Primary Reviewer: _____ **Signature:** _____
[Insert Name of Organization] **Date:** _____
Secondary Reviewer: _____ **Signature:** _____
[Insert Name of Organization] **Date:** _____

Template version 07/2011

DATA EVALUATION RECORD

STUDY TYPE: Steroidogenesis Assay (H295R Cells); OCSPP 890.1550

PC CODE: *(if applicable)* **DP BARCODE:** *(if applicable)*

TXR#: *(if applicable)* **CAS No.: [##]**

TEST MATERIAL (PURITY): *[use name of material tested as referred to in the study (common agency chemical name in parenthesis)]*

SYNONYMS: *(Other names and codes)*

CITATION: Author *[up to 3, see SOP for exact format]*. (*Study Year*). Title. Laboratory name and location. Laboratory report number, study completion date. MRID *[no hyphen]*. Unpublished. *(OR if published, list Journal name, vol.:pages)*

SPONSOR: *(Name of Study Sponsor)*

EXECUTIVE SUMMARY:

In a steroidogenesis assay (*if applicable* - MRID [number]), H295R cells cultured *in vitro* in 24-well *(or 48-well, if used)* plates were incubated with [chemical name, (% purity, batch/lot #)] at concentrations of [#, #, #, #, #, #, #, and #] µM in triplicate for 48 hours. The test chemical's vehicle was DMSO and its final concentration was 0.1%.

Testosterone and estradiol (*name other hormones if measured*) levels were measured using [ELISA, RIA, or other method]. [X] independent experiments were performed. A Quality Control (QC) plate was run concurrently with each independent run of a test chemical plate to demonstrate that the assay responded properly to positive control agents at two concentration levels; positive controls included the known inhibitor (prochloraz) and inducer (forskolin) of estradiol and testosterone production.

Include a brief summary of the results. Indicate increase or decrease of hormone levels for test compound and QC plates.

This study [satisfies/does not satisfy] the Test Order requirement for a Steroidogenesis Assay (OCSPP 890.1550). (*If it does not satisfy the requirement, concisely list only major deficiencies or refer to deficiency section.)*

COMPLIANCE: Signed and dated GLP Compliance and Quality Assurance statements [were /were not] provided. *(Discuss any deviations from regulatory requirements)*

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Facility: *Name of the Facility*
- **Location:** *Location of the Facility*
- **Study Director:** *Name*
- **Other Personnel:** *Name and study responsibility*
- **Study Period:** *Study start and end dates*

2. Test Substance: *Common name as used by Agency*
- **Description:** *e.g. technical, nature, color, molecular weight*
- **Lot / Batch #:** *include expiration date*
- **Purity:** %
- **Solubility (in Solvent):**
- **Volatility:**
- **Stability:**
- **Storage conditions:**
- **CAS #:** *CAS # or* Not available
- **Molecular weight:**
- **Structure:** *[Structure] or* Not available

3. Positive Control: Forskolin
- **Description:** *e.g. technical, nature, color, molecular weight*
- **Source:** *include catalog #*
- **Lot / Batch #:** *include expiration date*
- **Purity:**
- **Solubility (in Solvent):**
- **Storage conditions:**
- **CAS #:** 66428-89-5

4. Negative Control: Prochloraz
- **Description:** *e.g. technical, nature, color, molecular weight*
- **Source:** *Source/company (City, State [and Country, if outside U.S.A.])*
- **Lot / Batch #:** *include expiration date*
- **Purity:**
- **Solubility (in Solvent):**
- **Storage conditions:**
- **CAS #:** 67747-09-5

5. Solvent/Vehicle Control: Water, Ethanol, or Dimethyl Sulfoxide (DMSO)
- **Description:** *e.g. technical, nature, color, molecular weight*
- **Source:** *include catalog #*
- **Lot / Batch #:** *include expiration date*
- **Purity:**
- **Storage conditions:**
- **CAS #:**
- **Justification for choice of solvent:**
- **Final concentration:**
- **(% volume in assay)**

