Adopted:
28 July 2011

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

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 2002 OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals comprises five levels, each level corresponding to a different level of biological complexity (1). The *in vitro* H295R *S*teroidogenesis Assay (H295R) described in this Test Guideline (TG) utilises a human adreno-carcinoma cell line (NCI-H295R cells) and constitutes a level 2 "*in vitro* assay, providing mechanistic data", to be used for screening and prioritization purposes. Development and standardization of the assay as a screen for chemical effects on steroidogenesis, specifically the production of 17β-estradiol (E2) and testosterone (T), was carried out in a multi–step process. The H295R assay has been optimized and validated (2) (3) (4) (5).

2. The objective of the H295R Steroidogenesis Assay is to detect substances that affect production 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 E2 and/or T. 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; however, quantitative results may be obtained in some cases (see paragraphs 53 and 54). 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 specific mechanistic information concerning the interaction of the test substance with the endocrine system. Research has been conducted using the cell line to identify effects on specific enzymes and intermediate hormones such as progesterone (2).

3. Definitions and abbreviations used in this TG 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 Appendix I-III of the OECD document "*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. The H295R assay does not measure DHT (see paragraph 10).

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

7. The specificity of the lyase activity of CYP17 differs for the intermediate substrates among species. In the human, the enzyme favors substrates of the Δ^5-hydroxysteroid pathway (pregnenolone), whereas substrates in the Δ^4-ketosteroid pathway (progesterone) are favored in the rat (19). Such differences in the CYP17 lyase activity may explain some species-dependent differences in response to substances that alter steroidogenesis *in vivo* (6). The H295 cells have been shown to most closely reflect human adult adrenal enzyme expression and steroid production pattern (20), but are known to express enzymes for both the Δ^5-hydroxysteroid and Δ^4-ketosteroid pathways for androgen synthesis (7) (11) (13) (15).

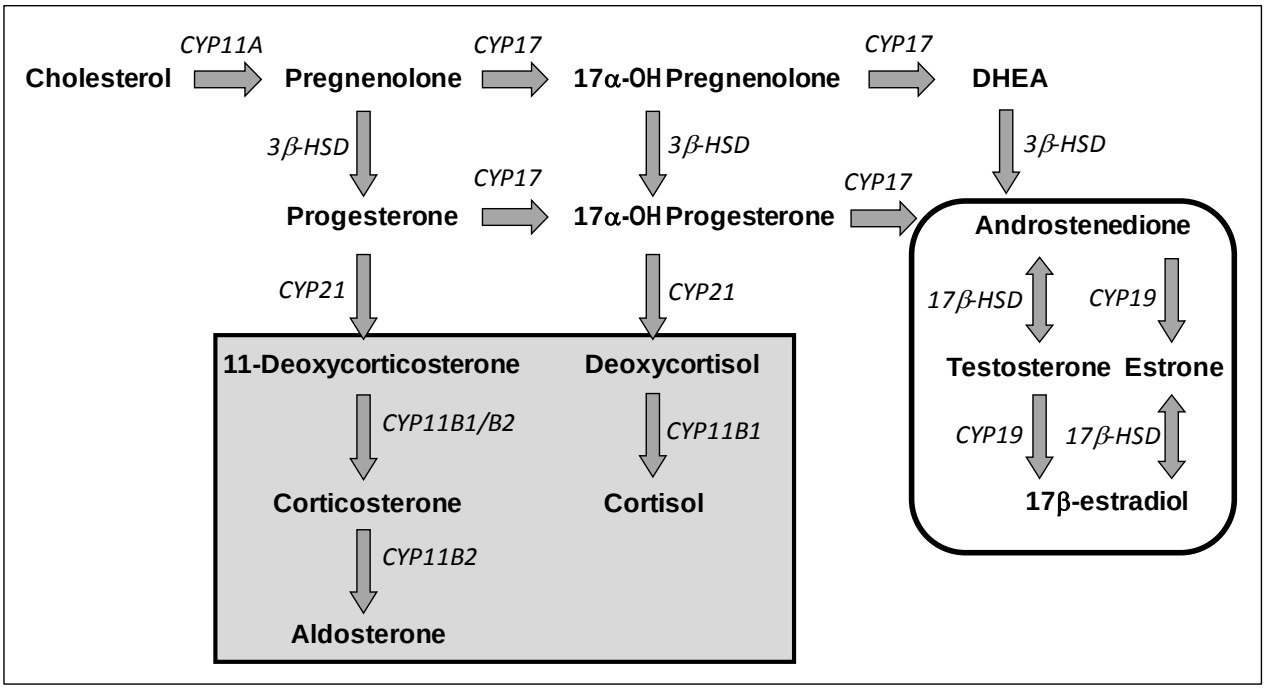


Figure 1: Steroidogenic pathway in H295R cells. Enzymes are in italics, hormones are bolded and arrows indicate the direction of synthesis. Gray background indicates corticosteroid pathways/products. Sex steroid pathways/products are circled. CYP = cytochrome P450; HSD = hydroxysteroid hydrogenase; DHEA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

