The following report presents results of a study conducted by a contract laboratory for the National Toxicology Program (NTP). The report may not have been peer reviewed. The findings and conclusions for this study should not be construed to represent the view of NTP or the U.S. Government.

CeeTox
In vitro models to predict toxicity

Estrogen Receptor Transcriptional Activation
(Human Cell Line (HeLa-9903))

Final Report

DATA REQUIREMENT(S): OECD 455 (2009)
OPPTS 890.1300 (2009)

AUTHOR(S): [redacted]

STUDY COMPLETION DATE: February 02, 2012

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LABORATORY PROJECT ID: Study Number: 9070-100107ERTA
Sponsor Contract No. HHSN273200900005C
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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study Number: 9070-100107ERTA

Study Title: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903))

I, the undersigned, hereby declare that this study was performed in accordance with the United States Environmental Protection Agency (US EPA) Good Laboratory Practice (GLP) regulations; Title 40 CFR 160 (for FIFRA) with the exception of section 160.113. Dose concentrations of test substance and control substances will not be verified by analytical methods.

The study was conducted according to the procedures herein described and this report represents a true and accurate record of the results obtained. There were no deviations that impacted the quality or integrity of the study data. Any deviations that occurred during the course of the study will be noted in this report, with the full write-ups included in the study binder.

[redacted]

Study Director

02 Feb 2012

Date

FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Title: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903))

Study Number: 9070-100107ERTA

In accordance with CeeTox, Inc.'s policies and Quality Assurance standard operating procedures for Good Laboratory Practice (GLP), the conduct of this study has been audited as follows:

Date(s) of Inspection/Audit	Inspection/Audit	Date(s) reported to Study Director	Date(s) reported to Management
27Jun11	Draft protocol audit	27Jun11	27Jun11
13Jul11	In-process assay audit	18Jul11	18Jul11
26Aug11	In-process assay audit	29Aug11	29Aug11
16Dec11	Data binder audit	16Dec11	16Dec11
30Jan12	Draft report audit	30Jan12	30Jan12

The signature below indicates the summary table is an accurate representation of Quality Assurance's involvement with this study.

[redacted] 02Feb2012

Date

Quality Assurance Auditor
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GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
[redacted]	Director of Project Management
	Director of Laboratory Operations
	Senior Scientist
	Scientist
	Scientist
	Lead Cell Culture Scientist
	Study Director

Study Dates

Study initiation date: June 24, 2011
Experimental start date: July 05, 2011
Experimental termination date: August 26, 2011
Study completion date: February 02, 2012

Deviations from the Protocol

See Appendix 2. There were five deviations however they did not impact the integrity of the data in this report.

Other

At the study closure, all study records including all original raw data and original final report, will be shipped to the sponsor at the following address:

NTP Archives
[redacted]
615 Davis Drive, Suite 300
Durham, NC 27713

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

The objective of this study was to evaluate the ability of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene to act as an agonists of human estrogen receptor alpha (hERα) using the hERα-HeLa-9903 cell line.

Preliminary assessments of cytotoxicity and precipitation were conducted in order to identify soluble and cytotoxic concentrations of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene in the transcriptional activation assays.

Five independent runs were conducted however the first three runs (11-July-2011, 13-July-2011 and 15-July-2011) were invalid because the reference control data did not fit the acceptance criteria outlined in the OPPTS 890.1300 guideline and in the table in section 3.2.2 (pg 13). A fresh vial of cells were brought up from cryopreservation and used for the last two runs, which were valid runs. The final concentrations of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene tested in the ER transcriptional activation assay were: 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M for both valid runs (23-August-2011 and 25-August-2011).

All concentrations were tested in replicates of 6/plate. In addition, for each concentration, 2 replicates/plate were prepared that incorporated the hERα antagonist ICI 182,780. Replicates incorporating the hERα antagonist allow for the identification of non-specific (i.e., non-hERα-mediated) induction of the luciferase gene. The duration of exposure was 24 h. A complete concentration response curve for each of 4 reference compounds (17β-estradiol, 17α-estradiol, corticosterone, and 17α-methyltestosterone) was run each time the transcriptional activation assay was performed.

1.2 Results

According to the range finder assay, the highest soluble concentration for use in the transcriptional activation assays was determined to be $10^{-4.5}$ M for oxybenzone, octylmethoxycinnamate, and octylsalate, and 10^{-4} M for octocrylene. Therefore, the exposure concentrations tested were 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M for all test substances for both valid runs. There was no cytotoxicity (≥20% reduction in cell viability) observed with oxybenzone during this study, however octylmethoxycinnamate, octylsalate and octocrylene all showed cytotoxicity at 10^{-5} M in the first valid run only. There was no cytotoxicity with any test substance in the second valid run.

In the first valid run (23-August-2011), oxybenzone resulted in an increase in luciferase activity to 14.9 ± 2.6% at 10^{-5} M. In the second valid run (25-August-2011), oxybenzone also resulted in an increase in luciferase activity to 20.9 ± 7.5% at 10^{-5} M. In the two valid runs of the transcriptional activation assay, octylmethoxycinnamate, octylsalate and

octocrylene did not result in an increase in luciferase activity at any of the viable concentrations tested (RPC_{max}<10%).

1.3 Conclusion

Octylmethoxycinnamate, octylsalate and octocrylene are not agonists of human estrogen receptor alpha (hERα) in the estrogen receptor transcriptional activation (Human Cell Line (HeLa-9903)) model system. Oxybenzone, however, is an agonist of human estrogen receptor alpha (hERα) in the HeLa-9903 model system resulting in a $LogPC_{10}$ of -5.4 Log[M] in the first valid run and -5.6 Log[M] in the second valid run.

2.0 INTRODUCTION

2.1 Purpose

The objective of this study was to evaluate the ability of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene to act as an agonist of human estrogen receptor alpha (hERα) using the hERα-HeLa-9903 cell line.

The hERα-HeLa-9903 cell line is derived from a human cervical tumor and has two stably inserted constructs: (i) the hERα expression construct (encoding the full-length human receptor) and (ii) a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin estrogen-responsive element driven by a mouse metallothionein (MT) promoter TATA element. The mouse MT TATA gene construct has been shown to have the best performance, and so is commonly used. Consequently, the hERα-HeLa-9903 cell line can measure the ability of a test substance to induce hERα-mediated transactivation of luciferase gene expression, i.e., the cell line can be used to assess the ability of a test substance to act as an agonist of hERα.

The results of this study are intended to be used in conjunction with results from other Tier 1 screening studies (OPPTS 890 test guideline series) that constitute the full screening battery under the Endocrine Disruptor Screening Program (EDSP). Together, the results from the screening battery will be used by the US EPA to identify substances that have the potential to interact with the estrogen, androgen, or thyroid system. Results of the Tier 1 screening battery, along with other scientifically relevant information, are to be used in a weight-of-evidence determination of a substance's potential to interact with these systems. The fact that a substance may interact with a hormone system does not mean that when the substance is used, it will cause adverse effects in humans or ecological systems. The Tier 1 battery is intended for screening purposes only and should not be used for endocrine classification or risk assessment.

2.2 Regulatory Citations

OECD guideline for the testing of chemicals number 455: Stably transfected human estrogen receptor-α transcriptional activation assay for detection of estrogenic agonist-activity of chemicals. 2009.

OPPTS 890.1300: Estrogen receptor transcriptional activation (human cell line (HeLa-9903)). 2009.

3.0 MATERIALS AND METHODS

3.1 Test Substance

3.1.1 Test substance details

Test Substance Name:	2-hydroxy-4-methoxybenzophenone (Oxybenzone)
Test Substance Manufacturer:	Ivy Fine Chemicals
CAS Number:	131-57-7
Description:	Light yellow solid
Solvent Used:	DMSO
Batch Number:	20100801
Expiry Date:	01-Aug-2012
Purity:	99.92%
Molecular Formula:	$C_{14}H_{12}O_3$
Molecular Weight:	228.25
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 3.

Test Substance Name:	2-ethylhexyl p-methoxycinnamate, octyl 4-methoxycinnamate (Octylmethoxycinnamate)
Test Substance Manufacturer:	Acros Organics
CAS Number:	5466-77-3
Description:	Clear colorless liquid
Solvent Used:	DMSO
Batch Number:	A0293319
Expiry Date:	Not Provided
Purity:	99.8%
Molecular Formula:	$C_{18}H_{26}O_3$
Molecular Weight:	290.39
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 3.

Test Substance Name:	Octyl salicylate, 2-ethylhexyl salicylate (Octylsalate)
Test Substance Manufacturer:	Sigma-Aldrich
CAS Number:	118-60-5
Description:	Colorless liquid
Solvent Used:	DMSO
Batch Number:	44698PJ
Expiry Date:	Not Provided
Purity:	99.6%
Molecular Formula:	$C_{15}H_{22}O_3$
Molecular Weight:	250.33
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 3.

Test Substance Name:	2-ethylhexyl 2-cyano-3,3-diphenylacrylate (Octocrylene)
Test Substance Manufacturer:	Sigma-Aldrich
CAS Number:	6197-30-4
Description:	Yellow viscous liquid
Solvent Used:	DMSO
Batch Number:	01697MJ
Expiry Date:	Not Provided
Purity:	99.2%
Molecular Formula:	$C_{24}H_{27}NO_2$
Molecular Weight:	361.48
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 3.

3.1.2 Vehicle selection

Dimethyl sulfoxide (DMSO) was selected as a suitable vehicle for oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene. Oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene solutions up to $10^{-4.5}$ M (the limit concentration for the assay) can be prepared while limiting the final concentration of DMSO in the assay medium to 0.1% (v/v). 17α-methyltestosterone, 17α-estradiol, corticosterone and 17β-estradiol were prepared on August 23, 2011 for use in this study. Oxybenzone, octylmethoxycinnamate, octylsalate, and octocrylene were prepared in DMSO on August 23, 2011 for use in this assay. Based upon historical data for reference compounds 17α-methyltestosterone, 17α-estradiol, corticosterone and 17β-estradiol, OPPTS 890.1300 guideline criteria for these reference compounds and the results of the reference compounds in this assay indicate they were stable over these times.

3.2 Cell Line

3.2.1 Source

The stably transfected hERα-HeLa-9903 cell line was used in this study. The cell line was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, 7-6-8 Asagi Saito, Ibaraki-shi, Osaka 567-0085, Japan. The cell line was certified to be free of mycoplasma (certification is presented in Appendix 4). The cells used in this study were passage 21 (rangefinder, run on 05-July-2011). New cells were brought up from cryo and maintained for the two valid runs (23-August-2011 and 25-August-2011) and were passages 19 and 20, respectively, prior to seeding into plates.

3.2.2 Stability of the cell line

The stability of the cell line was monitored by the use of the following reference chemicals: 17β-estradiol, 17α-estradiol, 17α-methyltestosterone and corticosterone. A complete concentration response curve for each reference compound was run each time the transcriptional activation assay was performed and the $LogPC_{50}$, $LogPC_{10}$, $LogEC_{50}$ and Hill slope values calculated and compared to the acceptable range values summarized below (values taken from the cited guidelines).

Name	$LogPC_{50}$	$LogPC_{10}$	$LogEC_{50}$	Hill slope	Test Range (M)
17β-Estradiol CAS No: 50-28-2	-11.4 ~ -10.1	<-11	-11.3 ~ -10.1	0.7 ~ 1.5	10^{-14} ~ 10^{-8}
17α-Estradiol CAS No: 57-91-0	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	10^{-12} ~ 10^{-6}
Corticosterone CAS No: 50-22-6	-	-	-	-	10^{-10} ~ 10^{-4}
17α-Methyltestosterone CAS No: 58-18-4	-6.0 ~ -5.1	-8.0 ~ -6.2	-	-	10^{-11} ~ 10^{-5}

3.2.3 Cell culture and plating conditions

Cells were maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with 60 mg/L of Kanamycin (antibiotic) and 10% dextran-coated-charcoal-treated fetal bovine serum (DCC-FBS), in a CO_2 incubator (5% CO_2) at 37±1°C. When the cells reached 75-90% confluency, they were subcultured at 10 mL of 0.4 X 10^5 – 1 X 10^5 cells/mL. The cells were suspended with 10% DCC-FBS in EMEM and plated into wells of a 96-well cell culture plate at a density of ~1 X 10^4 cells/100 µL/well. The cells were then placed into a 5% CO_2 incubator 37±1°C for at least 3 hours prior to chemical exposure.

3.3 Chemical Exposure and Assay Plate Organization

The reference chemicals, oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene were dissolved in 100% DMSO in brown glass vials by pipetting up and down, then serially

diluted as 1000x working stocks in DMSO in 96-well plates before further dilution in medium using deep-well 96-well blocks (which hold ~2 mL per well) to prepare 2x concentrations in media containing 0.2% DMSO (v/v). When added to the cell culture plates prepared as described in Section 3.2.3, the 2x concentrated media solutions would yield the final serial concentrations as specified in Section 3.2.2 for the reference chemicals and as determined for in the preliminary range finding assays for the test substance (see Section 3.5). The final concentration of DMSO in the medium was held constant at 0.1% (v/v).

After the three hour (minimum) post-seeding incubation, the plates were removed from the incubator and the media was aspirated. 75 µL of fresh media, followed by 75 µL of the 2x concentrated media solutions were added to wells containing ~1 X 10^4 cells/well for a final volume of 150 µL/well. Assay plates were organized as detailed below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank*	E2 (1 nM)	VC**	VC	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6	Conc. 7	Conc. 8
B	↓***	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	----------As above + antagonist (1 µM ICI 182,780)----------											
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

E2 = 17β-estradiol
*Blank wells contain media only (no cells)
**Vehicle control (VC) wells contain cells and media + 0.1% (v/v) DMSO
***↓ Indicates the composition of the well is identical to the well directly above it

After adding the reference chemicals/test substance, the plates were incubated in a 5% CO_2 incubator at 37±1°C for 24 ± 2 h.

All concentrations were tested in replicates of 6/plate. In addition, for each concentration, 2 replicates/plate were prepared that incorporated the hERα antagonist ICI 182,780. Replicates incorporating a hERα antagonist allow for the identification of non-specific (i.e., non-hERα-mediated) induction of the luciferase gene as true hERα-mediated induction is inhibited by addition of an antagonist whereas non-specific induction is not.

In view of the short-term nature of studies of this type, no analyses of stability, homogeneity or achieved concentration(s) were carried out on preparations of the test substance or positive control chemicals, either before or after the treatment phase. This is not considered to have affected the integrity of the study. For the reference control compounds, stability is demonstrated by an appropriate response in the assay system.

3.4 Assays

3.4.1 Cytotoxicity assay

Cell viability was monitored by a two-read propidium iodide (PI) uptake assay. PI is a light sensitive dye and all procedures were conducted under low light conditions. PI cannot cross the plasma membranes of intact and viable cells. Cells that are dead or dying have weakened plasma membranes which allows PI to enter the cytosol of the damaged cells. Once inside the cell, PI intercalates into DNA/RNA and yields a fluorescent signal. The intensity of the fluorescent signal is inversely proportional to cell viability, where a decrease in cell viability is detected by an increase in fluorescent signal. In the two-read procedure, the first read is taken immediately after full exposure to controls and test substances is completed. This measures "background" fluorescence and indicates cell spontaneous death and control/test material induced cytotoxicity. The cells are then lysed and a second read is taken, which indicates 100% cell death. The first read is then subtracted from the second read. The results of the subtracted reads are directly proportional to the viability of the cells. The control and test material data are normalized to vehicle control to generate percent cell viability.

