met and are provided in Table 7. Data should fall within the acceptable ranges defined for each parameter to be considered for further evaluation. If these criteria are not met, the spreadsheet should note that QC criteria were not met for the sample in question, and the sample should be re-analyzed or dropped from the data set.

Table 7: Acceptable ranges and/or variation (%) for H295R assay test plate parameters

	<i>Comparison Between</i>	<i>T</i>	<i>E2</i>
<i>Basal hormone production in SCs</i>	<i>Fold-greater than MDL</i>	$\geq 5\text{-fold}$	$\geq 2.5\text{-fold}$
<i>Exposure Experiments - Within Plate CV for SCs (Replicate Wells)</i>	<i>Absolute Concentrations</i>	$\leq 30\%$	$\leq 30\%$
<i>Exposure Experiments - Between Plate CV for SCs (Replicate Experiments)</i>	<i>Fold-Change</i>	$\leq 30\%$	$\leq 30\%$
<i>Hormone Measurement System – Sensitivity</i>	<i>Detectable fold-decrease relative to SC</i>	$\geq 5\text{-fold}$	$\geq 2.5\text{-fold}$
<i>Hormone Measurement System – Replicate Measure CV for SCs</i>	<i>Absolute Concentrations</i>	$\leq 25\%$	$\leq 25\%$
<i>Medium Extraction – Recovery of Internal ^3H Standard (If Applicable)</i>	<i>DPM</i>	$\geq 65\% \text{ Nominal}$	

Data Analysis

48. To evaluate the relative increase/decrease in chemically altered hormone production, the results should be normalized to the mean SC value of each test plate, and results expressed as changes relative to the SC in each test plate. All data are to be expressed as mean \pm 1 standard deviation (SD).

49. All concentrations that exhibit cytotoxicity greater than 20% are to be omitted for further

evaluation. Relative changes should be calculated as follows:

Relative Change = (Hormone concentration in each well)÷(Mean hormone concentration in all solvent control wells)

50. If by visual inspection of the well and the digital photographs described in paragraph 42 there appears to be an increase in cell number, the apparent increase needs to be verified. If an increase in cell numbers is verified, this should be stated in the test report, and the hormone data should be normalized by dividing hormone concentration by the relative change in the number of viable cells.

51. Prior to conducting statistical analyses, the assumptions of normality and variance homogeneity should be evaluated. Normality should be evaluated using standard probability plots or other appropriate statistical method (e.g. Shapiro-Wilk's test). If the data are not normally distributed, transformation of the data to approximate a normal distribution should be attempted. If the data are normally distributed or approximate normal distribution, differences between chemical treatments and SCs should be analyzed using a parametric test (e.g. Dunnett's Test). If data are not normally distributed, an appropriate non-parametric test should be used (e.g. Kruskal Wallis, Steel's Many-one rank test). Differences are considered significant at $p \leq 0.05$.

52. Results should be provided both in graphical (bar graphs representing mean \pm 1 SD) and tabular (LOEC/NOEC, direction of effect, and strength of maximum response that is part of the dose-response portion of the data) formats. Data assessment is only considered valid if it has been based on three independently conducted runs (see also paragraph 12). An experiment or run is considered independent if it has been conducted at a different date and with cells from a different culture plate. 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 (see paragraph 39).