6. Stock Medium: Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture
- **Description:**
- **Source:**
- **Lot / Batch #:** *include expiration date*

Sodium bicarbonate: *Source; purity*
Nu-Serum: Lot number [#]; tested for background hormone concentrations by performing laboratory
ITS+ premix: Lot number [#]

7. **Test Cells:** H295R human adrenocortical carcinoma cells (ATCC CLR-2128; lot [#]). *Include a brief description of culture procedures and conditions (cell passage number, temperature, atmosphere, frequency of medium renewal).*

The following performance criteria were met (indicated by an "x"):

	Cell passage identifier. Cell Passage #: [#]
	Cells frozen down at passage 5
	Frozen cells cultured for 4 additional passages
	Total number of passages does not exceed 10

B. METHODS

Document any deviations from the guideline methods along with any explanations provided.

1. Pre-Test Information: *(if applicable)*

a. Hormone Assay Interference Test: *Brief description of the test and results.*

b. Hormone Extraction: *Brief description and results if performed.*

c. Laboratory Proficiency Test: *State if previously performed, and provide date and study reference. If not performed, provide a rationale. Give a brief synopsis of methods and results including effective concentration, expressed as 50% of maximum response (EC50) for prochloraz and forskolin and compare EC50 to acceptable ranges for testosterone and estradiol (µM) as indicated on page 23 of guideline. In addition to the basic requirement of demonstrating proficiency of the laboratory in conducting the assay, check to see whether a new laboratory proficiency test was conducted whenever significant changes in personnel at the laboratory have occurred.*

TABLE 1. Dosing Schematic for the Positive Controls in the Laboratory Proficiency Study (µM).[a]

	1	2	3	4	5	6
Forskolin (Inducer plate)						
A	DMSO	DMSO	DMSO	10	10	10
B	0.03	0.03	0.03	1	1	1
C	0.3	0.3	0.3	0.1	0.1	0.1
D	3	3	3	Blank	Blank	Blank
Prochloraz (Inhibitor plate)						
A	DMSO	DMSO	DMSO	3	3	3
B	0.01	0.01	0.01	0.3	0.3	0.3
C	0.1	0.1	0.1	0.03	0.03	0.03
D	1	1	1	Blank	Blank	Blank

a Obtained from page [#] of the study report. Dosing was calculated based on a total volume of 1 mL per well.

2. Test Solutions: *Briefly summarize the procedures for preparation of the test solutions and positive control solutions. Note if the test material is insoluble at any of the concentrations, or if it is soluble at all concentrations. Example text is included below.*

A [100] mM stock solution was prepared by dissolving an appropriate amount ([MW x 0.00001]) of the test compound in [100] µL [name of solvent]. [Six] serial [1:10] dilutions were then prepared by adding [10] µL of the test solution to [90] µL *solvent*, yielding a range of [100 nM to 100 mM]. Forskolin and prochloraz solutions were prepared in a similar manner with three ([1-100 mM]) and four ([0.1-100 mM]) serial dilutions, respectively.

3. Cell Plating and Preincubation: *Briefly summarize the procedures for cell maintenance and culture. Example text is included below and altered according to the specific methods of the performing laboratory. When using a 48-well plate instead of a 24-well plate it is recommended that the laboratory follow the OECD guidance: "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 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."*

H295R cells (ATCC CLR-2128) were grown for [five] passages, frozen in liquid nitrogen, then thawed and cultured for [four] additional passages. The cells were then seeded in [#] [24]-well plates using [1] mL of cells at a concentration of [200,000 – 300,000] cells/mL, yielding approximately [#]% confluency at 24 hours. The seeded plates were incubated for 24 hours at 37°C in a 5% CO_2 atmosphere.

4. Exposure: *Describe the exposure methods for the steroidogenesis assy, using the following example text as a guide. Include information about the runs for the test chemical and the QC plates.*

The cells were checked microscopically for good attachment and proper morphology, and the medium was removed and replaced ([1 mL]). The cells were then exposed to [1] µL of each serial dilution of the test compound or [1] µL of [DMSO (0.1% DMSO)] in triplicate according to the schematic presented in Table 2.