8. The human H295R adreno-carcinoma cell line is a useful *in vitro* model for the investigation of effects on steroid hormone synthesis (2) (7) (8) (9) (10). The H295R cell line expresses genes that encode for all the key enzymes for steroidogenesis noted above (11) (15) (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 (2). H295R cells have physiological characteristics of zonally undifferentiated human fetal adrenal cells (11). 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. Changes recorded by the test system in the form of alteration in the production of T and E2 can be the result of a multitude of different interactions of test chemicals with steroidogenic functions that are expressed by the H295R cells. These include modulation of the expression, synthesis or function of enzymes involved in the production, transformation, or elimination of steroid hormones (12) (13) (14). Inhibition of hormone production can be due to direct competitive binding to an enzyme in the pathway, impact on co-factors such as NADPH (Nicotinamide Adenine Dinucleotide Phosphate) and cAMP (cyclic Adenosine Monophosphate), and/or increase in steroid metabolism or suppression of gene expression of certain enzymes in the steroidogenesis pathway. While inhibition can be a function of both direct or indirect processes involved with hormone production, induction is typically of an indirect nature, such as by affecting co-factors such as NADPH and cAMP (as in the case of forskolin), decreasing steroid metabolism (13), and or up-regulating steroidogenic gene expression.

9. The H295R assay has several advantages:

- It allows for the detection of both increases and decreases in the production of both T and E2;
- 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 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 mineral corticoids could influence the levels of T and E2 observed in the assay.
- It does not measure DHT and, therefore, would not be expected to detect substances that inhibit 5α-reductase in which case the Hershberger assay (16) can be used.
- The H295R assay will not detect substances that interfere with steroidogenesis by affecting the hypothalamic-pituitary-gonadal axis (HPG) axis as this can only be studied in intact animals.

PRINCIPLE OF THE TEST

11. The purpose of the assay is the detection of substances that affect T and E2 production. 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, other plate sizes can be used for conducting the assay; however, seeding and experimental conditions should be adjusted accordingly to maintain adherence to the performance criteria.

13. After an acclimation period of 24 h in multi-well plates, cells are exposed for 48 h to seven concentrations of the test chemical in at least 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 at least two 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 applicable, 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 (2), 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 then were split three times (4 passages counting the freshly thawed cells as passage 1) after they were cultured again would be labelled passage 4.5. An example of a numbering scheme is illustrated in Appendix I of the validation report (4).

16. Stock medium is used as the base for the supplemented and freezing mediums. 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 Quality Control (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 Appendix II of the validation report (4).

17 After initiation of an H295R cell culture from an original ATCC batch, cells should be grown for five 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 the previous passage four cells is run in a QC plate (See paragraph 36 and 37) to verify whether the basal production of 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[2] or frozen[3] batch of cells into culture that is acceptable for use in the H295R assay should not exceed 10. For example, acceptable passages for cultures of cells from a batch frozen at passage 5 would be 4.5 through 10.5. For cells started from these frozen batches, the procedure described in paragraph 19 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

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

[2] "New batch" refers to a fresh batch of cells received from ATCC.

[3] "Frozen batch" refers to cells that have been previously cultured and then frozen at a laboratory other than ATCC.

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 Appendix III of the validation report (4). Cells are removed from liquid nitrogen storage, thawed rapidly, placed in supplemented medium in a centrifuge tube, centrifuged at room temperature, re-suspended in supplemented medium, and transferred to a culture flask. The medium should be changed the following day. The H295R cells are cultivated in an incubator at 37°C with 5% CO_2 in air atmosphere and the medium is renewed 2-3 times per week. When the cells are approximately 85-90% 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 phosphate-buffered saline (PBS, without Ca^{2+} Mg^{2+}) and freed from the culture flask by the addition of an appropriate detachment enzyme, *e.g.* trypsin, in PBS (without Ca^{2+} Mg^{2+}). Immediately after the cells detach from the culture flask, the enzyme action should be stopped with the addition of supplemented medium at a ratio of 3X the volume used for the enzyme treatment. Cells are placed into a centrifuge tube, centrifuged at room temperature, the supernatant is removed and the pellet of cells is re-suspended in supplemented medium. The appropriate amount of cell solution is placed in the new culture 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 carefully labelled. The cells are now ready to be used in the assay and excess cells should be frozen in liquid nitrogen as described in paragraph 20.