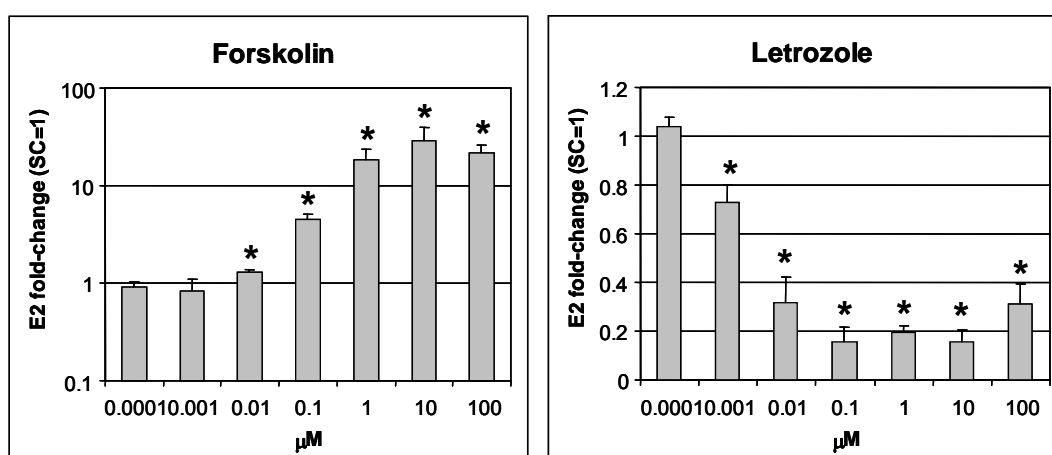


Figure 4: Example of the graphical presentation and evaluation of data obtained during the conduct of the H295R Assay. Asterisks indicate statistically significant differences from the solvent control ($p < 0.05$).

Data Interpretation Procedure

53. A chemical is judged to be positive if the fold induction is statistically different ($p \leq 0.05$) from the solvent control at two adjacent concentrations in at least two of the three tests, or when a single concentration data point is significantly different from the SC, and this can be confirmed by being significantly different in at least one more run within a +/- 1 concentration increment from the 1st positive run, following the decision tree in figure 4. 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 steroidogenesis pathway. Results exceeding the limits of solubility or at cytotoxic concentrations should not be included in the interpretation of results.

TEST REPORT

53. The test report should include the following information:

Testing facility:

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

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

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

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

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

Test results:

- Raw 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, DPM, etc.)
- Validation of normality or explanation of data transformation
- Mean responses +/- 1 SD for each well measured
- Cytotoxicity data
- Check that QC requirements were met
- Relative change compared with solvent control corrected for cytotoxicity
- A bar graph showing relative (fold change) at each concentration, SD and statistical significance as stated in paragraph 41

Data interpretation:

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

Discussion:

Conclusions:

REFERENCES

- (1) OECD (2002), OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals, in annex 2 of: OECD Guideline for the Testing of Chemicals No. 440, the Uterotrophic Bioassay in Rodents: A Short-term Screening Test for Oestrogenic Properties (2007). Available: [<http://www.oecd.org/env/testguidelines>]
- (2) Hecker, M., Newsted, J.L., Murphy, M.B., Higley, E.B., Jones, P.D., Wu, R. and Giesy, J.P. (2006), Human adrenocarcinoma (H295R) cells for rapid *in vitro* determination of effects on steroidogenesis: Hormone production. *Toxicol. Appl. Pharmacol.* 217, 114-124.
- (3) Hecker, M., Hollert, H., Cooper, R., Vinggaard, A.-M., Akahori, Y., Murphy, M., Nellemann, C., Higley, E., Newsted, J., Wu, R., Lam, P., Laskey, J., Buckalew, A., Grund, S., Nakai, M., Timm, G., and Giesy, J. (2007), The OECD validation program of the H295R steroidogenesis assay for the identification of *in vitro* inhibitors or inducers of testosterone and estradiol production. Phase 2: inter laboratory pre-validation studies. *Env. Sci. Pollut. Res.* 14, 23–30.
- (4) OECD (*Draft*), Multi-Laboratory Validation of the H295R Steroidogenesis Assay to Identify Modulators of Testosterone and Estradiol Production, OECD Series of Testing and assessment No. 1xx, Paris, France. Available at:
- (5) Battelle (2005), Detailed Review Paper on Steroidogenesis. Available at: [http://www.epa.gov/endo/pubs/edmv/steroidogenesis_drp_final_3_29_05.pdf]
- (6) Hecker, M., Newsted, J. L., Murphy, M. B., Higley, E. B., Jones, P. D., Wu, R. and Giesy, J. P. (2006), Human adrenocarcinoma (H295R) cells for rapid *in vitro* determination of effects on steroidogenesis: hormone production. *Toxicol. Appl. Pharmacol.* 217, 114-124.
- (7) Hilscherova, K., Jones, P. D., Gracia, T., Newsted, J. L., Zhang, X., Sanderson, J. T., Yu, R. M. K. , Wu, R. S. S. and Giesy, J. P. (2004), Assessment of the Effects of Chemicals on the Expression of Ten Steroidogenic Genes in the H295R Cell Line Using Real-Time PCR. *Toxicol. Sci.* 81, 78-89.
- (8) Sanderson, J. T., Boerma, J., Lansbergen, G. and Van den Berg, M. (2002), Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. *Toxicol. Appl. Pharmacol.* 182, 44-54.
- (9) Gazdar, A. F., Oie, H. K., Shackleton, C. H., Chen, T. R., Triche, T. J., Myers, C. E., Chrousos, G. P., Brennan, M. F., Stein, C. A. and La Rocca, R. V. (1990), Establishment and characterization of a human adrenocortical carcinoma cell line that expresses Multiple pathways of steroid biosynthesis. *Cancer Res.* 50, 5488-5496.
- (10) Rainey, W. E., Bird, I. M., Sawetawan, C., Hanley, N. A., Mccarthy, J. L., Mcgee, E. A., Wester, R. and Mason, J. I. (1993), Regulation of human adrenal carcinoma cell (NCI-H295) production of C19 steroids. *J. Clin. Endocrinol. Metab.* 77, 731-737.
- (11) OECD (2009), Hershberger Bioassay in Rats: A short-term Screening Assay for (Anti)Androgenic Properties. OECD Guideline for the Testing of Chemicals No. 441, Paris, France. Available: [<http://www.oecd.org/env/testguidelines>]
- (12) Shapiro, R., and Page, L.B. (1976), Interference by 2,3-dimercapto-1-propanol (BAL) in angiotensin I radioimmunoassay. *J. Lab. Clin. Med.* 2, 222-231.
- (13) Mosmann, T., (1983), Rapid colorimetric assay for growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65, 55-63.