Cells were seeded as described in Section 3.2.3, with the exception that a black-walled 96-well cell culture plate was used. The cells were exposed to the test chemicals in replicates of 6 (rows A-F) while the last 2 rows (G and H) received 125 μM digitonin as a positive control for cell death. Following chemical exposure, the growth medium was removed and 50 μL of a PI working solution (44 μM in phosphate buffered saline or cell culture medium [see Section 3.2.3]) was added to each well. Background fluorescence was evaluated by measuring fluorescence immediately on a Packard Fusion fluorescence plate reader at an excitation wavelength of 544 nm and an emission wavelength of 612 nm. Following this determination, 50 μL of a 2% (v/v) Triton X-100 solution was added to each well and the plate was incubated at room temperature for a minimum of 15 minutes to fully lyse all cells in the wells before measuring fluorescence at the same wavelengths.

The background-corrected fluorescence was calculated for each well by subtracting the results of the first read from the results of the second read. The change in cell viability was determined by comparing treated wells to the vehicle control wells. A ≥20% reduction in cell viability was considered evidence of cytotoxicity.

3.4.2 Precipitation assessment

Limit of solubility was determined by visual inspection of the test materials and controls after preparation of the final 1x dosing solutions in culture media. A sample of the 1x dosing solution was placed into wells of a clear 96-well plate and an endoscope was used to assess precipitation in each sample.

3.4.3 Transcriptional activation assay

A luciferase assay was performed as described in CeeTox Standard Operating Protocol (SOP) 2041 using the reagents listed below. Luciferase assay reagent was prepared as described in CeeTox SOP 2041 (proprietary information).

Reagent	Supplier	Catalog #
Trisma Base	Sigma	T6066
Magnesium Chloride	Sigma	M2393
EDTA	Sigma	E5134
Dithiothreitol	Sigma	D9779
ATP	Sigma	A2383
Coenzyme A	Sigma	C3019
AMP	Sigma	A1752
Luciferin	Promega	E160E
Glycerol	Sigma	G5516
Triton-X100	Sigma	T8787
Bovine Serum Albumin	Sigma	A9418
CDTA	Sigma	D0922

3.5 Preliminary Range Finding

In order to identify soluble and cytotoxic concentrations in the transcriptional activation assays, preliminary cytotoxicity and precipitation assays were conducted with oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene. These preliminary assays were conducted as described in Sections 3.4.1 and 3.4.2 and assessed cytotoxicity and precipitation, respectively, at the following concentrations: $10^{-6.5}$, 10^{-6}, $10^{-5.5}$, 10^{-5}, $10^{-4.5}$, 10^{-4}, $10^{-3.5}$ and 10^{-3} M.

3.6 Transcriptional Activation Assay Data Analysis and Interpretation

In order to determine the relative transcriptional activity as compared to the positive control (PC), 1 nM 17β-estradiol, the luminescence data from each plate were analyzed according to the steps outlined below. Wells incorporating ICI 182,780 were analyzed in an identical fashion to wells not incorporating ICI 182,780, except that the data were normalized by subtracting the mean value for the ICI 182,780-containing vehicle control (VC) wells.

1. Any cytotoxic concentrations (as defined in Section 3.4.1) were excluded from data analysis.
2. The mean value for the VC wells was calculated.
3. The mean value for the VC wells was subtracted from each well to correct the data for background transcriptional activity in control cells.
4. The mean value for the background-corrected PC wells was calculated and this value was defined as 100% transcriptional activation.

5. The background-corrected value for each well was normalized to the mean value of the background-corrected PC wells to determine the relative transcriptional activity.

The data were then interpreted according to the following steps:

1. Where appropriate, $LogPC_{50}$, $LogPC_{10}$, $LogEC_{50}$ and Hill slope values were calculated.
2. For the test substance, the maximum response relative to the positive control (RPC_{Max}) was determined. In each individual run of the transcriptional activation assay, if RPC_{max} was less than 10%, the test substance was considered to have given a negative response for hERα agonism.
3. For each individual run of the transcriptional activation assay, the acceptability of the data was evaluated using the following criteria:

    - The mean background-corrected luciferase signal of the PC (1 nM 17β-estradiol) should be at least 4-fold that of the mean VC on each plate.

    - The results of the 4 reference chemicals should be within the acceptable ranges (see Section 3.2.2).

4. If the acceptability criteria outlined above were met, that run of the transcriptional activation assay was considered to be valid.
5. The test substance was considered negative if RPC_{Max} <10% in at least 2 definitive runs of the transcriptional activation assay. The test substance was considered positive if $RPC_{Max} \geq 10\%$ in at least 2 definitive runs of the transcriptional activation assay. If the results are not reproducible, a deciding third run would be performed.

4.0 RESULTS AND DISCUSSION

4.1 Concentration Range for the Test Substance

In order to identify a range of test substance exposure, preliminary assessments of cytotoxicity and precipitation were conducted as described in Sections 3.4.1 and 3.4.2, respectively.

According to the rangefinder, the highest soluble concentration for use in the transcriptional activation assays was determined to be $10^{-4.5}$ M for oxybenzone, octylmethoxycinnamate and octylsalate, and 10^{-4} M for octocrylene. There was cytotoxicity observed at 10^{-3} M oxybenzone, 10^{-3} M octylmethoxycinnamate, $10^{-3.5}$ M octylsalate and $10^{-3.5}$ M and 10^{-4} M octocrylene.

The final concentrations of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene tested in the transcriptional activation assays were: 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M for both valid runs (23-August-2011 and 25-August-2011).

4.2 Transcriptional Activation Assay Acceptance Criteria

In both valid independent runs of the assay, the mean luciferase activity of the PC (1 nM 17β-estradiol) was greater than 4-fold that of the mean luciferase activity of the VC on each plate (see Table 6). In addition, in both independent runs of the assay the $LogPC_{50}$, $LogPC_{10}$, $LogEC_{50}$ and Hill slope values for the 4 reference compounds (17β-estradiol, 17α-estradiol, 17α-methyltestosterone and corticosterone) were within the acceptable ranges specified in Section 3.2.2 (see Table 6), with the following minor exceptions:

- In the first valid run of the assay, the $LogPC_{10}$ value for 17α-methyltestosterone was not reached due to cytotoxicity observed at the highest concentration ($LogPC_{10}$ = -8.0 ~ -6.2 according to the criteria)
- In both valid runs of the assay, the $LogPC_{50}$ value for 17α-methyltestosterone was not reached ($LogPC_{50}$ = -6.0 ~ -5.1 according to the criteria)
- In the second valid run of the assay, the $LogPC_{10}$ value for 17α-methyltestosterone was marginally greater than the specified range ($LogPC_{10}$ = -5.9; compared to the specified range of -8.0 ~ -6.2)

These variations from the ranges suggested in the OPPTS guideline were minor and not considered to impact the interpretation of results as the assay response with 17β-estradiol, 17α-estradiol, 17α-methyltestosterone and corticosterone were characteristic of a strong estrogen, a weak estrogen, a weak agonist, and a negative compound, respectively. The results of 17α-methyltestosterone exposure listed above, though outside of the test guideline criteria, are typical and fit within CeeTox historical values. Therefore, both independent runs of the assay were considered to have met the assay acceptance criteria and were considered to be definitive.

4.3 Transcriptional Activation Assay Results

Five independent runs of the transcriptional activation assay were conducted because in the first three runs, the reference control data did not fit the acceptance criteria and was substantially different from historical data sets. The data is located in the study binder but is not included in the analysis of this report. The issue was determined to be the HeLa 9903 cells. Cells were brought up from cryo and used for the last two runs (23-August-2011 and 25-August-2011), which were valid runs.

In the first valid run (23-August-2011), oxybenzone resulted in an increase in luciferase activity to 14.9 ± 2.6% at 10^{-5} M. In the second valid run (25-August-2011), oxybenzone also resulted in an increase in luciferase activity to 20.9 ± 7.5% at 10^{-5} M. In the two valid runs of the transcriptional activation assay, octylmethoxycinnamate, octylsalate and octocrylene did not result in an increase in luciferase activity at any of the viable concentrations tested (RPC_{max}<10%).

4.4 Discussion

According to the range finder assay, the highest soluble concentration for use in the transcriptional activation assays was determined to be $10^{-4.5}$ M for oxybenzone, octylmethoxycinnamate and octylsalate, and 10^{-4} M for octocrylene. Therefore, the exposure concentrations tested were 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M for both valid runs. There was no cytotoxicity (≥20% reduction in cell viability) observed with oxybenzone during this study, however octylmethoxycinnamate, octylsalate and octocrylene all showed cytotoxicity at 10^{-5} M in the first valid run only. There was no cytotoxicity with any test substance in the second valid run.

In the first valid run (23-August-2011), oxybenzone resulted in an increase in luciferase activity to 14.9 ± 2.6% at 10^{-5} M. In the second valid run (25-August-2011), oxybenzone also resulted in an increase in luciferase activity to 20.9 ± 7.5% at 10^{-5} M. In the two valid runs of the transcriptional activation assay, octylmethoxycinnamate, octylsalate and octocrylene did not result in an increase in luciferase activity at any of the viable concentrations tested (RPC_{max}<10%).

5.0 CONCLUSIONS

Octylmethoxycinnamate, octylsalate and octocrylene are not agonists of human estrogen receptor alpha (hERα) in the estrogen receptor transcriptional activation (Human Cell Line (HeLa-9903)) model system. Oxybenzone, however, is an agonist of human estrogen receptor alpha (hERα) in the HeLa-9903 model system resulting in a $LogPC_{10}$ of -5.4 Log[M] in the first valid run and -5.6 Log[M] in the second valid run.

6.0 REFERENCES

Endocrine Disruptor Screening Program Test Guidelines. *OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903)).* EPA 70-C-09-006. October, 2009.

OECD Guideline fof the Testing of Chemicals. *OECD 455: Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals.* September, 2009.

TABLES SECTION

TABLE 1 Range finder - Results of Preliminary Cytotoxicity and Precipitation Assays

Test Substance	Concentration (M)	Cell Viability (% of VC)		Precipitation
		Mean	SD	
Oxybenzone	$10^{-6.5}$	105	4	-
	10^{-6}	102	7	-
	$10^{-5.5}$	103	4	-
	10^{-5}	98	7	-
	$10^{-4.5}$	96	7	-
	10^{-4}	86	5	+
	$10^{-3.5}$	83	3	+
	10^{-3}	79	7	+
Octylmethoxycinnamate	$10^{-6.5}$	98	2	-
	10^{-6}	97	4	-
	$10^{-5.5}$	96	6	-
	10^{-5}	95	5	-
	$10^{-4.5}$	93	6	-
	10^{-4}	90	13	+
	$10^{-3.5}$	87	15	+
	10^{-3}	71	19	+
Octylsalate	$10^{-6.5}$	97	5	-
	10^{-6}	92	5	-
	$10^{-5.5}$	99	4	-
	10^{-5}	91	2	-
	$10^{-4.5}$	90	6	-
	10^{-4}	86	4	+
	$10^{-3.5}$	77	12	+
	10^{-3}	81	6	+
Octocrylene	$10^{-6.5}$	94	6	-
	10^{-6}	93	7	-
	$10^{-5.5}$	97	7	-
	10^{-5}	96	5	-
	$10^{-4.5}$	86	7	-
	10^{-4}	67	11	-
	$10^{-3.5}$	63	13	+
	10^{-3}	85	7	+

VC = Vehicle Control
+ = Precipitation observed
- = No precipitation observed
SD = Standard Deviation
Red lettering = Viability < 80% and considered cytotoxic

TABLE 2 Results of 1st Valid Transcriptional Activation Assay - Controls

Chemical	Concentration (M)	RTA (% of PC)		RTA with ICI (% of PC)		Cell Viability (% of VC)		Precipitation
		Mean	SD	Value 1	Value 2	Mean	SD	
17β-Estradiol	10^{-15}	1.0	1.7	1.6	-1.7	99.7	9.0	-
	10^{-14}	1.0	1.8	3.0	-1.3	100.2	9.6	-
	10^{-13}	3.4	2.4	3.1	-1.1	99.2	8.4	-
	10^{-12}	3.3	1.6	2.3	-1.0	96.7	8.0	-
	10^{-11}	17.5	5.8	6.7	1.1	97.5	8.6	-
	10^{-10}	101.2	27.3	6.1	3.0	96.5	9.0	-
	10^{-9}	167.4	66.7	5.4	3.1	93.7	9.7	-
	10^{-8}	132.4	24.8	6.4	-0.1	88.6	7.6	-
17α-Estradiol	10^{-13}	1.6	1.2	-0.1	-0.4	99.9	10.0	-
	10^{-12}	0.7	1.6	1.6	-1.5	101.6	8.6	-
	10^{-11}	1.6	1.2	0.9	-0.7	101.0	7.4	-
	10^{-10}	2.6	2.0	0.8	-1.7	98.4	9.9	-
	10^{-9}	27.2	6.9	3.0	1.5	97.0	9.0	-
	10^{-8}	96.8	19.0	1.7	1.4	98.4	8.9	-
	10^{-7}	110.4	42.0	3.0	0.7	94.7	8.3	-
	10^{-6}	97.6	27.8	2.4	0.2	86.0	7.3	-
Corticosterone	10^{-11}	0.8	1.3	0.9	-0.5	101.3	6.2	-
	10^{-10}	0.4	1.8	1.4	-0.8	103.2	8.8	-
	10^{-9}	1.3	1.4	1.3	0.1	100.2	8.2	-
	10^{-8}	0.7	1.4	1.5	-1.2	100.5	8.9	-
	10^{-7}	2.3	1.3	3.0	-0.2	99.5	7.1	-
	10^{-6}	2.1	1.4	1.5	-0.1	99.3	8.6	-
	10^{-5}	-2.0	0.8	-2.1	-2.2	89.3	8.0	-
	10^{-4}	*	*	-4.3	-3.7	78.2	6.7	-
17α-Methyltestosterone	10^{-12}	1.0	0.8	0.2	-0.3	98.5	8.1	-
	10^{-11}	1.1	1.5	2.2	-0.4	99.0	7.4	-
	10^{-10}	2.1	1.3	1.9	-0.1	95.5	9.2	-
	10^{-9}	1.8	2.0	1.9	0.0	94.1	8.7	-
	10^{-8}	5.4	2.7	3.1	2.1	95.0	7.4	-
	10^{-7}	5.9	3.6	4.0	2.0	93.8	6.6	-
	10^{-6}	7.9	3.5	3.3	1.0	90.1	8.0	-
	10^{-5}	*	*	-0.6	-1.1	75.9	5.7	-

RTA = Relative Transcriptional Activation
PC = Positive Control (1 nM 17β-Estradiol)
VC = Vehicle Control
SD = Standard Deviation
+ = Precipitation observed
- = No precipitation observed
* = Cytotoxixity observed so data not assessed
Red lettering = Viability < 80% and considered cytotoxic