Freezing H295R Cells (preparing cells for liquid nitrogen storage)

20. To prepare H295R cells for freezing, the procedure described above for splitting cells should be followed until the step for re-suspending the pellet of cells in the bottom of the centrifuge tube. Here, the pellet of cells is re-suspended in freezing medium. The solution is transferred to a cryogenic vial, labelled appropriately, and frozen at -80°C for 24 hours after which the cryogenic vial is transferred to liquid nitrogen for storage. Details for this procedure are set forth in Appendix III of the validation report (4).

Plating and Pre-incubation of Cells for Testing

21. The number of 24-well plates, prepared as outlined in Paragraph 19, 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 culture flask (75 cm^2) of 80-90% confluent cells will supply sufficient cells for one to 1.5 (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 typically the optimal cell density for hormone production in the assay. At higher densities, 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.

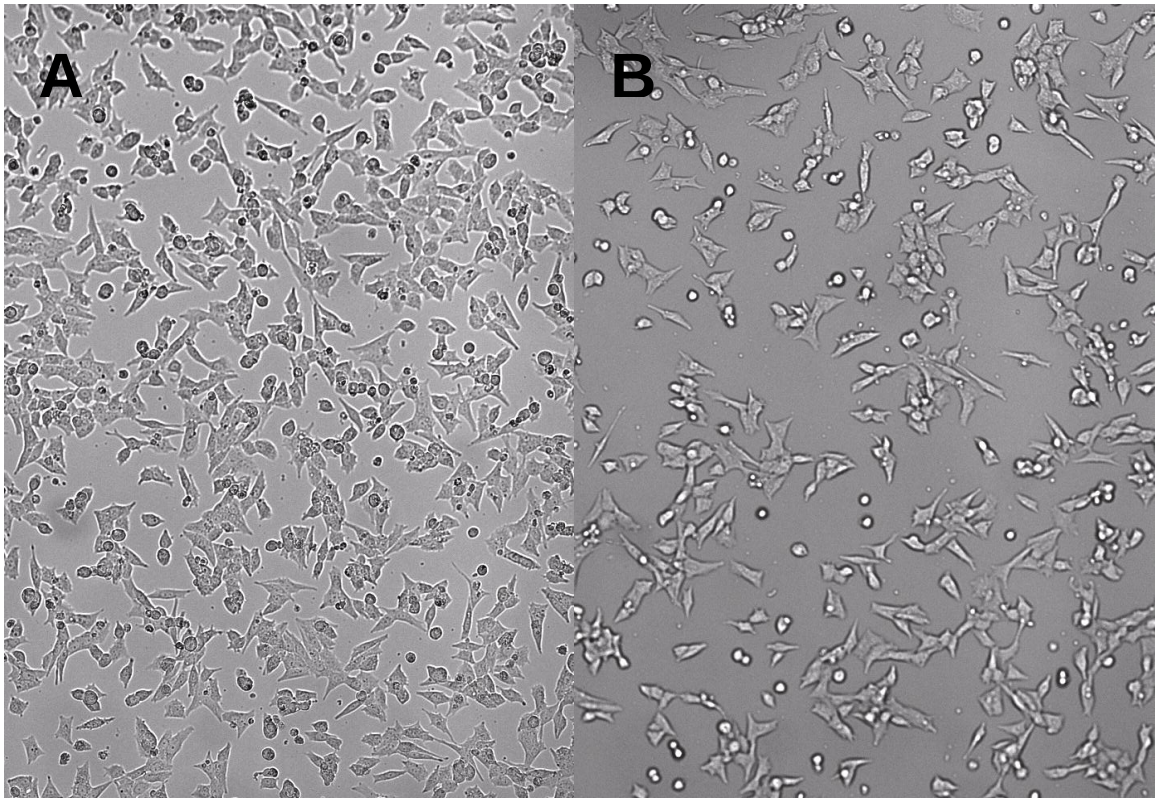


Figure 2: Photomicrograph of H295R cells at a seeding density of 50% in a 24 well culture plate at 24 hours taken at the edge (A) and centre (B) of a well.