TABLE 2. Dosing Schematic for the Exposure of H295R Cells to [Test Chemical](Final Concentrations in µM).[a]

	1	2	3	4	5	6
A	DMSO	DMSO	DMSO	0.1	0.1	0.1
B	100	100	100	0.01	0.01	0.01
C	10	10	10	0.001	0.001	0.001
D	1	1	1	0.0001	0.0001	0.0001

a Data were obtained from page [#] of the study report. Dosing was calculated based on a total volume of 1 mL per well.

A concurrent quality control (QC) plate was included with each of the three independent runs of the test chemical plates to demonstrate the assay's response to forskolin (an inducer of testosterone and estradiol production) and prochloraz (an inhibitor of testosterone and estradiol production). The QC plate was prepared and dosed in the same manner with [1] µL of either forskolin or prochloraz according to the schematic presented in Table 3.

TABLE 3. Dosing Schematic for the QC Plate for Positive Controls (Final Concentrations in µM).[a]

	1	2	3	4	5	6
A	Blank[b]	Blank	Blank	Blank + MeOH[c]	Blank + MeOH	Blank + MeOH
B	DMSO	DMSO	DMSO	DMSO + MeOH	DMSO + MeOH	DMSO + MeOH
C	Forskolin 1µM	Forskolin 1µM	Forskolin 1µM	Prochloraz 0.1µM	Prochloraz 0.1µM	Prochloraz 0.1µM
D	Forskolin 10µM	Forskolin 10µM	Forskolin 10µM	Prochloraz 1µM	Prochloraz 1µM	Prochloraz 1µM

a Data were obtained from page [#] of the study report. Dosing was calculated based on a total volume of 1 mL per well.
b Blank wells received medium only.
c MeOH = 70% methanol was added to these wells for 30 minutes at room temperature following medium removal.

Following dosing, the plates were incubated for [48] hours under the conditions previously described. The medium from each well was removed, split into two equal volume aliquots, and frozen at −80°C until hormone measurements.

5. Cell Viability/Cytotoxicity Assay: *Describe the assay used for determining cell viability/cytotoxicity. Acceptable assays include the LIVE/DEAD® Viability/Cytotoxicity assay and the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] test described by Mosman (1983)[1]. Briefly describe the methods, including incubation time, temperature, equipment (cite manufacturer and location) used, settings, etc. Example text for the LIVE/DEAD® Viability assay is included below.*

Cell viability was determined using the [*LIVE/DEAD®* Viability/Cytotoxicity kit (Molecular Probes, Eugene, OR)] immediately after removal of the culture medium. The plate wells were rinsed once with [300] µL of phosphate buffered saline (PBS, with Ca^{2+} and Mg^{2+}), and then covered with [300] µL of PBS; wells treated with methanol on the QC plate were rinsed three times.

6. Hormone Measurement System: [ELISA, RIA, other (if commercial, name and source)].

Briefly describe the methods used, including the number of analyses for each sample and how the method was validated for use in the study. If a commercial kit is used, cite the manufacturer and location. If an extraction step is needed, it is recommended that the percent recovery be documented as described on pages 33-35 of the guideline.

The following performance criteria were met (indicated by an "x"):

	Method detection limit (100 pg/mL testosterone; 10 pg/mL estradiol)
	Spiked sample recovery acceptable for two concentrations of testosterone and estradiol (mean measured amount from triplicate samples ≤30% of nominal concentration)
	Hormone cross-reactivity (antibody-based assays only; ≤30% of basal production of the respective hormone)
	Solvent control within 75% range below maximum response on standard curve
	Test compound tested for interference with measurement system

C. DATA ANALYSIS: *Describe the statistical methods used. Include a statement that the Reviewer considers the analyses used to be appropriate; if inappropriate, provide alternative/rationale. If inappropriate statistical methods were used, the reviewer should perform the appropriate statistics and discuss the results.*

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

Evaluate whether the guideline was followed. The guideline recommends results be normalized to the mean solvent control (SC) for each 24-well plate used to test a given chemical as follows:

Relative change = (hormone concentration in each well) ÷ (mean SC hormone concentration).