TABLE 3 Results of 1st Valid Transcriptional Activation Assay – Test Substances

Chemical	Concentration (M)	RTA (% of PC)		RTA with ICI (% of PC)		Cell Viability (% of VC)		Precipitation
		Mean	SD	Value 1	Value 2	Mean	SD	
Oxybenzone	10^{-12}	0.6	1.7	1.6	-1.1	99.8	6.5	-
	10^{-11}	0.2	1.2	0.6	0.0	98.1	5.4	-
	10^{-10}	2.3	1.7	1.1	0.6	98.3	4.9	-
	10^{-9}	0.2	1.9	0.5	-0.3	95.3	5.2	-
	10^{-8}	3.8	1.2	3.5	1.6	93.8	5.9	-
	10^{-7}	2.8	1.4	3.1	3.0	93.9	3.8	-
	10^{-6}	3.9	2.4	4.1	1.4	88.4	5.8	-
	10^{-5}	14.9	2.6	8.9	4.2	82.4	3.9	-
Octyl-methoxycinnamate	10^{-12}	0.6	1.2	1.1	-0.7	96.2	6.0	-
	10^{-11}	0.3	1.6	1.2	-0.7	95.3	5.9	-
	10^{-10}	1.6	1.4	1.6	-1.0	93.8	5.0	-
	10^{-9}	1.1	1.2	1.6	-0.7	91.7	7.3	-
	10^{-8}	4.1	2.1	3.1	1.7	88.7	5.3	-
	10^{-7}	3.2	1.0	2.8	1.9	89.5	8.0	-
	10^{-6}	2.9	2.3	1.8	2.1	87.3	7.5	-
	10^{-5}	*	*	0.7	-0.3	77.8	7.3	-
Octylsalate	10^{-12}	0.6	1.5	1.7	-0.2	96.6	5.9	-
	10^{-11}	1.2	1.9	2.3	-0.5	99.0	5.6	-
	10^{-10}	2.8	2.9	2.1	0.1	97.5	5.4	-
	10^{-9}	0.5	1.7	2.3	-0.3	96.0	4.3	-
	10^{-8}	3.7	1.1	4.6	2.4	93.8	4.4	-
	10^{-7}	3.5	1.1	4.4	2.9	95.3	4.0	-
	10^{-6}	3.1	1.7	5.0	1.9	87.1	4.1	-
	10^{-5}	*	*	3.3	0.7	79.9	6.1	-
Octocrylene	10^{-12}	0.2	1.8	0.6	-0.2	97.4	8.5	-
	10^{-11}	0.9	1.8	2.3	-0.5	99.0	8.7	-
	10^{-10}	1.4	1.8	2.1	-0.6	97.4	7.6	-
	10^{-9}	0.6	1.4	2.1	-1.2	95.7	6.9	-
	10^{-8}	4.4	1.7	3.6	0.9	93.0	5.5	-
	10^{-7}	3.2	1.4	2.8	2.6	91.8	5.8	-
	10^{-6}	2.2	1.7	2.7	0.4	89.6	8.4	-
	10^{-5}	*	*	0.8	-1.0	79.1	4.4	-

RTA = Relative Transcriptional Activation
PC = Positive Control (1 nM 17β-Estradiol)
VC = Vehicle Control
SD = Standard Deviation
+ = Precipitation observed
- = No precipitation observed
* = Cytotoxixity observed so data not assessed
Red lettering = Viability < 80% and considered cytotoxic

TABLE 4 Results of 2nd Valid Transcriptional Activation Assay – Controls

Chemical	Concentration (M)	RTA (% of PC)		RTA with ICI (% of PC)		Cell Viability (% of VC)		Precipitation
		Mean	SD	Value 1	Value 2	Mean	SD	
17β-Estradiol	10^{-15}	1.4	2.0	2.2	-1.5	100.3	3.1	-
	10^{-14}	1.7	1.8	2.0	-1.5	98.4	4.2	-
	10^{-13}	3.9	2.6	3.7	-1.1	100.3	2.6	-
	10^{-12}	3.0	2.0	3.1	-1.4	97.2	3.2	-
	10^{-11}	18.6	5.8	7.1	0.0	98.2	5.0	-
	10^{-10}	91.2	14.1	6.5	2.6	100.0	6.3	-
	10^{-9}	158.6	44.3	6.4	1.5	93.1	4.5	-
	10^{-8}	131.8	32.0	5.0	-1.0	92.1	3.9	-
17α-Estradiol	10^{-13}	1.4	2.1	1.3	-2.4	96.5	5.4	-
	10^{-12}	0.6	1.8	1.8	-2.0	98.4	6.1	-
	10^{-11}	3.3	2.0	2.9	-1.4	95.7	5.2	-
	10^{-10}	4.0	3.9	3.6	-1.9	93.1	5.8	-
	10^{-9}	22.1	8.8	5.5	1.2	92.9	6.1	-
	10^{-8}	101.5	17.2	6.7	2.8	98.9	4.0	-
	10^{-7}	133.0	33.9	5.2	2.5	91.2	6.6	-
	10^{-6}	109.5	27.5	5.5	-0.6	85.2	6.9	-
Corticosterone	10^{-11}	0.6	1.6	1.3	-2.1	93.0	3.7	-
	10^{-10}	0.5	1.7	1.6	-1.9	95.9	5.4	-
	10^{-9}	1.7	1.5	1.9	-2.1	93.7	3.9	-
	10^{-8}	0.4	1.9	1.2	-2.6	91.7	3.7	-
	10^{-7}	2.2	1.3	3.3	-0.8	89.6	6.2	-
	10^{-6}	1.3	1.0	3.3	0.8	88.5	6.3	-
	10^{-5}	-1.6	0.8	0.1	-1.2	80.8	4.2	-
	10^{-4}	*	*	-2.6	-3.3	78.5	4.1	-
17α-Methyltestosterone	10^{-12}	0.7	1.6	1.9	-1.5	95.8	4.0	-
	10^{-11}	1.4	2.1	2.7	-1.8	96.4	5.3	-
	10^{-10}	1.9	1.7	1.1	-2.3	93.1	4.0	-
	10^{-9}	1.5	1.3	2.8	-1.6	91.9	3.6	-
	10^{-8}	4.4	2.1	5.6	-0.1	93.9	4.6	-
	10^{-7}	4.9	0.9	5.3	0.7	94.3	3.1	-
	10^{-6}	7.2	2.3	5.0	0.4	90.5	4.8	-
	10^{-5}	35.4	9.7	1.7	-1.9	83.3	3.6	-

RTA = Relative Transcriptional Activation
PC = Positive Control (1 nM 17β-Estradiol)
VC = Vehicle Control
SD = Standard Deviation
+ = Precipitation observed
- = No precipitation observed
* = Cytotoxixity observed so data not assessed
Red lettering = Viability < 80% and considered cytotoxic

TABLE 5 Results of 2nd Valid Transcriptional Activation Assay – Test Substances

Chemical	Concentration (M)	RTA (% of PC)		RTA with ICI (% of PC)		Cell Viability (% of VC)		Precipitation
		Mean	SD	Value 1	Value 2	Mean	SD	
Oxybenzone	10^{-12}	1.0	1.0	0.9	-2.5	92.3	6.2	-
	10^{-11}	0.4	1.8	1.8	-1.9	91.0	4.2	-
	10^{-10}	2.7	1.7	2.5	-1.4	90.2	5.3	-
	10^{-9}	0.5	1.7	1.4	-2.4	88.1	4.5	-
	10^{-8}	3.7	1.8	4.2	1.0	88.5	7.6	-
	10^{-7}	4.0	1.3	5.1	2.5	89.8	5.8	-
	10^{-6}	4.0	1.8	3.4	1.4	85.4	6.8	-
	10^{-5}	20.9	7.5	14.3	6.0	84.9	4.9	-
Octyl-methoxycinnamate	10^{-12}	0.2	1.0	1.1	-1.8	96.4	6.7	-
	10^{-11}	1.3	2.3	0.6	-1.9	96.0	6.9	-
	10^{-10}	1.3	1.6	2.0	-1.8	95.2	4.6	-
	10^{-9}	0.6	1.5	2.6	-1.5	92.3	6.0	-
	10^{-8}	3.2	1.0	4.7	-0.1	92.0	6.7	-
	10^{-7}	2.7	0.6	2.6	1.6	92.9	5.4	-
	10^{-6}	2.1	1.0	3.6	1.0	91.3	5.5	-
	10^{-5}	1.9	2.3	2.4	-1.7	87.9	6.6	-
Octylsalate	10^{-12}	0.4	1.0	1.3	-2.2	96.7	5.5	-
	10^{-11}	1.0	1.2	2.4	-1.8	96.2	5.8	-
	10^{-10}	1.7	1.5	2.1	-2.3	95.6	4.4	-
	10^{-9}	1.1	1.7	2.7	-2.5	90.9	5.0	-
	10^{-8}	4.5	1.1	6.6	-0.7	91.2	6.8	-
	10^{-7}	4.2	1.2	5.9	1.7	92.8	6.4	-
	10^{-6}	3.0	2.4	5.7	1.1	88.2	5.9	-
	10^{-5}	4.6	3.6	5.2	-1.0	87.3	2.6	-
Octocrylene	10^{-12}	1.4	1.3	2.1	-1.7	96.7	1.4	-
	10^{-11}	0.8	1.7	0.7	-2.3	96.1	6.8	-
	10^{-10}	2.1	1.8	2.2	-2.4	95.7	6.6	-
	10^{-9}	0.9	1.6	1.8	-2.3	93.0	5.8	-
	10^{-8}	3.9	2.0	3.4	-0.2	92.8	6.0	-
	10^{-7}	4.6	0.8	4.7	1.1	95.0	7.1	-
	10^{-6}	2.2	0.8	3.0	0.7	91.8	3.9	-
	10^{-5}	1.3	2.0	2.1	-1.5	85.9	5.1	-

RTA = Relative Transcriptional Activation
PC = Positive Control (1 nM 17β-Estradiol)
VC = Vehicle Control
SD = Standard Deviation
+ = Precipitation observed
- = No precipitation observed
* = Cytotoxixity observed so data not assessed
Red lettering = Viability < 80% and considered cytotoxic

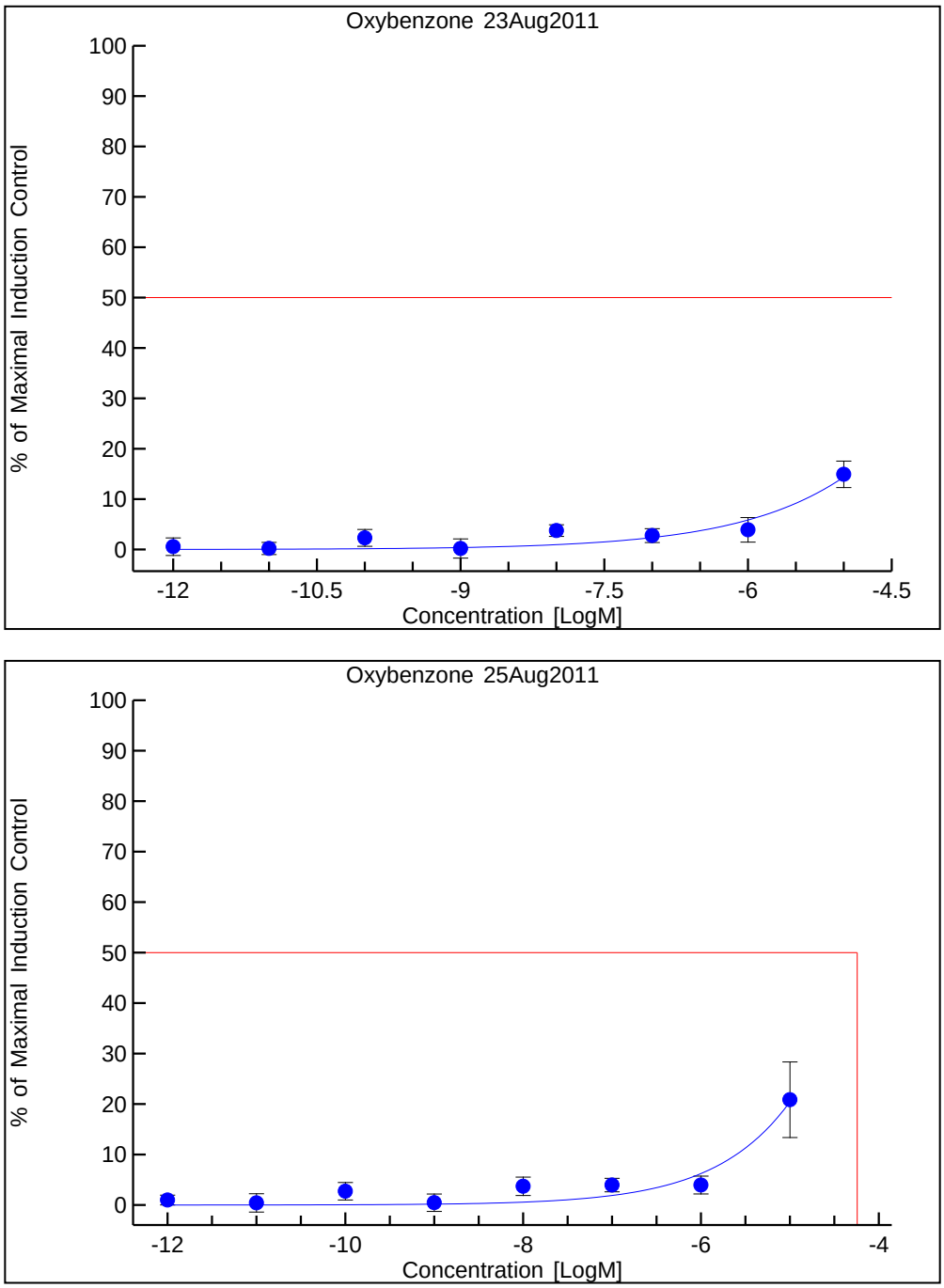
TABLE 6 $LogPC_{50}$, $LogPC_{10}$, $LogEC_{50}$ and Hill Slope Values for the Reference Chemicals

Name	$LogPC_{50}$		$LogPC_{10}$		$LogEC_{50}$		Hill Slope		PC-Induced Fold Induction	
	1st Valid Assay	2nd Valid Assay	1st Valid Assay	2nd Valid Assay	1st Valid Assay	2nd Valid Assay	1st Valid Assay	2nd Valid Assay	1st Valid Assay	2nd Valid Assay
17β-Estradiol	-10.6	-10.6	-11.5	-11.6	-10.5	-10.4	1.4	1.2	17.4	12.9
17α-Estradiol	-8.7	-8.6	-9.7	-9.7	-8.7	-8.6	1.6	1.4	20.4	13.9
Corticosterone	-	-	-	-	-	-	-	-	18.2	15.3
17α-Methyltestosterone	-	-	-	-5.9	-	-	-	-	20.9	14.0

PC = Positive Control (1 nM 17β-Estradiol)