22. The medium is pipetted off the culture flask, and the cells are rinsed 3 times with sterile PBS (without $Ca^{2+}Mg^{2+}$). An enzyme solution (in PBS) is added to detach the cells from the culture flask. Following an appropriate time for detachment of the cells, the enzyme action should be stopped with the addition of supplemented medium at a ratio of 3X the volume used for the enzyme treatment. Cells are placed into a centrifuge tube, centrifuged at room temperature, the supernatant is removed, and the pellet of cells is re-suspended in supplemented medium. The cell density is calculated using *e.g.* a haemocytometer or cell counter. 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 5% CO_2 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.

24. Prior to the initiation of cell culture and any subsequent testing, each laboratory should demonstrate the sensitivity of its hormone measurement system (paragraphs 29-31).

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 32 prior to initiating testing.

26. DMSO is the recommended solvent for the assay. If an alternative solvent is utilized, the following should be determined:

- The solubility of the test chemical, forskolin and prochloraz in the solvent; and
- The cytotoxicity as a function of the concentration of solvent.

It is recommended that the maximum allowable solvent concentration should not exceed a 10x dilution of the least cytotoxic concentration of the solvent.

27. 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 33-35.

28. When initiating testing using a new batch, a control plate should be run before using a new batch of cells to evaluate the performance of the cells as described in paragraphs 36 and 37.

Performance of the Hormone Measurement System

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 Limit of Quantification (LOQ). 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 5 by analyzing supplemented medium spiked with an internal hormone control. Supplemented medium should be spiked with at least three concentrations of each hormone (*e.g.* 100, 500 and 2500 pg/mL of T; 10, 50 and 250 pg/mL of E2; or the lowest possible concentrations based upon the detection limits of the chosen hormone measurement system can be used for the lowest spike concentrations for T and 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 8 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 8 are not fulfilled, a significant matrix effect may be occurring, and an experiment with extracted spiked medium should be conducted. An example of an extraction procedure is described in Appendix II of the validation report (4). Measurements of the hormone concentrations in the extracted samples should be made in triplicate.[4] 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 system is not suitable for the purpose of the H295R Steroidogenesis Assay, and an alternative hormone detection method should be used.

Standard curve

31. The hormone concentrations of the solvent controls (SC) should be within the linear portion of the standard curve. Preferably, the SC values should fall close to the centre of the linear portion to ensure that induction and inhibition of hormone synthesis can be measured. Dilutions of medium (or extracts) to be measured are to be selected accordingly. The linear relationship is to be determined by a suitable statistical approach.

[4] Note: If extraction is required, three replicate measurements are made for each extract. Each sample will be extracted only once.

Chemical interference test

32. 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 (Appendix III of the validation report (4)) because some chemicals can interfere with these tests (17). If interference occurs that is ≥ 20% of basal hormone production for T and/or E2 as determined by hormone analysis, the Chemical Hormone Assay Interference Test (such as described in Appendix III of the validation report (4) section 5.0) should be run on all test chemical stock solution dilutions to identify the threshold dose at which significant (≥ 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. In order to avoid interference from contaminating substances it is recommended that hormones are extracted from the medium using suitable solvent, possible methods can be found in the validation report (4).

Table 1: Performance criteria for hormone measurement systems

Parameter	***Criterion***
Measurement Method Sensitivity	Limit of Quantification (LOQ) T: 100 pg/mL; E2: 10 pg/mL [a]
Hormone Extraction Efficiency (only when extraction is needed)	The average recovery rates (based on triplicate measures) for the spiked amounts of hormone should not deviate more than 30% from amount that was added.
Chemical Interference (only antibody based systems)	No substantial (≥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 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.

[c] These include: cholesterol, pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17α-pregnenolone,17α-progesterone, deoxycortisol, cortisol, DHEA, androstenedione, estrone.

Laboratory Proficiency Test

33. 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 by running the laboratory proficiency test. As the performance of an assay is directly linked to the laboratory personnel conducting the assay, these procedures should be partly repeated if a change in laboratory personnel occurs.

34. This proficiency test will be conducted under the same conditions listed in paragraphs 38 through 40 by exposing cells to 7 increasing concentrations of strong, moderate and weak inducers and inhibitors as well as a negative chemical (see Table 2). Specifically, chemicals to be tested include the strong inducer forskolin (CAS no. 66575-29-9); the strong inhibitor prochloraz (CAS no. 67747-09-5); the moderate inducer atrazine (CAS no. 1912-24-9); the moderate inhibitor aminoglutethimide (CAS no. 125-84-8); the

weak inducer (E2 production) and weak inhibitor (T production) bisphenol A (CAS no. 80-05-7); and the negative chemical human chorionic gonadotropin (HCG) (CAS no. 9002-61-3) as shown in Table 2. Separate plates are run for all chemicals using the format as shown in Table 6. One QC plate (Table 4, paragraphs 36-37) should be included with each daily run for the proficiency chemicals.