ANNEX 1

Definitions

Confluency refers to the coverage or proliferation that the cells are allowed over or throughout the culture medium.

CYP stands for cytochrome P450 mono-oxygenases, a family of genes and the enzymes produced from them that are involved in catalyzing a wide variety of biochemical reactions including the synthesis and metabolism of steroid hormones.

E2 is 17 β -oestradiol, the most important estrogen in mammalian systems.

H295R cells are human adreno-carcinoma cells which have the physiological characteristics of zonally undifferentiated human fetal adrenal cells and which express all of the enzymes of the steroidogenesis pathway. They are available from the ATCC.

Freeze medium is used to freeze and to store frozen cells. It consists of stock medium plus BD NuSerum and dimethyl sulfoxide..

Passage is the number of times that cells are split after initiation of a culture from frozen stock. The initial passage that was started from the frozen stock is assigned the number one (1). Cells that were split 1 time are labeled passage 2, etc.

LOEC is the Lowest Observed Effect Concentration, the lowest concentration level at which the assay response is statistically different from that of the solvent control.

NOEC is the No Observed Effect Concentration, which is the highest concentration tested if the assay does not provide a positive response.

PBS is Dulbecco's phosphate buffered saline.

Quality Control, abbreviated QC, refers to the measures needed to assure valid data.

Quality control plate is a 24 well plate containing two concentrations of the positive and negative controls to monitor the performance of a new batch of cells or to provide the positive controls for the assay when testing chemicals.

Run is an independent experiment characterized by a new set of solutions and controls.

Stock medium is the base for the preparation of other reagents. It consists of a 1:1 mixture of Dubecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture (DME/F12) in 15 mM HEPES buffer without phenol red or sodium bicarbonate. Sodium bicarbonate is added as the buffer, see Annex 3.

Supplemented medium consist of stock medium plus BD Nu-Serum and ITS+ premium mix, see Annex 3.

Steroidogenesis is the synthetic pathway leading from cholesterol to the various steroid hormones. Several intermediates in the steroid synthesis pathway such as progesterone and testosterone are important hormones in their own right but also serve as precursors to hormones farther down the synthetic pathway.

T stands for testosterone, one of the two most important androgens in mammalian systems.

Test plate is the plate on which H295R cells are exposed to test chemicals. Test plates contain the solvent control and two test chemicals at six concentration levels in triplicate.

Trypsin 1X is a dilute solution of the enzyme trypsin, a pancreatic serine protease, used to loosen cells from a cell cultivation plate, see Annex 3, section 4.