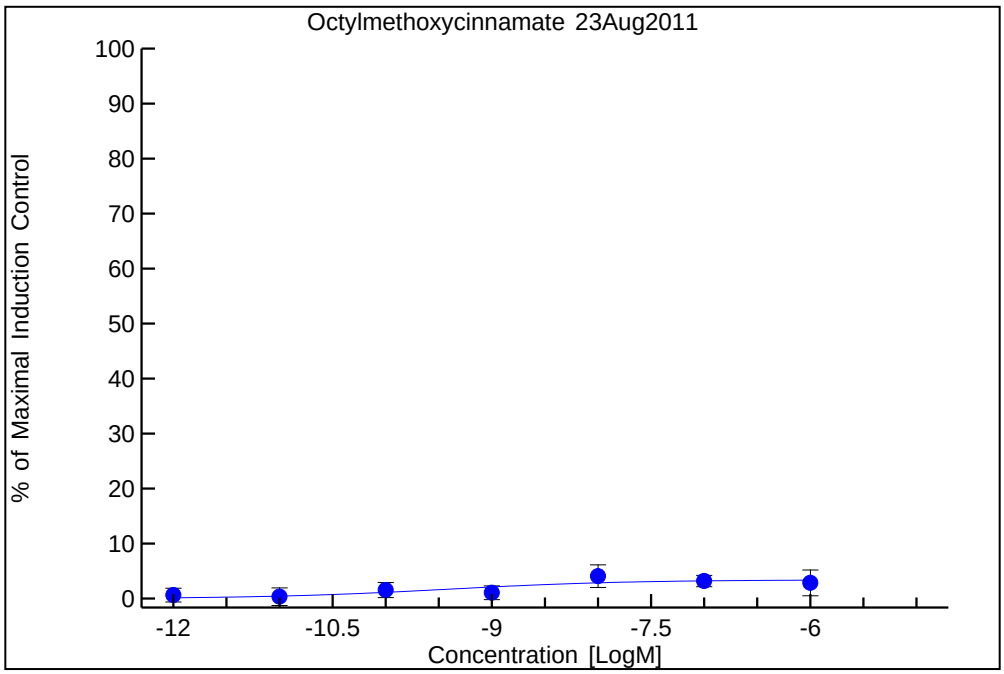
FIGURES SECTION

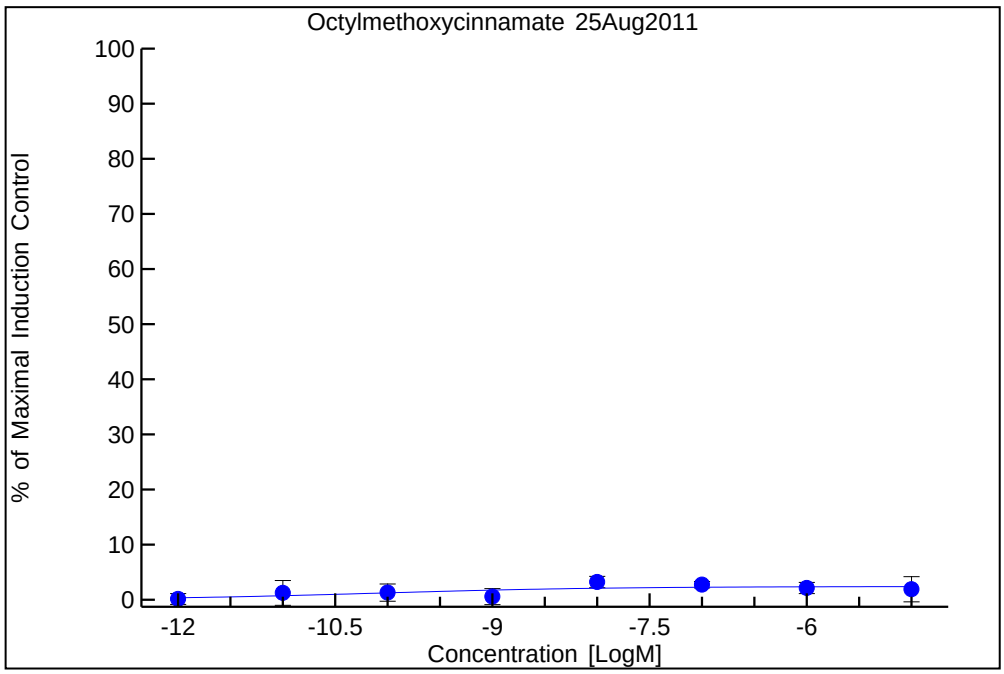
FIGURE 1 Oxybenzone – Relative Transcriptional Activation



The two separate graphs represent the data (Means±Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

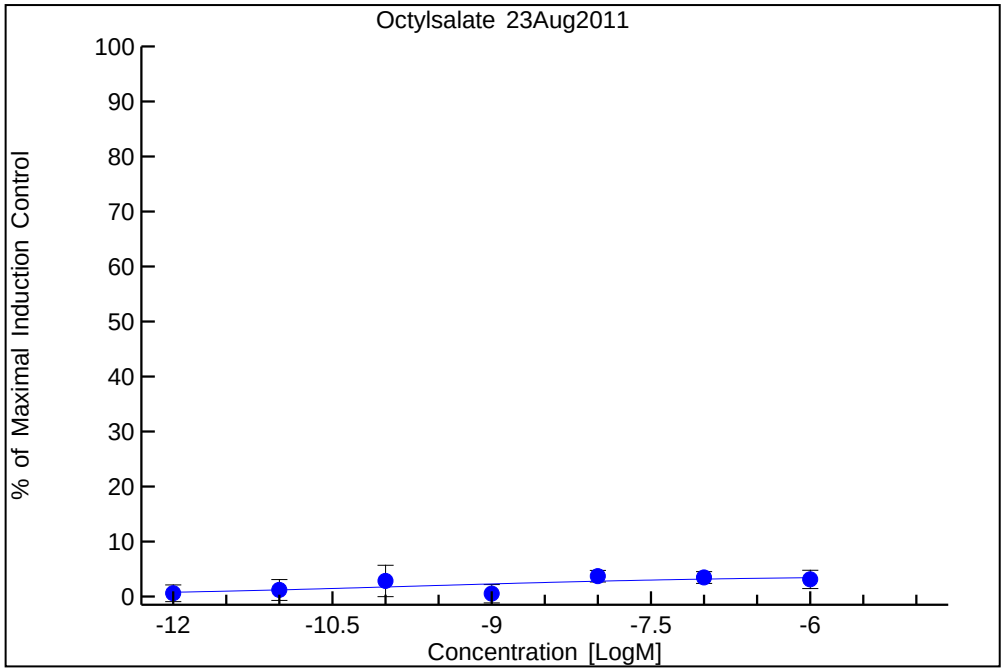
FIGURE 2 Octylmethoxycinnamate – Relative Transcriptional Activation

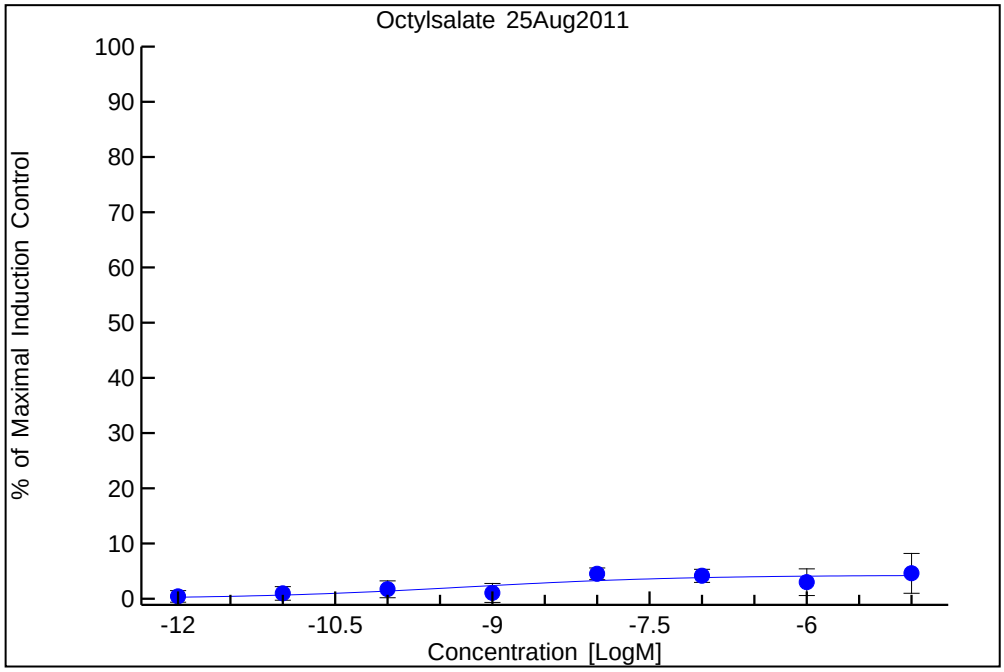




The two separate graphs represent the data (Means±Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

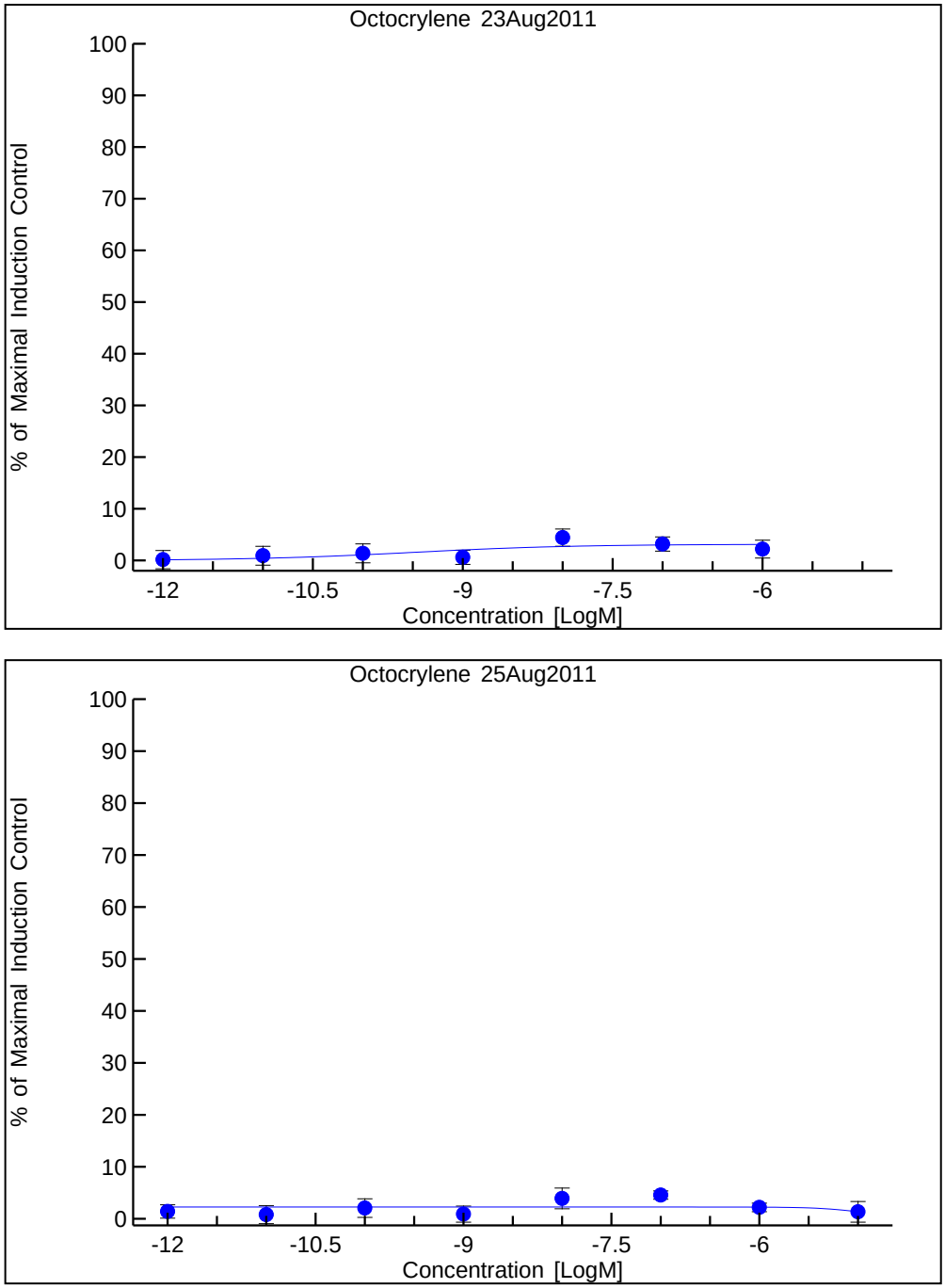
FIGURE 3 Octylsalate – Relative Transcriptional Activation





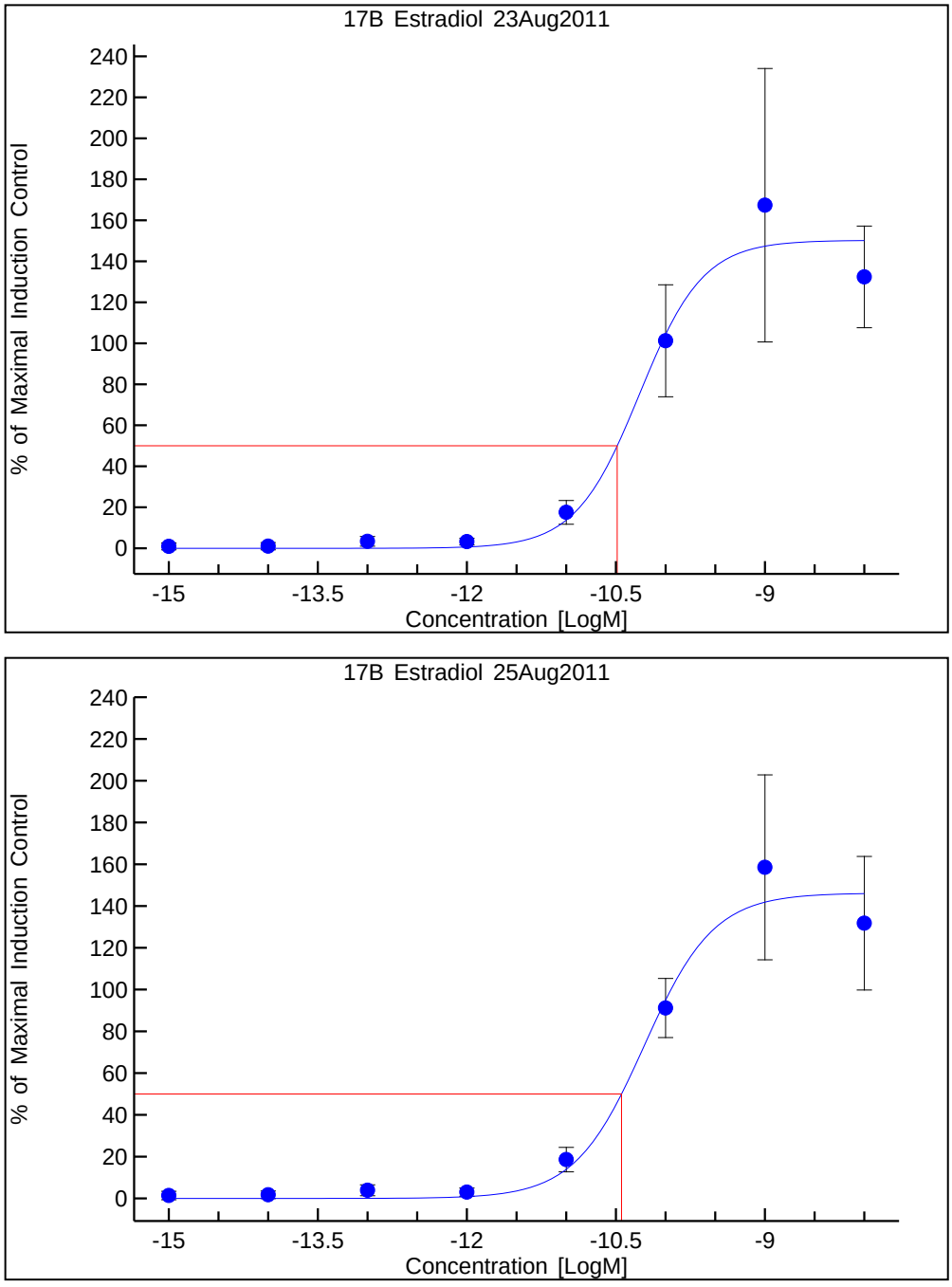
The two separate graphs represent the data (Means±Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