Table 2: Proficiency chemicals and exposure concentrations

Chemical	Test Concentrations [µM]
Prochloraz	0[a], 0.01, 0.03, 0.1, 0.3, 1, 3, 10
Forskolin	0 [a], 0.03, 0.1, 0.3, 1, 3, 10, 30
Atrazine	0 [a], 0.03, 0.1, 1, 3, 10, 30, 100
Aminoglutethimide	0 [a], 0.03, 0.1, 1, 3, 10, 30, 100
Bisphenol A	0 [a], 0.03, 0.1, 1, 3, 10, 30, 100
HCG	0 [a], 0.03, 0.1, 1, 3, 10, 30, 100

[a]Solvent (DMSO) control (0), 1 µL DMSO/well

Exposure of H295R to proficiency chemicals should be conducted in 24 well plates during the laboratory proficiency test. Dosing is in µM for all test chemical doses. Doses should be administered in DMSO at 0.1% v/v per well. All test concentrations should be tested in triplicate wells (Table 6). Separate plates are run for each chemical. One QC plate is included with each daily run.

35. Cell viability and hormone analyses should be conducted as provided in paragraphs 42 through 46. The threshold value (lowest observed effect concentration, LOEC) and classification decision should be reported and compared with the values in Table 3. The data are considered acceptable if they meet the LOEC and decision classification in Table 3.

Table 3: Threshold values (LOECs) and decision classifications for Proficiency Substances

	CAS no.	LOEC [µM]		Decision Classification	
		T	***E2***	***T***	***E2***
Prochloraz	67747-09-5	≤0.1	≤1.0	+ [a] (Inhibition)	+ (Inhibition)
Forskolin	66575-29-9	≤10	≤0.1	+ (Induction)	+ (Induction)
Atrazine	1912-24-9	≤100	≤10	+ (Induction)	+ (Induction)
Aminoglutethimide	125-84-8	≤100	≤100	+ (Inhibition)	+ (Inhibition)
Bisphenol A	80-05-7	≤10	≤10	+ (Inhibition)	+ (Induction)
HCG	9002-61-3	n/a	n/a	Negative	Negative

[a] +, positive

n/a: not applicable as no changes should occur after exposure to non-cytotoxic concentrations of negative control.

Quality Control Plate

36. The quality control (QC) plate is used to verify the performance of the H295R cells under standard culture conditions, and to establish a historical database for hormone concentrations in solvent controls, positive and negative controls, as well as other QC measures over time.

- H295R cell performance should be assessed using a QC plate for each 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.

- A QC plate provides a complete assessment of the assay conditions (*e.g.* cell viability, solvent controls, negative and positive controls, as well as intra- and inter-assay variability) when testing chemicals and should be part of each test run.

37. 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 38 through 46 for testing chemicals. The QC plate contains blanks, solvent controls, and two concentrations of a known inducer (forskolin, 1, 10 µM) and inhibitor (prochloraz, 0.1, 1 µM) of E2 and T synthesis. In addition, MeOH is used in select wells as a positive control for the viability/cytotoxicity assay. 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 for T and E2 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. After termination of the exposure experiment and removal of medium, a 70% methanol solution will be added to all MeOH wells to serve as a positive control for cytotoxicity (see cytotoxicity assay in Appendix III of the validation report (4)).

	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 [c] 1 µL	DMSO [c] 1 µL	DMSO [c] 1 µL	DMSO [c] 1 µL (+ MeOH) [b]	DMSO [c] 1 µL (+ MeOH)[b]	DMSO [c] 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] Cells in Blank wells receive medium only (*i.e.* no solvent).

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

[c]DMSO sovent control (1 µL/well).