Express data as mean ± SD. The guideline recommends that concentrations that exhibit approximately 20% cytotoxicity be omitted from calculations. It is recommended that data be evaluated for normal distribution and homogeneity of variance, and transformations applied as necessary. Use parametric (e.g., Dunnett's) or non-parametric (e.g., Kruskal-Wallis, Steel's Many-one rank) tests as appropriate. Significance is attained at $p \leq 0.05$. See Table 8 on page 38 of the guideline for data criteria regarding:

- *Statistical significance*
- *Dose-response*
- *Interference*
- *Solubility and cell viability.*

II. RESULTS

A. <u>TEST COMPOUND</u>: *Present the hormone level results (See Table 4). The guideline recommends that the SC be within the upper 75% range of the standard curve for the hormone assay for inducers, and within the lower 75% range for inhibitors. Calculate the fold difference relative to the SC. Data are to be reported in both tabular and graphic formats. The column graph should present mean (±SD) relative (-fold) change for each concentration, with statistical significance indicated (See Figure 1).*

Discuss the data in terms of meeting QC parameters and hormone production criteria:

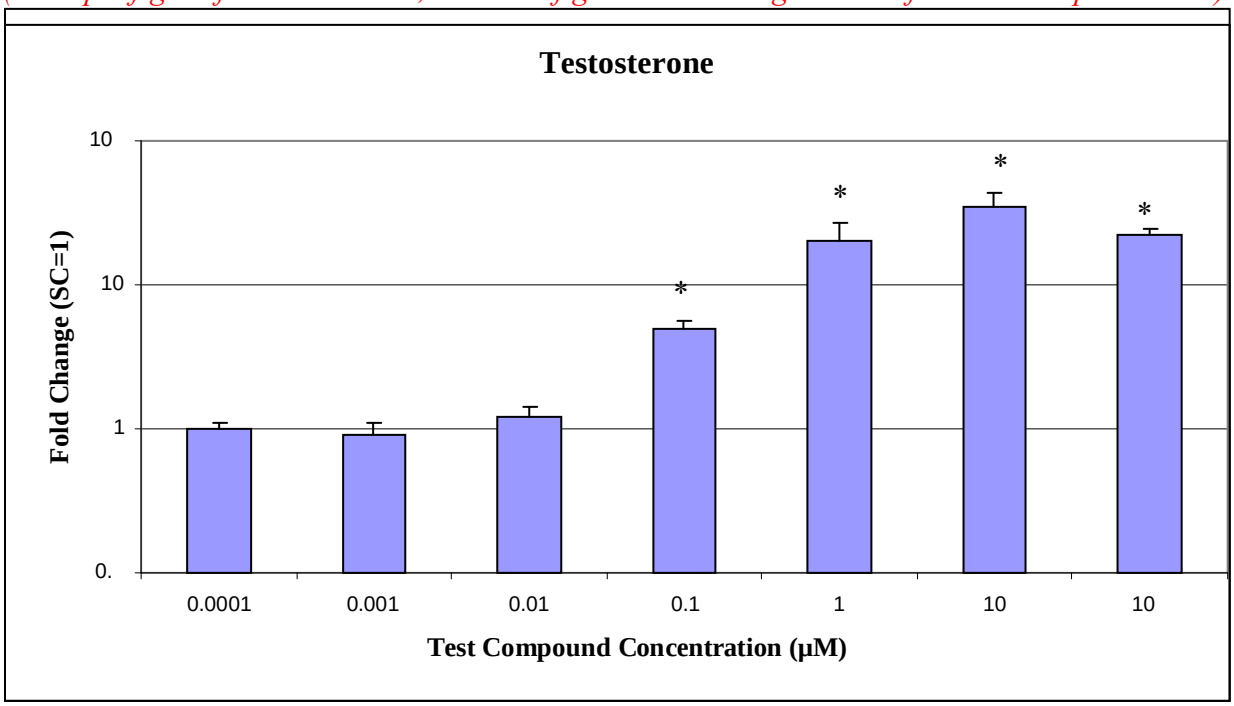
- *Basal hormone production in SC is ≥5-fold for testosterone and ≥2.5-fold for estrogen above the minimum detection level (MDL) of the assay (See Table 6 in SEP).*
- *Replicate wells CV ≤30% within a plate (absolute concentrations)*
- *Between plate SC CV ≤30% (fold change)*