FIGURE 4 Octocrylene – Relative Transcriptional Activation



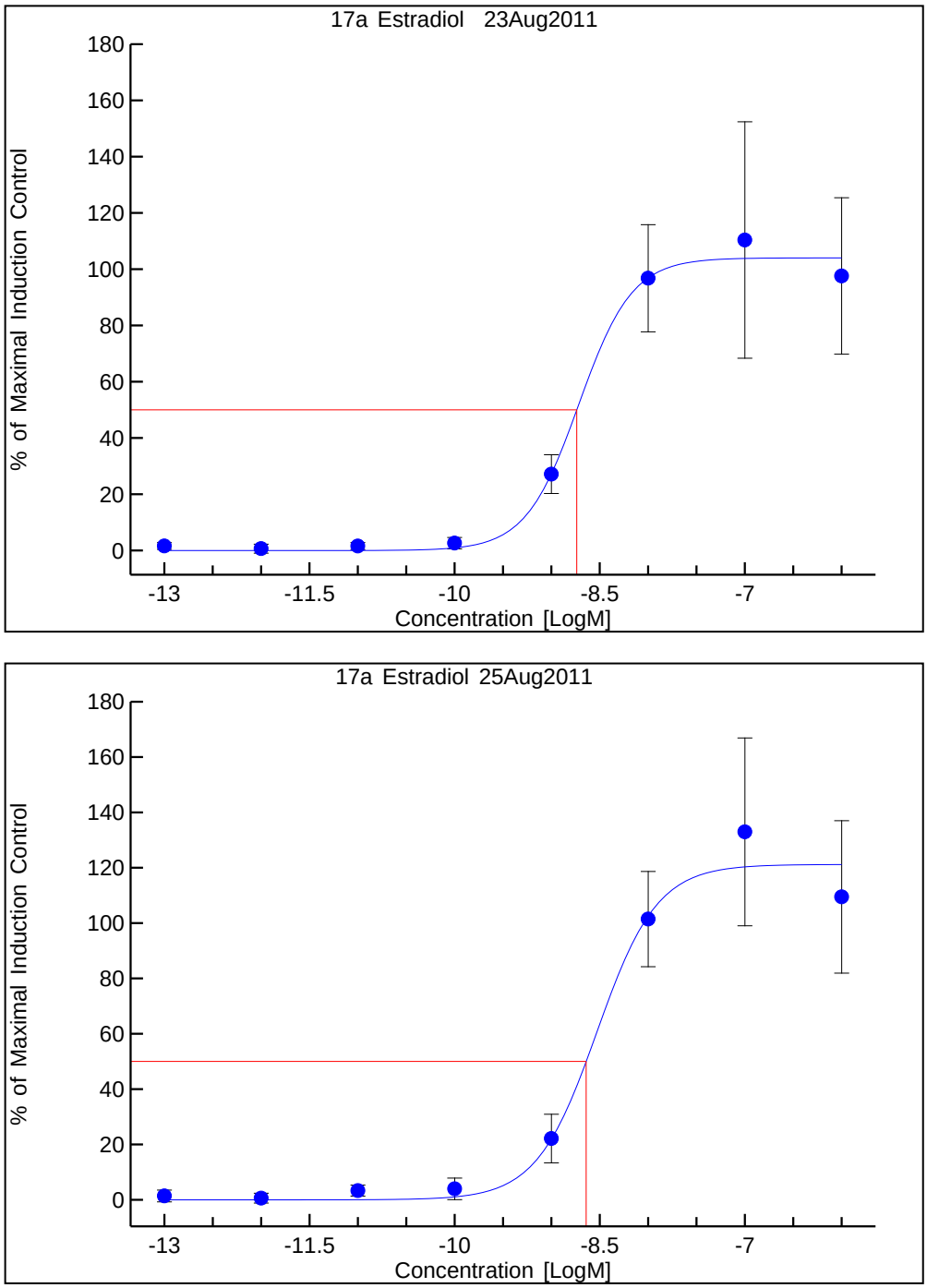
The two separate graphs represent the data (Means±Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

FIGURE 5 17β-Estradiol – Relative Transcriptional Activation



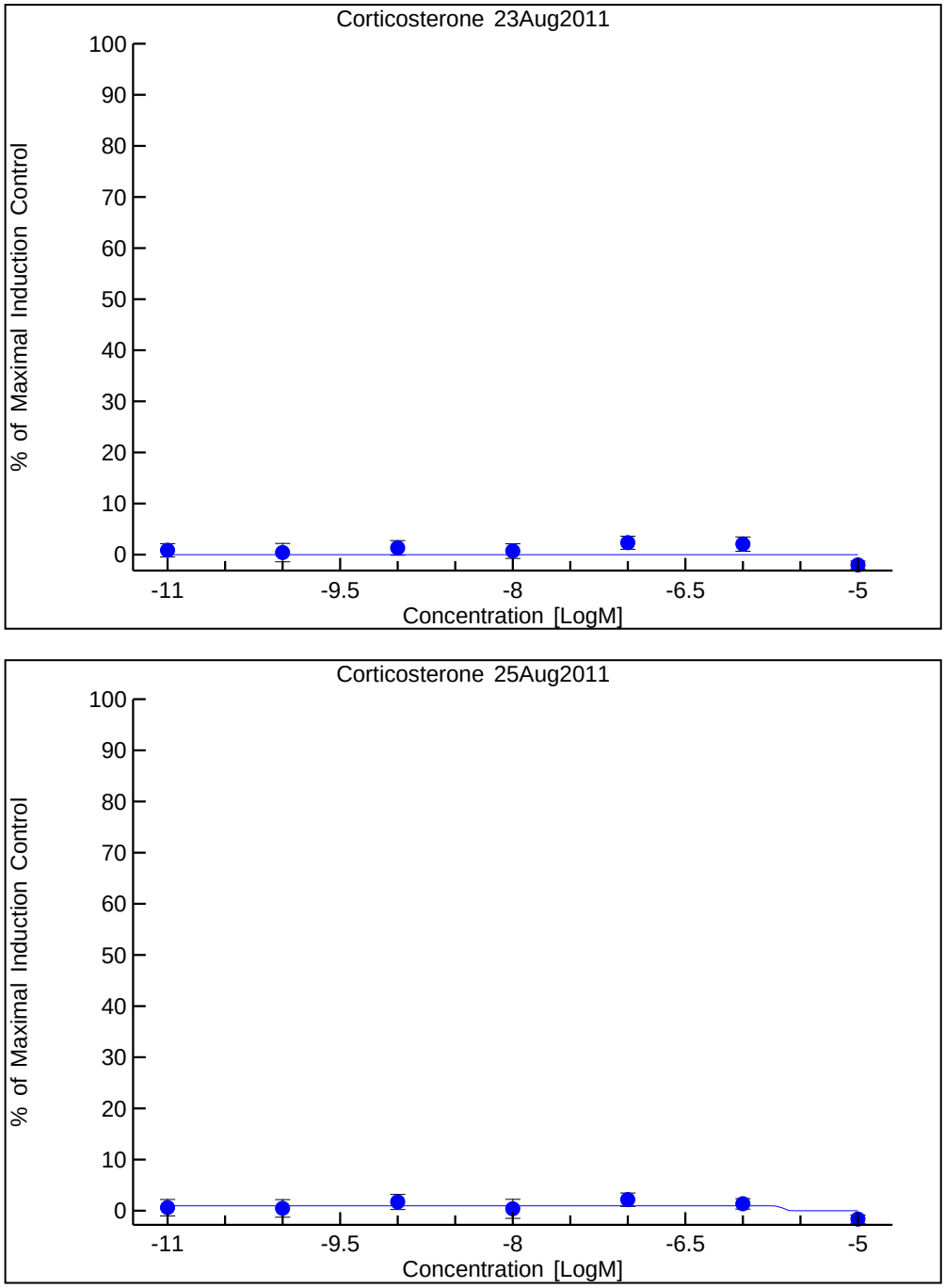
The two separate graphs represent the data (Means±Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

FIGURE 6 17α-Estradiol – Relative Transcriptional Activation



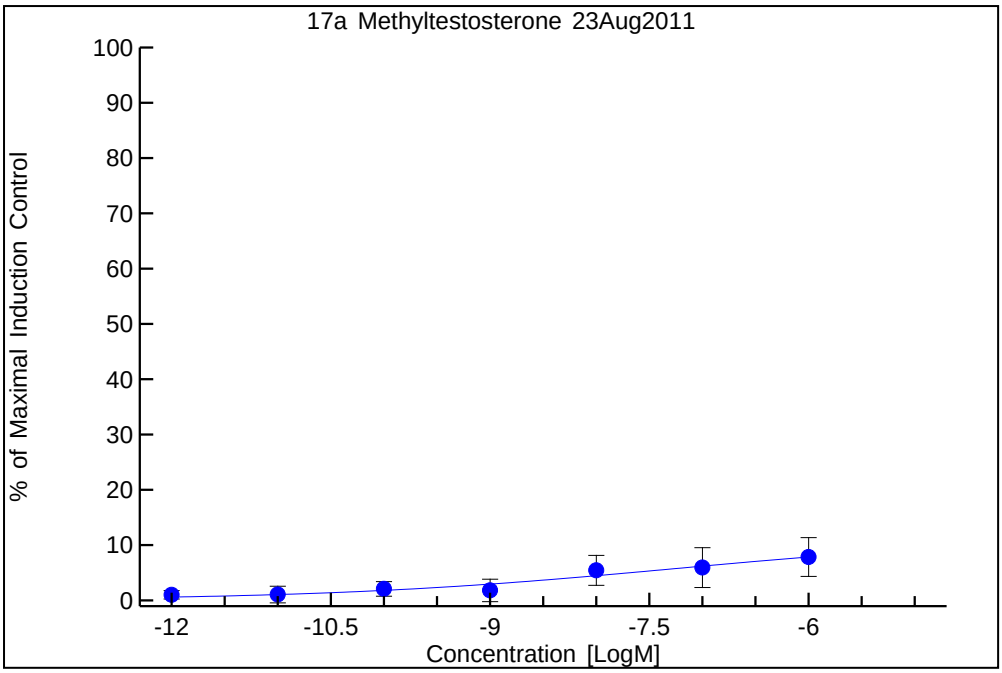
The two separate graphs represent the data (Means±Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

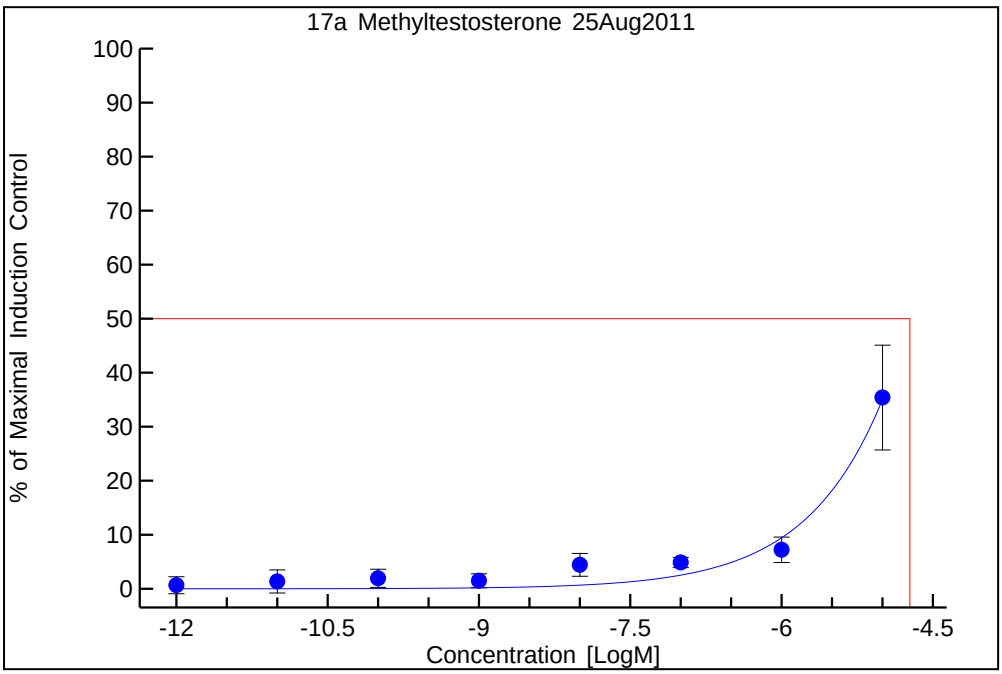
FIGURE 7 Corticosterone – Relative Transcriptional Activation



The two separate graphs represent the data (Means±Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

FIGURE 8 17α-Methyltestosterone – Relative Transcriptional Activation





The two separate graphs represent the data (Means±Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

APPENDICES SECTION

APPENDIX 1 Raw and Normalized Luminescence Data

VALID RUN 1
August 23, 2011

					Concentration [LogM]							
Oxybenzone (Raw Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	157500	8150	6250	8500	8450	22150	6650	14950	13350	11400	40450
B	0	173150	12350	10150	10300	9900	12950	8950	17400	14000	16500	39500
C	50	212150	12200	13350	13300	11800	14500	13000	20550	16750	24150	37550
D	50	199000	16000	12600	18200	13400	15100	17100	18450	18200	18000	45150
E	0	198150	9550	10600	13450	13950	15150	12900	20250	20100	21750	45300
F	0	273900	14000	12750	11700	13900	15600	12650	20400	18250	21950	31950
G	50	11650	10000	10100	12250	10300	11300	10150	15850	15100	17000	26100
H	0	11700	8250	8350	7100	9100	10250	8550	12200	14950	11850	17250

Mean Vehicle Control (VC): 11496

Mean ICI 182,780 Control: 9175

					Concentration [LogM]							
Oxybenzone (Normalized Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	146004	-3346	-5246	-2996	-3046	10654	-4846	3454	1854	-96	28954
B	0	161654	854	-1346	-1196	-1596	1454	-2546	5904	2504	5004	28004
C	50	200654	704	1854	1804	304	3004	1504	9054	5254	12654	26054
D	50	187504	4504	1104	6704	1904	3604	5604	6954	6704	6504	33654
E	0	186654	-1946	-896	1954	2454	3654	1404	8754	8604	10254	33804
F	0	262404	2504	1254	204	2404	4104	1154	8904	6754	10454	20454
G	50	2475	825	925	3075	1125	2125	975	6675	5925	7825	16925
H	0	2525	-925	-825	-2075	-75	1075	-625	3025	5775	2675	8075

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
August 25, 2011

					Concentration [LogM]							
Oxybenzone (Raw Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	135300	8950	8900	10250	7150	10650	6700	11100	16550	12650	22400
B	0	160000	12300	7400	11650	10850	17400	12700	17400	14850	16950	38950
C	0	152450	12450	10250	14350	9700	12800	10950	19200	17250	17650	41800
D	50	171850	12450	11300	13600	14350	16250	13450	17300	15700	20450	46600
E	0	175350	11850	10000	11750	13950	16650	13850	16450	15750	15000	55450
F	0	178150	12800	10750	12050	12550	15800	11250	17000	20550	18000	49000
G	0	11750	11200	9300	9900	11300	12400	10650	14950	16250	13800	30200
H	50	8100	8000	5900	4800	5750	6550	4900	10100	12400	10650	17650