Table 5: Performance criteria for the Quality Control Plate

	T	**E2**
Basal Production of hormone in the solvent control (SC)	≥ 5 times the LOQ	≥ 2.5 times the LOQ
Induction (10 µM forskolin)	≥ 1.5 times the SC	≥ 7.5 times the SC
Inhibition (1µM prochloraz)	≤ 0.5 times the SC	≤ 0.5 times the SC

CHEMICAL EXPOSURE PROCEDURE

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

39. The cells are placed in a bio-safety cabinet and the supplemented medium removed and replaced with new supplemented medium (1 mL/well). DMSO is the preferred solvent for this Test Guideline. However, if there are reasons for using other solvents the scientific rationale should be described. Cells are exposed to the test chemical by adding 1 µL of the appropriate stock solution in DMSO (see Appendix II of the validation report (4)) per 1 mL supplemented medium (well volume). This results in a final concentration of 0.1% DMSO in the wells. To assure adequate mixing it is generally preferred that the appropriate stock solution of the test chemical in DMSO is mixed with supplemented medium to yield the desired final concentration for each dose, and the mixture added to each well immediately after removal of old medium. If this option is used, the concentration of DMSO (0.1%) should remain consistent among all wells. The wells containing the greatest two concentrations are visually assessed for formation of precipitates or cloudiness as an indication of incomplete solubility of the test compound by using a stereo microscope. If such conditions (cloudiness, formation precipitates) are observed, wells containing the next lesser concentrations are examined as well (and so forth) and concentrations that did not completely go into solution are to be excluded from further evaluation and analysis. The plate is returned to the incubator at 37°C under a 5% CO_2 in air atmosphere for 48 hours. The test chemical plate layout is shown in Table 6. Stocks 1 -7 show placement of increasing doses of test chemical.

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

40. 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 µL each) and transferred to two separate vials appropriately labeled (*i.e.* one aliquot to provide a spare sample for each well). To prevent cells from drying out, medium is removed a row or column at a time and replaced with the medium for the cell viability/cytotoxicity assay. If cell viability/cytotoxicity is not to be measured immediately, 200 µL PBS with Ca^{2+} and Mg^{2+} is added to each well. The media are frozen at -80ºC until further processing to analyze hormone concentrations (see paragraphs 44-46). While T and E2 in medium kept at -80°C are generally stable for at least 3 months, hormone stability during storage should be documented within each laboratory.

41. Immediately after removing the medium, cell viability/cytotoxicity is determined for each exposure plate.

Cell Viability Determination

42. 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 (see Appendix III 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 (18). 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. If by visual inspection or as demonstrated by the viability/cytotoxicity assay 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. Cell viability will be expressed relative to the average response in the SCs, which is considered 100% viable cells, and is calculated as appropriate for the cell viability/cytotoxicity assay that is used. For the MTT assay, the following formula may be used:

% viable cells = (response in well – average response in MeOH treated [=100% dead] wells) ÷ (average response in SC wells - average response in MeOH treated [=100% dead] wells)

43. Wells with viability lower than 80%, relative to the average viability in the SCs (=100% viability), should not be included in the final data analysis. Inhibition of steroidogenesis occurring in the presence of almost 20% cytotoxicity should be carefully evaluated to ensure that cytotoxicity is not the cause for the inhibition.

Hormone Analysis

44. Each laboratory can use a hormone measurement system of its choice for the analysis of T and E2. Spare aliquots of medium from each treatment group may be used to prepare dilutions to bring the concentration within the linear part of the standard curve. As noted in paragraph 29, each laboratory should demonstrate the conformance of their hormone measurement system (*e.g.* ELISA, RIA, LC-MS, LC-MS/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 may need to be extracted from the media prior to their measurement (see paragraph 30 for the conditions under which an extraction is or is not required). It is recommended to conduct extraction following the procedures in Appendix III of the validation report (4).

45. 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 conducted. Dilutions of samples 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 (Appendix III of the validation report (4)). Values outside of the linear portion of the standard curve should be rejected.

46. 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 linear range of the standard curve)
Hormone Concentration in Assay:	150 pg/mL (already adjusted to concentration per mL sample assayed)
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

47. A minimum of two independent runs of the assay should be conducted. Unless prior information such as information on solubility limits or cytotoxicity provides a basis for selecting test concentrations, it is recommended that the test concentrations for the initial run be spaced at $\log_{10}$ intervals with 10^{-3} M being