TABLE 4. Mean (±SD) Hormone Concentrations Following Treatment with [Test Chemical] for 48 Hours.[a]

Nominal Concentration (µM)	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Mean	± SD	Statistical Significance
	Testosterone (pg/mL)			Fold Difference					
DMSO									
0.0001									
0.001									
0.01									
0.1									
1									
10									
100									
	Estradiol (pg/mL)			Fold Difference					
DMSO									
0.0001									
0.001									
0.01									
0.1									
1									
10									
100									

a Data were obtained from page [#] of the study report.

Figure 1. Change in Testosterone Production Relative to [test compound] Concentration.
(Example figure for testosterone, a similar figure should be generated for estradiol production)



* Significantly different from the solvent control at p≤0.05.

B. CYTOTOXICITY: *Present viability (cytotoxicity) data. For example, if using Live/Dead® cell viability/cytotoxicity assay, include the following:*

- *Divide the average calcein fluorescence by its ethidium bromide homodimer fluorescence to obtain a live to dead cell ratio.*
- *Include a graph of the mean (±SD) calcein AM fluorescence for the negative control, solvent control, and each concentration tested.*
- *If visual examination of the graph indicates that the blank has greater viability than the treated groups, then the solvent may be cytotoxic.*
- *If the graph indicates that viability decreases with increasing dose, then the test material may be cytotoxic and preclude determination of any treatment-related effects on steroidogenesis.*

C. QC PLATE: *Present QC plate results as described for test compound (see Table 5).*

Discuss the data in terms of meeting QC parameters and hormone production criteria:

- *Minimum basal hormone production is met in both blank and SC wells (500 pg/mL for testosterone, 40 pg/mL for estradiol).*
- *Basal hormone production in SC is ≥5-fold for testosterone and ≥2.5-fold for estrogen above the minimum detection level of the assay.*
- *10 µM forskolin induces testosterone ≥1.5-fold and estradiol ≥7.5-fold over SC.*
- *1 µM prochloraz inhibits synthesis of testosterone and estradiol ≤0.5-fold (i.e., ≥50% inhibition) compared to SC.*

TABLE 5. Mean (±SD) Hormone Concentrations Following Treatment with Forskolin or Prochloraz for 48 Hours.[a]								
Concentration (µM)	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 1**	**Trial 2**	**Trial 3**	**Mean**	**± SD**
	Testosterone (pg/mL)			**Fold Difference**				
Blank								
DMSO								
1 µM Forskolin								
10 µM Forskolin								
0.1 µM Prochloraz								
1 µM Prochloraz								
	Estradiol (pg/mL)			**Fold Difference**				
Blank								
DMSO								
1 µM Forskolin								
10 µM Forskolin								
0.1 µM Prochloraz								
1 µM Prochloraz								

a Data were obtained from page [#] of the study report. .

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: *Concisely summarize the investigator's conclusions regarding the effect of the test compound on hormone levels.*

B. AGENCY COMMENTS: *Summarize the results for the test compound and the QC plate. Discuss any discrepancy with investigators' conclusions; include rationale for acceptability or not; necessity for repeat.*

C. STUDY DEFICIENCIES: *List each deficiency (distinguishing between major and minor ones) with the data required to resolve the deficiency. If no data can be provided to satisfy the deficiency, indicate the need for a repeat study. If unacceptable, is the study potentially upgradable to acceptable, how?*