Mean Vehicle Control (VC): 10783

Mean ICI Control: 182,780 8600

					Concentration [LogM]							
Oxybenzone (Normalized Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	124517	-1833	-1883	-533	-3633	-133	-4083	317	5767	1867	11617
B	0	149217	1517	-3383	867	67	6617	1917	6617	4067	6167	28167
C	0	141667	1667	-533	3567	-1083	2017	167	8417	6467	6867	31017
D	50	161067	1667	517	2817	3567	5467	2667	6517	4917	9667	35817
E	0	164567	1067	-783	967	3167	5867	3067	5667	4967	4217	44667
F	0	167367	2017	-33	1267	1767	5017	467	6217	9767	7217	38217
G	0	3150	2600	700	1300	2700	3800	2050	6350	7650	5200	21600
H	50	-500	-600	-2700	-3800	-2850	-2050	-3700	1500	3800	2050	9050

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
August 23, 2011

					Concentration [LogM]							
Octylmethoxy-cinnamate (Raw Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	219100	7700	7100	8000	6100	9500	9050	13200	14800	12050	9700
B	50	171950	12950	9550	11400	9650	12500	11450	25350	16300	17150	13000
C	0	219350	12350	12850	11900	13000	13650	12850	20050	14800	14600	15150
D	0	225100	10600	13350	14900	12150	17250	16100	18400	18750	14700	34800
E	0	204650	9600	10800	14150	14600	14500	14300	18400	19350	14600	16100
F	0	189850	12550	10200	11900	13350	15500	13450	16900	18050	25050	16900
G	0	10000	12750	8750	11850	12200	12850	12900	15850	15200	13350	11150
H	0	10750	9650	8000	8350	8450	7850	8450	13150	13500	13950	9250

Mean Vehicle Control (VC): 10800

Mean ICI 182,780 Control: 9788

					Concentration [LogM]							
Octylmethoxy-cinnamate (Normalized Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	208300	-3100	-3700	-2800	-4700	-1300	-1750	2400	4000	1250	-1100
B	50	161150	2150	-1250	600	-1150	1700	650	14550	5500	6350	2200
C	0	208550	1550	2050	1100	2200	2850	2050	9250	4000	3800	4350
D	0	214300	-200	2550	4100	1350	6450	5300	7600	7950	3900	24000
E	0	193850	-1200	0	3350	3800	3700	3500	7600	8550	3800	5300
F	0	179050	1750	-600	1100	2550	4700	2650	6100	7250	14250	6100
G	0	213	2963	-1038	2063	2413	3063	3113	6063	5413	3563	1363
H	0	963	-138	-1788	-1438	-1338	-1938	-1338	3363	3713	4163	-538

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
August 25, 2011

					Concentration [LogM]							
Octylmethoxy-cinnamate (Raw Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	131050	9000	6600	8050	7150	10550	8600	14150	16550	12500	7250
B	0	189000	13100	10150	12100	11700	13500	9350	15600	14900	13650	14850
C	100	217350	13000	12450	11700	14500	12800	13700	17850	17300	14700	14050
D	0	235600	11900	12450	13000	19300	15150	14300	16750	17000	17450	18300
E	100	201100	9450	13850	11850	13250	18100	14350	18350	14950	16400	14950
F	100	158450	11350	11350	12250	14750	11200	13200	19000	15850	15450	18350
G	50	13200	12800	9650	11300	10300	12950	14000	17650	13950	15700	13500
H	50	7850	8200	6600	6100	6000	6200	6650	9100	12200	11150	6350

Mean Vehicle Control (VC): 11221

Mean ICI 182,780 Control: 9313

					Concentration [LogM]							
Octylmethoxy-cinnamate (Normalized Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	119829	-2221	-4621	-3171	-4071	-671	-2621	2929	5329	1279	-3971
B	0	177779	1879	-1071	879	479	2279	-1871	4379	3679	2429	3629
C	100	206129	1779	1229	479	3279	1579	2479	6629	6079	3479	2829
D	0	224379	679	1229	1779	8079	3929	3079	5529	5779	6229	7079
E	100	189879	-1771	2629	629	2029	6879	3129	7129	3729	5179	3729
F	100	147229	129	129	1029	3529	-21	1979	7779	4629	4229	7129
G	50	3888	3488	338	1988	988	3638	4688	8338	4638	6388	4188
H	50	-1463	-1113	-2713	-3213	-3313	-3113	-2663	-213	2888	1838	-2963

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
August 23, 2011

					Concentration [LogM]							
Octylsalate (Raw Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	164200	7100	7900	9050	7850	9800	7050	19200	14950	13850	18300
B	0	232600	11100	9200	11250	12650	14200	11850	16950	18050	16700	20850
C	50	221000	15550	11850	10600	15600	16050	11800	16700	18850	16250	23300
D	50	223100	13400	15750	15550	13450	17800	15250	21850	19700	21900	27950
E	0	197400	14100	11050	15850	17650	26600	15650	19950	20550	22400	19400
F	0	245700	11450	13200	15850	18250	20850	15600	21150	20800	17700	20100
G	0	13250	10000	10850	12850	14000	13600	14000	18600	18200	19450	16000
H	0	10950	7600	9150	9000	8400	9700	8750	14250	15300	13300	10850

Mean Vehicle Control (VC): 11804

Mean ICI 182,780 Control: 9400

					Concentration [LogM]							
Octylsalate (Normalized Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	152396	-4704	-3904	-2754	-3954	-2004	-4754	7396	3146	2046	6496
B	0	220796	-704	-2604	-554	846	2396	46	5146	6246	4896	9046
C	50	209196	3746	46	-1204	3796	4246	-4	4896	7046	4446	11496
D	50	211296	1596	3946	3746	1646	5996	3446	10046	7896	10096	16146
E	0	185596	2296	-754	4046	5846	14796	3846	8146	8746	10596	7596
F	0	233896	-354	1396	4046	6446	9046	3796	9346	8996	5896	8296
G	0	3850	600	1450	3450	4600	4200	4600	9200	8800	10050	6600
H	0	1550	-1800	-250	-400	-1000	300	-650	4850	5900	3900	1450

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
August 25, 2011

					Concentration [LogM]							
Octylsalate (Raw Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	167600	8750	8450	10150	10000	10650	7800	15450	15850	10600	7950
B	0	138650	13650	10350	9350	10950	11350	11400	15700	14300	13500	23400
C	0	149300	9600	8900	11300	11150	11900	12500	17450	16650	14500	19400
D	0	180000	10500	13100	12450	14850	15650	13700	19200	16650	17700	18450
E	0	147500	14400	8550	11600	12950	15150	14750	17450	18400	13900	16000
F	0	153200	12450	10700	13550	13200	14950	13750	18800	19050	20600	19650
G	50	13050	10800	10200	10100	11650	11200	12150	17850	16750	16450	15800
H	50	7500	7000	4850	5050	5550	4800	4550	7150	10700	9800	6800

Mean Vehicle Control (VC): 10783

Mean ICI 182,780 Control: 8213

					Concentration [LogM]							
Octylsalate (Normalized Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	156817	-2033	-2333	-633	-783	-133	-2983	4667	5067	-183	-2833
B	0	127867	2867	-433	-1433	167	567	617	4917	3517	2717	12617
C	0	138517	-1183	-1883	517	367	1117	1717	6667	5867	3717	8617
D	0	169217	-283	2317	1667	4067	4867	2917	8417	5867	6917	7667
E	0	136717	3617	-2233	817	2167	4367	3967	6667	7617	3117	5217
F	0	142417	1667	-83	2767	2417	4167	2967	8017	8267	9817	8867
G	50	4838	2588	1988	1888	3438	2988	3938	9638	8538	8238	7588
H	50	-713	-1213	-3363	-3163	-2663	-3413	-3663	-1063	2488	1588	-1413

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
August 23, 2011

					Concentration [LogM]							
Octocrylene (Raw Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	50	124950	7350	6500	6450	9000	7750	7900	18850	11900	9850	9900
B	0	182700	10850	10450	8850	9850	11100	10350	17650	16650	13250	16150
C	50	195900	10000	9400	9450	9050	13300	11800	15100	16000	13050	12450
D	100	233700	12050	13900	15150	15150	14900	14400	18150	17500	18400	15600
E	50	258400	12700	11550	12450	15850	14550	14550	18300	17250	16750	13550
F	0	166450	11100	11850	13300	15050	17400	11600	24350	19500	16700	12850
G	0	10750	10700	11500	10350	13500	13100	13100	15850	14450	14150	10650
H	50	8550	7800	7050	8900	8400	8150	7150	10850	14050	9950	7500

Mean Vehicle Control (VC): 10642

Mean ICI Control: 182,780 9263

					Concentration [LogM]							
Octocrylene (Normalized Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	50	114308	-3292	-4142	-4192	-1642	-2892	-2742	8208	1258	-792	-742
B	0	172058	208	-192	-1792	-792	458	-292	7008	6008	2608	5508
C	50	185258	-642	-1242	-1192	-1592	2658	1158	4458	5358	2408	1808
D	100	223058	1408	3258	4508	4508	4258	3758	7508	6858	7758	4958
E	50	247758	2058	908	1808	5208	3908	3908	7658	6608	6108	2908
F	0	155808	458	1208	2658	4408	6758	958	13708	8858	6058	2208
G	0	1488	1438	2238	1088	4238	3838	3838	6588	5188	4888	1388
H	50	-713	-1463	-2213	-363	-863	-1113	-2113	1588	4788	688	-1763

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
August 25, 2011

					Concentration [LogM]							
Octocrylene (Raw Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	111650	9900	9850	13100	8050	10350	7950	13000	18000	13850	8800
B	0	172150	11300	9850	12150	10000	12700	12500	17400	17300	13000	18450
C	0	156150	12050	10200	10900	13700	14200	13900	19550	20450	14700	14150
D	0	194900	11250	12450	16750	14550	16950	15150	20800	17150	14500	13350
E	0	144500	10450	11750	14850	14400	18300	13600	19850	19400	15450	13800
F	50	249250	14400	11000	13050	14200	14550	12800	14350	18850	16800	11600
G	50	10700	10500	10700	11150	8900	11400	10700	13250	15350	12700	11250
H	0	7150	5700	4400	5050	4200	3900	4200	7450	9550	8950	5400

Mean Vehicle Control (VC): 11204

Mean ICI Control: 182,780 7825

					Concentration [LogM]							
Octocrylene (Normalized Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	100446	-1304	-1354	1896	-3154	-854	-3254	1796	6796	2646	-2404
B	0	160946	96	-1354	946	-1204	1496	1296	6196	6096	1796	7246
C	0	144946	846	-1004	-304	2496	2996	2696	8346	9246	3496	2946
D	0	183696	46	1246	5546	3346	5746	3946	9596	5946	3296	2146
E	0	133296	-754	546	3646	3196	7096	2396	8646	8196	4246	2596
F	50	238046	3196	-204	1846	2996	3346	1596	3146	7646	5596	396
G	50	2875	2675	2875	3325	1075	3575	2875	5425	7525	4875	3425
H	0	-675	-2125	-3425	-2775	-3625	-3925	-3625	-375	1725	1125	-2425

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
August 23, 2011

					Concentration [LogM]							
17β-Estradiol (Raw Data)	blank	1 nM E2	VC	VC	-15.0	-14.0	-13.0	-12.0	-11.0	-10.0	-9.0	-8.0
A	50	181200	8600	7950	8150	9800	12200	13000	33500	223150	195250	213450
B	0	196200	13450	8800	14650	11500	15500	19100	39050	143600	285150	273100
C	100	224800	14850	13300	14250	15250	22000	21050	53550	246050	360600	291350
D	0	288150	16000	14950	17450	19800	20950	20600	60850	196250	535600	333600
E	100	190500	14800	13550	17300	17100	24700	22500	63600	217850	273400	263300
F	50	246400	13450	13100	16500	15900	23600	20900	45300	315950	520050	357950
G	0	13000	12350	12250	12050	15000	15200	13400	22650	21300	19850	22050
H	0	7950	4450	5600	5100	6000	6400	6500	11000	14950	15100	8400

Mean Vehicle Control (VC): 12733

Mean ICI 182,780 Control: 8663

					Concentration [LogM]							
17β-Estradiol (Normalized Data)	blank	1 nM E2	VC	VC	-15.0	-14.0	-13.0	-12.0	-11.0	-10.0	-9.0	-8.0
A	50	168467	-4133	-4783	-4583	-2933	-533	267	20767	210417	182517	200717
B	0	183467	717	-3933	1917	-1233	2767	6367	26317	130867	272417	260367
C	100	212067	2117	567	1517	2517	9267	8317	40817	233317	347867	278617
D	0	275417	3267	2217	4717	7067	8217	7867	48117	183517	522867	320867
E	100	177767	2067	817	4567	4367	11967	9767	50867	205117	260667	250567
F	50	233667	717	367	3767	3167	10867	8167	32567	303217	507317	345217
G	0	4338	3688	3588	3388	6338	6538	4738	13988	12638	11188	13388
H	0	-713	-4213	-3063	-3563	-2663	-2263	-2163	2338	6288	6438	-263

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
August 25, 2011

					Concentration [LogM]							
17β-Estradiol (Raw Data)	blank	1 nM E2	VC	VC	-15.0	-14.0	-13.0	-12.0	-11.0	-10.0	-9.0	-8.0
A	50	225200	15750	12250	13900	16250	17750	19100	39700	233800	254950	211800
B	0	223650	19850	22550	19900	18550	23750	21900	50750	178400	362250	342700
C	50	204800	20050	19200	20750	23050	31300	24950	64000	255150	336800	423900
D	0	287350	19250	18050	26100	26000	31000	27250	62550	212150	400800	288350
E	0	284200	19400	18300	25200	25700	32750	31550	77500	248150	314350	306500
F	0	209850	20250	17000	23550	24400	26150	26250	62700	190700	541200	282900
G	0	15100	15950	11700	15050	14550	18350	17100	25900	24600	24350	21300
H	0	8350	6650	6500	7000	6800	7800	7150	10200	16000	13400	7900

Mean Vehicle Control (VC): 18492

Mean ICI 182,780 Control: 10200

					Concentration [LogM]							
17β-Estradiol (Normalized Data)	blank	1 nM E2	VC	VC	-15.0	-14.0	-13.0	-12.0	-11.0	-10.0	-9.0	-8.0
A	50	206708	-2742	-6242	-4592	-2242	-742	608	21208	215308	236458	193308
B	0	205158	1358	4058	1408	58	5258	3408	32258	159908	343758	324208
C	50	186308	1558	708	2258	4558	12808	6458	45508	236658	318308	405408
D	0	268858	758	-442	7608	7508	12508	8758	44058	193658	382308	269858
E	0	265708	908	-192	6708	7208	14258	13058	59008	229658	295858	288008
F	0	191358	1758	-1492	5058	5908	7658	7758	44208	172208	522708	264408
G	0	4900	5750	1500	4850	4350	8150	6900	15700	14400	14150	11100
H	0	-1850	-3550	-3700	-3200	-3400	-2400	-3050	0	5800	3200	-2300

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
August 23, 2011

					Concentration [LogM]							
17α-Estradiol (Raw Data)	blank	1 nM E2	VC	VC	-13.0	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0
A	100	267150	8600	8400	14550	6400	9550	9900	54750	166200	146450	143200
B	0	155000	9900	10450	10900	11050	12450	12450	93300	169450	195100	313100
C	0	254550	13100	9900	11800	11000	14850	19600	63450	232200	192100	187700
D	0	220100	10700	14200	16550	14750	14800	20100	63300	237300	295250	200600
E	100	244150	10750	10250	13450	14800	16150	15900	55550	196900	218950	235600
F	50	163550	9800	11950	16950	14300	16050	18700	70650	262750	385600	194700
G	50	11550	12950	9700	10600	14000	12550	12300	16850	14150	17000	15750
H	0	11250	10250	9950	9950	7550	9350	7200	13900	13550	12150	11050