the maximum concentration. If the chemical is soluble, and not cytotoxic at any of the tested concentrations, and the first run was negative for all concentrations, then it is to be confirmed in one more run using the same conditions as the first run was conducted (Table 7). If the results of the first run are *equivocal* (i.e. the fold-change is statistically significant from the SC at only one concentration) or positive (i.e. the fold change at two or more adjacent concentrations is statistically significant), the test should be repeated as indicated in Table 7 by refining the selected test concentrations. Test concentrations in runs two and three (if applicable) should be adjusted on the basis of the results of the initial run bracketing concentrations that elicited an effect using ½-log concentration spacing (*e.g.* if the original run of 0.001, 0.01, 0.1, 1, 10, 100, 1000 µM resulted in inductions at 1 and 10 µM, the concentrations tested in the second run should be 0.1, 0.3, 1, 3, 10, 30, 100 µM), unless lower concentrations need to be employed to achieve a LOEC. In the latter case, at least five concentrations below the lowest concentration tested in the first run should be used in the second run using a ½-log scale. If the second run does not confirm the first run (*i.e.* statistical significance does not occur at the previously positively tested concentration ± 1 concentration-increment), a third experiment is to be conducted using the original testing conditions. Equivocal results in the first run are considered negative if the observed effect could not be confirmed in any of the two subsequent runs. Equivocal results are considered as positive responses (effect) when the response can be confirmed in at least one more run within a ± 1 concentration increment (see section 55 for the Data Interpretation Procedure).

Table 7: Decision matrix for possible outcome scenarios

Run 1	Run 2		Run 3		Decision	
Scenario	**Decision**	**Scenario**	**Decision**	**Scenario**	**Positive**	**Negative**
Negative	Confirm[a]	Negative	Stop			X
Negative	Confirm[a]	Positive	Refine[b]	Negative		X
Equivocal[c]	Refine[b]	Negative	Confirm[a]	Negative		X
Equivocal[c]	Refine[b]	Negative	Confirm[a]	Positive	X	
Equivocal[c]	Refine[b]	Positive			X	
Positive	Refine[b]	Negative	Confirm[a]	Positive	X	
Negative	Confirm[a]	Positive	Refine[b]	Positive	X	
Positive	Refine[b]	Positive	Stop		X	

[a] Confirm previous run using the same experimental design.
[b] Re-run assay at ½-log concentration spacing (bracketing the concentration that tested significantly different in the preceding experiment).
[c] Fold-change at one concentration is statistically significantly different from the SC

Quality Control of the Test Plate

48. 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 (if applicable; see Paragraph 30 regarding extraction requirements) should be met and are provided in Table 8. 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 8: Acceptable ranges and/or variation (%) for H295R assay test plate parameters. LOQ: Limit of Quantification of the hormone measurement system. CV: Coefficient of variation; SC: Solvent Control; DPM: Disintegrations per minute.

	Comparison Between	T	E2
Basal hormone production in SCs	Fold-greater than LOQ	≥ 5-fold	≥ 2.5-fold
Exposure Experiments - Within Plate CV for SCs (Replicate Wells)	Absolute Concentrations	≤ 30%	≤ 30%
Exposure Experiments - Between Plate CV for SCs (Replicate Experiments)	Fold-Change	≤ 30%	≤ 30%
Hormone Measurement System – Sensitivity	Detectable fold-decrease relative to SC	≥ 5-fold	≥ 2.5-fold
Hormone Measurement System – Replicate Measure CV for SCs[a]	Absolute Concentrations	≤ 25%	≤ 25%
Medium Extraction – Recovery of Internal ^{3}H Standard (If Applicable)	DPM	≥ 65% Nominal	

[a] Refers to replicate measures of the same sample

DATA ANALYSIS AND REPORTING

Data Analysis

49. 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 ± 1 standard deviation (SD).

50. Only hormone data from wells where cytotoxicity was less than 20% should be included in the data analysis. Relative changes should be calculated as follows:

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

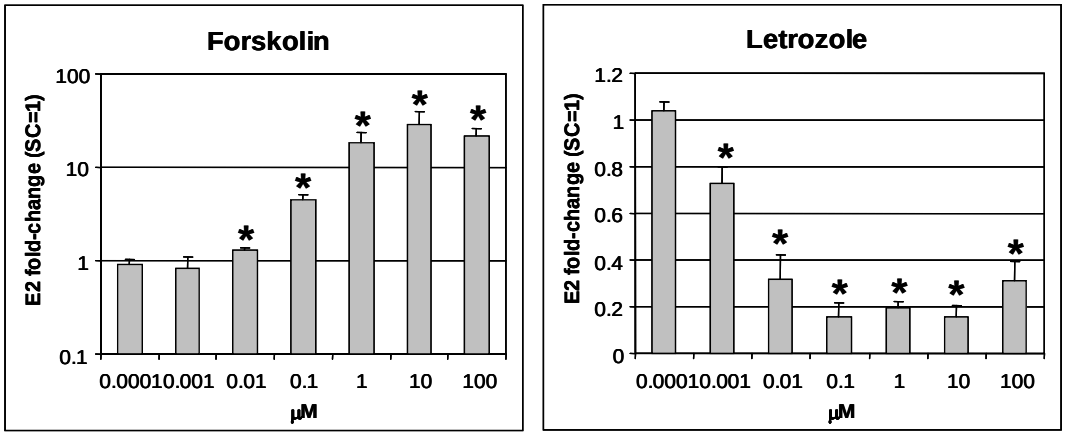
51. If by visual inspection of the well or as demonstrated by the viability/cytotoxicity assay described in paragraph 42 there appears to be an increase in cell number, the apparent increase needs to be verified. If an increase in cell numbers is verified, this should be stated in the test report.

52. 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 (fold changes) are not normally distributed, transformation of the data should be attempted to approximate a normal distribution. If the data are normally distributed or approximate a normal distribution, differences between chemical concentration groups and SCs should be analyzed using a parametric test (*e.g.* Dunnett's Test) with *concentration* being the independent, and *response* (fold-change) being the dependent variable. 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 $p \leq 0.05$. Statistical evaluations are done based on average values for each well that represent independent replicate data points. It is anticipated that due to the large spacing of doses in the first run ($\log_{10}$ scale) in many cases it will not be possible to describe clear concentration-response relationships where the two greatest doses will be on the linear portion of the

sigmoid curve. Therefore, for the first run or any other data sets where this condition occurs (*e.g.* where no maximum efficacy can be estimated) type I fixed variable statistics as described above will be applied.

53. If more than two data points lie on the linear portion of the curve and where maximum efficacies can be calculated - as is anticipated for some of the 2nd runs that are conducted using a semi-log spacing of exposure concentrations - a probit, logit or other appropriate regression model should be utilized to calculate effective concentrations (*e.g.* EC50 and EC20).

54. Results should be provided both in graphical (bar graphs representing mean +/- 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 (see Figure 3 for an example). Data assessment is only considered valid if it has been based on at least two independently conducted runs. An experiment or run is considered independent if it has been conducted at a different date using a new set of solutions and controls. The concentration range used in runs 2 and 3 (if necessary) may be tailored on the basis of the results of run 1 to better define the dose response range containing the LOEC (see paragraph 47).



Chemical	*LOEC*	*Max Change*
Forskolin	0.01	29-fold
Letrozole	0.001	0.15-fold

Figure 3: Example of the presentation and evaluation of data obtained during the conduct of the H295R Assay in graphical and tabular format. Asterisks indicate statistically significant differences from the solvent control ($p<0.05$). LOEC: Lowest observed effective concentration; Max Change: Maximum strength of the response observed at any concentration relative to the average SC response (=1).

Data Interpretation Procedure

55. 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 independent runs (Table 7). A chemical is judged to be negative following two independent negative runs, or in three runs, comprising two negative runs and one equivocal or positive run. If the data generated in three independent experiments does not meet the decision criteria listed in Table 7, the experimental results are not interpretable. Results at concentrations exceeding the limits of solubility or at cytotoxic concentrations should not be included in the interpretation of results.

TEST REPORT

56. 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;
- Detection limits for the selected analytical assays;

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 (limit of solubility, if determined);
- 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 (test concentrations that caused cytotoxicity);
- Confirmation 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 49-54;

Data interpretation:

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

Discussion:

- Are there any indications from the study regarding the possibility that the T/E2 data could be influenced by indirect effects on the gluco-, and mineral-corticoid pathways?

Conclusions:

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ANNEX 1

Definitions

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

CV refers to the coefficient of variation, and is defined as the ratio of the standard deviation of a distribution to its arithmetic mean.

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.

DPM are disintegration per minute. It is the number of atoms in a given quantity of radioactive material that is detected to have decayed in one minute.

E2 is 17β-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.

Linear Range is the range within the standard curve for a hormone measurement system where the results are proportional to the concentration of the analyte present in the sample.

LOQ stands for "*Limit of Quantification*", and is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) within a stated confidence limit. For the purpose of this guideline, the LOQ is typically defined by the manufacturer of the test systems if not specified differently.

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.

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.

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 (DMEM/F12) in 15 mM HEPES buffer without phenol red or sodium bicarbonate. Sodium bicarbonate is added as the buffer, see Appendix II of the validation report (4).

Supplemented medium consist of stock medium plus BD Nu-Serum and ITS+ premium mix, see Appendix II of the validation report (4).

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 seven 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 Appendix III of the validation report (4).