Mean Vehicle Control (VC): 10667

Mean ICI 182,780 Control: 10713

					Concentration [LogM]							
17α-Estradiol (Normalized Data)	blank	1 nM E2	VC	VC	-13.0	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0
A	100	256483	-2067	-2267	3883	-4267	-1117	-767	44083	155533	135783	132533
B	0	144333	-767	-217	233	383	1783	1783	82633	158783	184433	302433
C	0	243883	2433	-767	1133	333	4183	8933	52783	221533	181433	177033
D	0	209433	33	3533	5883	4083	4133	9433	52633	226633	284583	189933
E	100	233483	83	-417	2783	4133	5483	5233	44883	186233	208283	224933
F	50	152883	-867	1283	6283	3633	5383	8033	59983	252083	374933	184033
G	50	838	2238	-1013	-113	3288	1838	1588	6138	3438	6288	5038
H	0	538	-463	-763	-763	-3163	-1363	-3513	3188	2838	1438	338

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
August 25, 2011

					Concentration [LogM]							
17α-Estradiol (Raw Data)	blank	1 nM E2	VC	VC	-13.0	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0
A	0	147200	11500	12100	11500	9900	13250	14400	33750	176600	194850	152750
B	0	173600	15700	12500	13200	13450	23200	16000	52100	155450	233800	258000
C	50	184350	16550	15400	19850	17350	21450	21700	48950	233500	261800	279500
D	0	254550	13200	14700	18800	18250	22450	22300	71900	205250	262150	172650
E	0	181500	16300	14150	21850	18500	21900	22050	78600	232050	246600	200600
F	50	270350	17150	14550	17450	16300	22150	35150	50700	225100	383250	254600
G	100	15400	12700	10850	11750	12600	14700	15950	19500	21850	18950	19600
H	0	6900	7350	6200	4850	5550	6700	5700	11500	14550	13950	8150

Mean Vehicle Control (VC): 14483

Mean ICI 182,780 Control: 9275

					Concentration [LogM]							
17α-Estradiol (Normalized Data)	blank	1 nM E2	VC	VC	-13.0	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0
A	0	132717	-2983	-2383	-2983	-4583	-1233	-83	19267	162117	180367	138267
B	0	159117	1217	-1983	-1283	-1033	8717	1517	37617	140967	219317	243517
C	50	169867	2067	917	5367	2867	6967	7217	34467	219017	247317	265017
D	0	240067	-1283	217	4317	3767	7967	7817	57417	190767	247667	158167
E	0	167017	1817	-333	7367	4017	7417	7567	64117	217567	232117	186117
F	50	255867	2667	67	2967	1817	7667	20667	36217	210617	368767	240117
G	100	6125	3425	1575	2475	3325	5425	6675	10225	12575	9675	10325
H	0	-2375	-1925	-3075	-4425	-3725	-2575	-3575	2225	5275	4675	-1125

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
August 23, 2011

					Concentration [LogM]							
Corticosterone (Raw data)	blank	1 nM E2	VC	VC	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0	-4.0
A	0	186550	8550	9100	11000	6850	10000	8700	13150	23150	6800	2800
B	0	244950	11600	11800	11850	10850	15100	12500	15950	16150	7050	4650
C	0	244700	14000	12600	15450	14750	15050	15500	20550	17000	7950	9400
D	0	246650	15900	13800	18100	17050	17350	15800	19800	16100	7250	5400
E	0	190300	13100	13900	17000	16100	16400	17500	19200	14450	9200	3800
F	100	269900	14000	13550	13650	15550	19250	15050	17800	16050	11150	4350
G	0	13000	12750	12900	12800	13950	13550	14050	17400	14050	6300	1550
H	0	12250	8850	8750	9750	9050	11050	8300	10300	10650	6050	2850

Mean Vehicle Control (VC): 12658

Mean ICI 182,780 Control: 10813

					Concentration [LogM]							
Corticosterone (Normalized Data)	blank	1 nM E2	VC	VC	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0	-4.0
A	0	173892	-4108	-3558	-1658	-5808	-2658	-3958	492	10492	-5858	-9858
B	0	232292	-1058	-858	-808	-1808	2442	-158	3292	3492	-5608	-8008
C	0	232042	1342	-58	2792	2092	2392	2842	7892	4342	-4708	-3258
D	0	233992	3242	1142	5442	4392	4692	3142	7142	3442	-5408	-7258
E	0	177642	442	1242	4342	3442	3742	4842	6542	1792	-3458	-8858
F	100	257242	1342	892	992	2892	6592	2392	5142	3392	-1508	-8308
G	0	2188	1938	2088	1988	3138	2738	3238	6588	3238	-4513	-9263
H	0	1438	-1963	-2063	-1063	-1763	238	-2513	-513	-163	-4763	-7963

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
August 25, 2011

					Concentration [LogM]							
Corticosterone (Raw Data)	blank	1 nM E2	VC	VC	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0	-4.0
A	50	152250	11450	10100	11150	10100	13350	10300	15800	16450	9050	2850
B	0	300000	14550	11450	13600	12400	16150	12550	18700	14100	12050	7750
C	0	188850	15400	15800	16150	17300	20300	16650	19150	20350	11200	8100
D	100	214450	15400	17800	16350	18000	20750	17100	21500	18200	9750	7300
E	100	205650	22950	12250	20150	18500	21350	21650	23650	18700	13550	11250
F	50	298800	14600	16600	19400	18600	19050	15600	17850	18500	12700	9100
G	50	15150	14150	12500	12400	13000	13650	12250	16700	16600	9800	4150
H	0	8650	7250	4750	5200	5550	5300	4050	8000	11400	7200	2600

Mean Vehicle Control (VC): 14863

Mean ICI 182,780 Control: 9663

					Concentration [LogM]							
Corticosterone (Normalized Data)	blank	1 nM E2	VC	VC	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0	-4.0
A	50	137388	-3413	-4763	-3713	-4763	-1513	-4563	938	1588	-5813	-12013
B	0	285138	-313	-3413	-1263	-2463	1288	-2313	3838	-763	-2813	-7113
C	0	173988	538	938	1288	2438	5438	1788	4288	5488	-3663	-6763
D	100	199588	538	2938	1488	3138	5888	2238	6638	3338	-5113	-7563
E	100	190788	8088	-2613	5288	3638	6488	6788	8788	3838	-1313	-3613
F	50	283938	-263	1738	4538	3738	4188	738	2988	3638	-2163	-5763
G	50	5488	4488	2838	2738	3338	3988	2588	7038	6938	138	-5513
H	0	-1013	-2413	-4913	-4463	-4113	-4363	-5613	-1663	1738	-2463	-7063

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
August 23, 2011

					Concentration [LogM]							
17α-Methyl-Testosterone (Raw Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	179150	10700	7450	12500	9450	14300	11100	38900	21450	25650	80450
B	50	295700	11700	8050	13550	11850	13950	12800	25050	26050	25400	95100
C	0	371650	17850	13250	16150	18100	20200	16900	23950	23300	29750	198000
D	0	287000	13900	15150	15900	17900	20050	18950	20450	24800	32400	80150
E	50	221050	13500	14000	17550	17300	22200	25650	30200	25600	34000	173300
F	0	252400	13650	14250	16600	18350	17900	19000	21400	46400	49650	122150
G	0	12500	13150	13700	12600	17600	16850	16800	19900	22050	20400	10450
H	50	13000	9700	11350	11100	11050	11650	11900	17250	17150	14400	9100

Mean Vehicle Control (VC): 12788

Mean ICI 182,780 Control: 11975

					Concentration [LogM]							
17α-Methyl-Testosterone (Normalized Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	166363	-2088	-5338	-288	-3338	1513	-1688	26113	8663	12863	67663
B	50	282913	-1088	-4738	763	-938	1163	13	12263	13263	12613	82313
C	0	358863	5063	463	3363	5313	7413	4113	11163	10513	16963	185213
D	0	274213	1113	2363	3113	5113	7263	6163	7663	12013	19613	67363
E	50	208263	713	1213	4763	4513	9413	12863	17413	12813	21213	160513
F	0	239613	863	1463	3813	5563	5113	6213	8613	33613	36863	109363
G	0	525	1175	1725	625	5625	4875	4825	7925	10075	8425	-1525
H	50	1025	-2275	-625	-875	-925	-325	-75	5275	5175	2425	-2875

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
August 25, 2011

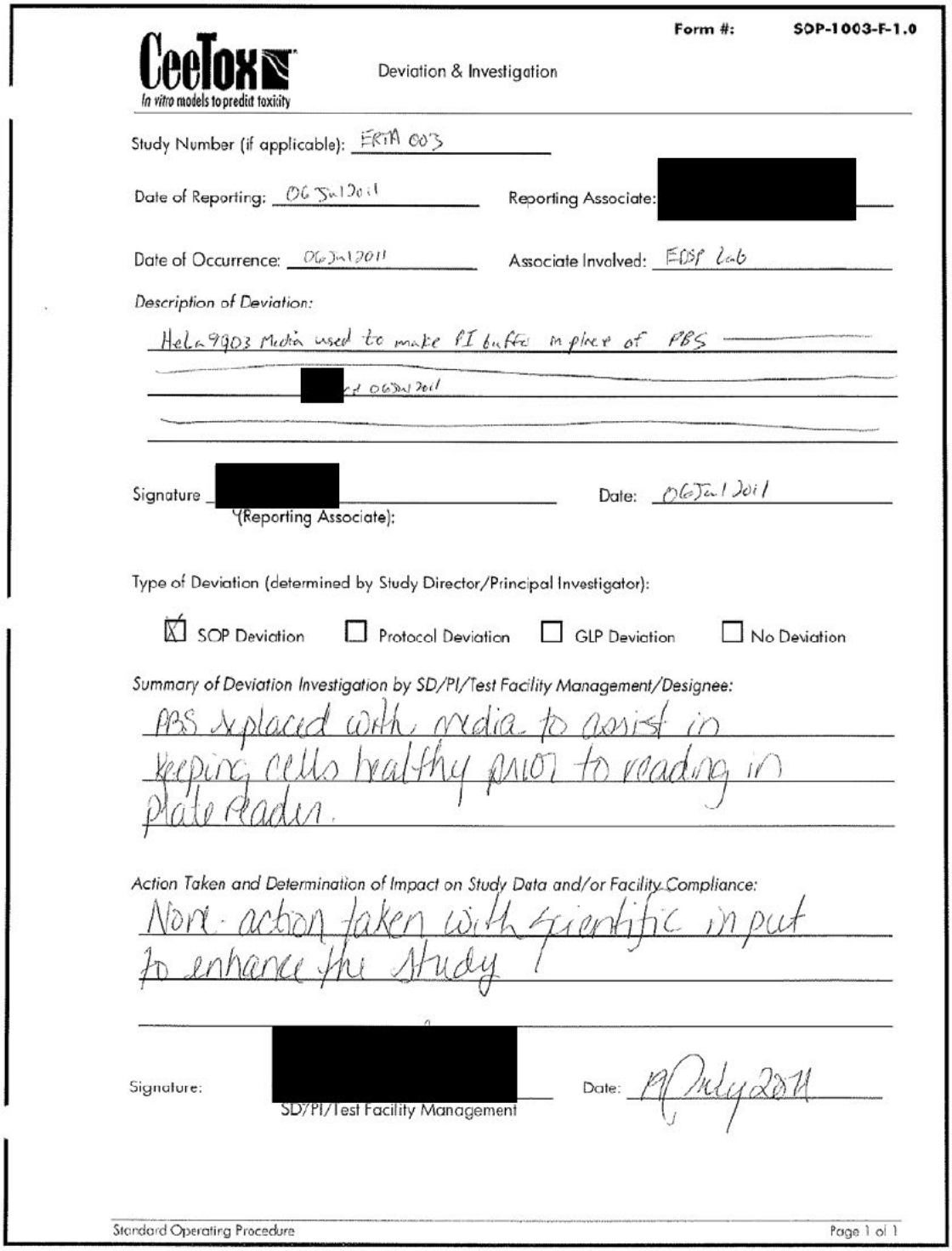
					Concentration [LogM]							
17α-Methyl-Testosterone (Raw Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	151550	10600	9700	11000	11000	14650	13600	16900	22950	22800	54300
B	50	216850	15000	12600	14550	14650	16350	15500	22650	25250	26100	90000
C	0	188850	15000	15200	14950	17050	18600	19400	26350	21950	29250	80500
D	50	242850	15050	19650	18800	18850	22350	19450	27650	22450	35500	111550
E	0	208700	17000	16450	18150	19200	22100	19550	25100	26550	26100	79800
F	0	227900	15550	14250	18200	22850	16300	17650	20250	24850	31250	78550
G	0	13300	10450	11800	11500	12900	10000	13200	18600	17950	17350	11000
H	0	5200	4600	4400	4950	4450	3500	4700	7600	9200	8600	4100

Mean Vehicle Control (VC): 14671

Mean ICI 182,780 Control: 7813

					Concentration [LogM]							
17α-Methyl-Testosterone (Normalized Data)	blank	1 nM E2	VC	VC	-12.0	-11.0	-10.0	-9.0	-8.0	-7.0	-6.0	-5.0
A	0	136879	-4071	-4971	-3671	-3671	-21	-1071	2229	8279	8129	39629
B	50	202179	329	-2071	-121	-21	1679	829	7979	10579	11429	75329
C	0	174179	329	529	279	2379	3929	4729	11679	7279	14579	65829
D	50	228179	379	4979	4129	4179	7679	4779	12979	7779	20829	96879
E	0	194029	2329	1779	3479	4529	7429	4879	10429	11879	11429	65129
F	0	213229	879	-421	3529	8179	1629	2979	5579	10179	16579	63879
G	0	5488	2638	3988	3688	5088	2188	5388	10788	10138	9538	3188
H	0	-2613	-3213	-3413	-2863	-3363	-4313	-3113	-213	1388	788	-3713

APPENDIX 2 Deviation Forms



Form #: SOP-1003-F-1.0

CeeTox
In vitro models to predict toxicity

Deviation & Investigation

Study Number (if applicable): ERTA 003

Date of Reporting: 06 Jan 2011 Reporting Associate: [redacted]

Date of Occurrence: 06Jan2011 Associate Involved: EDSP lab

Description of Deviation:

Hela9903 Media used to make PI buffer in place of PBS

[redacted] 06Jan 2011

Signature [redacted] Date: 06Jan 2011
(Reporting Associate):

Type of Deviation (determined by Study Director/Principal Investigator):

☒ SOP Deviation ☐ Protocol Deviation ☐ GLP Deviation ☐ No Deviation

Summary of Deviation Investigation by SD/PI/Test Facility Management/Designee:

PBS replaced with media to assist in keeping cells healthy prior to reading in plate reader.

Action Taken and Determination of Impact on Study Data and/or Facility Compliance:

None - action taken with scientific input to enhance the study

Signature: [redacted] Date: 19 July 2011
SD/PI/Test Facility Management

Standard Operating Procedure Page 1 of 1

Form #: SOP-1003-F-1.0

CeeTox
In vitro models to predict toxicity

Deviation & Investigation

Study Number (if applicable): __~~9146V-100337STER~~ ①_____

Date of Reporting: __22 Jul 2011___ Reporting Associate: _QA Director_ in process audit_____

Date of Occurrence: __20 and 21st Jul___2011___ Associate Involved: _n/a_____

Description of Deviation:

The temperatures for refridgerators 1, 2, 3, 7, 9 and freezers 4,5 6, 8 were not recorded on July 20 and July 21, 2011. The impact of this deviation for this study is specific to Freezer # 8 that contained matierials for study number 9146V-100337STER. The contents of the #8 minus 80 freezer were examined for signs of freeze/thaw and no sign was found. Thus it can be expected that the temperature remained in range for the July 20th and July 21st. It was determined that there was no impact on this study and other studies due to the missed temperature recording of freezer #8 on these two days. The min/max temperatures were examined for refridgerators 1,2,3,7,9 and freezers 4,5, additionally. It was determined from the min/max readings that these refridgerators and freezers were within the determined range for the 24 hour time period before the first missed reading and the 24 hour period after the second missed reading time period. The contents of the freezers were examined for signs of freeze/thaw and none were identified. The # 6 minus 80 freezer log recorder was examined for temperature excursions during the July 20th and July 21st time period. No excursions were identified.

Type of Deviation (determined by Study Director/Principal Investigator):

Facility Deviation from SOP-4007

Summary of Deviation Investigation by SD/PI/Test Facility Management/Designee:

The records of the temperatures of the listed refrigerators and freezers were examined. All contents of freezers were examined for signs of freeze/thaw.

Action Taken and Determination of Impact on Study Data and/or Facility Compliance:

The result of the above listed investigation concluded there was no GLP study impact due to possible temperature excursions that could have been a result of the missed temperature monitoring for the July 20th and July 21st time period.

Signature: [redacted] Date: 18-Aug-2011

SD/PI/Test Facility Management

① This was the study number that the deviation was identified, but as a facility deviation, it applies to all studies ongoing during those days. [redacted] 26 Aug 2011

COPY

Form #: SOP-1003-F-1.0

CeeTox
In vitro models to predict toxicity

Deviation & Investigation

Study Number (if applicable): 9070-100107ERTA

Date of Reporting: 26 Aug 2011 Reporting Associate: [redacted]

Date of Occurrence: 05 July 2011, 11 July 2011, 13 July 2011, 15 July 2011, 23 Aug 2011 and 25 Aug 2011 Associate Involved: [redacted]

Description of Deviation:

Oxybenzone (2-hydroxy-4-methoxybenzone) lot # in protocol was 20080801 however lot supplied was 20100801. No C of A for lot 20100801 and C of A for lot 20080801 says it expired 04 Aug 2010

Signature [redacted] Date: 26 Aug 2011

(Reporting Associate):

Type of Deviation (determined by Study Director/Principal Investigator):

☐ SOP Deviation ☒ Protocol Deviation ☐ GLP Deviation ☐ No Deviation

Summary of Deviation Investigation by SD/PI/Test Facility Management/Designee:

Incorrect lot and C of A provided

Action Taken and Determination of Impact on Study Data and/or Facility Compliance:

Asked sponsor to supply proper C of A.

Signature: [redacted] Date: 26 Aug 2011

SD/PI/Test Facility Management

Standard Operating Procedure Page 1 of 1

Form #: SOP-1003-F-1.0

CeeTox
In vitro models to predict toxicity

Deviation & Investigation

Study Number (if applicable): 9070-100107ERTA

Date of Reporting: 04-Jan-12 Reporting Associate: [redacted]

Date of Occurrence: 05-Jul-11 and 23-Aug-11 Associate Involved: [redacted]

Description of Deviation:

Wrong purity was used for methoxycinnamate. Used 98% instead of 99.8%.

Signature [redacted] Date: 04-Jan-12

(Reporting Associate)

Type of Deviation (determined by Study Director/Principal Investigator):

☐SOP Deviation ☒Protocol Deviation ☐GLP Deviation ☐No Deviation

Summary of Deviation Investigation by SD/PI/Test Facility Management/Designee:

Wrong purity was used for methoxycinnamate.

Action Taken and Determination of Impact on Study Data and/or Facility Compliance:

None. After dilutions, the difference is negligible.

Signature: [redacted] Date: 04-Jan-12

SD/PI/Test Facility Management

Standard Operating Procedure Page 1 of 1

Form #: SOP-1003-F-1.0

CeeTox
In vitro models to predict toxicity

Deviation & Investigation

Study Number (if applicable): 9070-100107ERTA

Date of Reporting: 04-Jan-12 Reporting Associate: [redacted]

Date of Occurrence: 06-Oct-11 and 18-Oct-11 Associate Involved: [redacted]

Description of Deviation:

Sponsor was not asked to sign amendments according to the protocol.

Signature [redacted] Date: 04-Jan-12

(Reporting Associate)

Type of Deviation (determined by Study Director/Principal Investigator):

☐SOP Deviation ☒Protocol Deviation ☐GLP Deviation ☐No Deviation

Summary of Deviation Investigation by SD/PI/Test Facility Management/Designee:

Sponsor was notified of pending Amendments but were not asked to sign the amendments as stated in the protocol.

Action Taken and Determination of Impact on Study Data and/or Facility Compliance:

None. Sponsor signature and date will be required for all future amendments, if any, for this study.

Signature: [redacted] Date: 04-Jan-12

SD/PI/Test Facility Management

Standard Operating Procedure Page 1 of 1

APPENDIX 3 Certificate of Analysis

IVYCHEM

IVY FINE CHEMICALS

http://www.ivychem.com

CERTICATE OF ANALYSIS

Product Name	**2-HYDROXY-4-METHOXYBENZOPHENONE**		
Synonym	Oxybenzone		
Catalog Number	HH13-026		
CAS Number	131-57-7		
Batch Number	20100801	**Quantity**	200 KG
Manu. Date	August 2, 2010	**Expiry Date**	August 1, 2012
Date of Report	August 2, 2010	**Package**	
Quality Specifications	Specifications (In house)		

Test	Standard	Results
Appearance	Light yellow to green crystalline powder	Light yellow crystalline powder
Assay (HPLC)	98% min	99.92%
Melting Point	62 °C to 65 °C	63.8 °C to 64.8 °C
Loss on Drying	0.5% max	0.07%
Heavy Metals	<= 5 ppm	2.9 ppm
Conclusion: **Conform**		

CERTIFICATE OF ANALYSIS

[redacted]

Product 29116 **Octyl 4-methoxycinnamate,98%,stabilized**

Specifications

Appearance	CLEAR COLOURLESS TO YELLOW LIQUID
Infrared spectrometry	AUTHENTIC
Separat. techn. GC	>97.5 %
Acid value	<1 mg KOH/g
Specific abs. A (1%/1cm)	>830 (at 307 to 308 nm in methanol)
Specific gravity	(25/25°C) 1.007 to 1.012
Refractive index	1.5430 to 1.5470 (20°C, 589 nm)
Stabilizer	0.05 to 0.1 % BHT

General Product Data

Version	00
CAS No.	5466-77-3
Molecular weight	290.39
Molecular formula	C18 H26 O3
Linear formula	
Flash point (°C)	193

Lot Specific Data for Lot No.: A0293319

Appearance	CLEAR COLOURLESS LIQUID
Infrared spectrometry	AUTHENTIC
Separat. techn. GC	99.8 %
Acid value	0.1 mg KOH/g
Specific abs. A (1%/1cm)	865 (at 307 to 308 nm in methanol)
Specific gravity	(25/25°C) 1.0096
Refractive index	1.5453 (20°C, 589 nm)
Stabilizer	0.09 % BHT

ACROS ORGANICS

[redacted]

Issued: 10-08-10

[redacted] **Quality Assurance Manager**

Acros Organics Geel West Zone 2, Janssen Pharmaceuticalaan 3a, B-2440 Geel, Belgium Tel +32 14/57.52.11 - Fax +32 14/58.34.34 Internet: http://www.acros.com
1 Reagent Lane, Fair Lawn, NJ 07410,USA Fax 201-796-1329

[redacted]

MRS-N TDVTech 1492

A-1

Certificate of Analysis

SIGMA-ALDRICH

Product Name	2-Ethylhexyl salicylate, ≥99%
Product Number	W514600
Product Brand	ALDRICH
CAS Number	118-60-5
Molecular Formula	$(HO)C_6H_4CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3$
Molecular Weight	250.33

TEST	SPECIFICATION	LOT 44698PJ RESULTS
Appearance (Color)	Colorless	Colorless
Appearance (Form)	Liquid	Liquid
Refractive Index at 20 °C	1.500 - 1.504	1.502
Infrared spectrum	Conforms to Structure	Conforms
Purity (GC)	≥99.0 %	99.6 %
Color Test	≤100 APHA	10 APHA
Arsenic (As)	≤3.0 ppm	< 1.0 ppm
Cadmium (Cd)	≤1.0 ppm	< 1.0 ppm
Mercury (Hg)	≤1.0 ppm	< 1.0 ppm
Lead (Pb)	≤10.0 ppm	< 1.0 ppm
Specification Date:		DEC 2008
Date of QC Release:		DEC 2008
Print Date:		DEC 18 2009

[redacted] Supervisor
Quality Control
Milwaukee, Wisconsin USA

Certificate of Analysis

SIGMA-ALDRICH

Product Name	2-Ethylhexyl 2-cyano-3,3-diphenylacrylate, 97%
Product Number	415820
Product Brand	ALDRICH
CAS Number	6197-30-4
Molecular Formula	$(C_6H_5)_2C=C(CN)CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3$
Molecular Weight	361.48

TEST	SPECIFICATION	LOT 01697NJ RESULTS
Appearance (Color)	Yellow	Yellow
Appearance (Form)	Viscous Liquid	Viscous Liquid
Infrared spectrum	Conforms to Structure	Conforms
Purity (GC)	≥96.5 %	99.2 %
Specification Date:		OCT 2008
Date of QC Release:		OCT 2008
Print Date:		OCT 22 2008

[redacted] Supervisor
Quality Control
Milwaukee, Wisconsin USA

APPENDIX 4 Certification of Mycoplasma-Free Status of HeLa-9903 Cell Line

bionique Testing Laboratories **CELLshipper®**

Mycoplasma Testing Services
156 Fay Brook Drive • Saranac Lake NY 12983
Phone: 518-891-2356 • Fax: 518-891-5753

Please enclose this completed form with each slide to avoid delays in processing.

Date Sent: 05 04 2010 *Sample Designation or #:* Hela0129 free

Name [redacted]
(Bionique will submit results only to the person named above)

Company/University Cectar, Inc.

Complete Mailing Address: *(Results are mailed 1st class USPS)*
4717 Campus Dr.
Kalamazoo, MI 49008

Optional: FAX #: _____
(one Fax # only)

Cell Type: ✓ *Adherent* ____ *Nonadherent*

Normal ___ *Transfect* ___ *Monoclonal* ___ *Tumor* ✓

Flask ✓ T150 *Roller Bottle* ___ *≤ 2 liter suspension*

Bioreactor ___ *Other* _____

For Research Use Only

www.bionique.com 100499

Date received at Bionique Testing Labs: 10/6/10 Code #: 45924

M-100 CELLSHIPPER DNA FLUOROCHROME ASSAY RESULTS:

X **NEGATIVE:** A reaction with staining limited to the nuclear region, which indicates no mycoplasmal contamination.

_____ **POSITIVE:** A significant amount of extranuclear staining which strongly suggests mycoplasmal contamination.

_____ **INCONCLUSIVE:**

_____ A significant amount of extranuclear staining consistent with low - level mycoplasmal contamination or nuclear degeneration.

_____ A significant amount of extranuclear staining consistent with bacterial, fungal or other microbial contaminant or viral CPE. Morphology not consistent for mycoplasmal contamination.

COMMENTS: _____

Date Processed: 10/6/10 **By:** [redacted]

Thank you for allowing us to assist you, and for using the CELLshipper. **(dc: 3003 att # 2: 10/9/2003)**

APPENDIX 5 Protocol and Protocol Amendments

CeeTox®
In vitro models to predict toxicity

FINAL PROTOCOL

Estrogen Receptor Transcriptional Activation
(Human Cell Line (HeLa-9903))

Data Requirements: *OPPTS 890.1300*
Test Guidelines: *OECD 455*

Author
[redacted]

Study Number:
9070-100107ERTA

Sponsor:
NIEHS
530 Davis Drive, MD K2-12
PO BOX 12233
Durham, NC 27713

Test Facility:
CeeTox
4717 Campus Drive
Kalamazoo, MI 49008

TEST PROTOCOL

TO BE COMPLETED BY THE STUDY SPONSOR:	
Study Sponsor:	NIEHS/NTP ([redacted] Chief Toxicology Branch)
Address:	P.O. Box 12233
	Phone: [redacted]
	Research Triangle Park, NC
Study Monitor: [redacted]	**E-mail:** [redacted]
Sponsor Protocol/Project No.:	
Test Substance Name(s): Octyl Salicylate, 2-Ethylhexyl p-methoxycinnamate, 2-Ethylhexyl 2-cyano-3,3-diphenylacrylate, 2-Hydroxy-4-methoxybenzophenone	

NIEHS/NTP Investigator
[redacted]
Telephone No.: [redacted]
Facsimile No.:
E-mail:
Contract Office Technical Representative
[redacted]
(Contract No. HHSN273200900005C; NIEHS Control No. N01-ES-00005)

Study Monitor
[redacted] (ILS, Inc, Durham, NC)
Telephone No.: [redacted]
Facsimile No.:
E-mail:

Table of Contents

Signatures

[redacted]

Study Sponsor

6/24/14
Date

[redacted]

Study Monitor

6/24/14
Date

[redacted]

Study Director (Director of Project Management)

6/24/2014
Date

1. Title of Study

Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903))

2. Purpose of Study

The objective of this study is to evaluate test substances for human estrogen receptor alpha transcriptional activation activity using the hERα–HeLa-9903 reporter cell line. The hERα–HeLa-9903 cell line is derived from a human cervical tumor and has two stably inserted constructs: (i) the human estrogen receptor alpha (hERα) expression construct (encoding the full-length human receptor) and (ii) a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin estrogen-responsive element driven by a mouse metallothionein (MT) promoter TATA element. The mouse MT TATA gene construct has been shown to have the best performance, and so is commonly used. Consequently, the hERα–HeLa-9903 cell line can measure the ability of a test substance to induce hERα-mediated transactivation of luciferase gene expression (agonism).

3. Compliance Statement

This study will be conducted in compliance with EPA GLP regulations (Title 40 Part 160) with the exception of section 160.113. Dose concentrations of test and control substances will not be verified using analytical methods.

4. Quality Assurance

This study will be subjected to periodic inspections and the draft and final reports will be reviewed by the Quality Assurance Unit of CeeTox in accordance with CeeTox SOP.

5. Regulatory Citations / Guidelines

- OECD guideline for the testing of substances number 455: Stably transfected human estrogen receptor-α transcriptional activation assay for detection of estrogenic agonist-activity of substances. 2009.
- OPPTS 890.1300: Estrogen receptor transcriptional activation (human cell line (HeLa-9903)). 2009.

6. Test Facility

CeeTox, Inc.
4717 Campus Drive
Kalamazoo, MI 49008
USA

7. Test Substance

7.1 Preparation of Test Substance

Test substances will be prepared as a stock in DMSO, or appropriate vehicle and serially diluted to prepare solutions for dilutions with media (to a final concentration of ≤ 0.1% (v/v)). Fresh dilutions of the stock solutions will be prepared on the day of use in the assay. Dose concentrations of test and control substances will not be verified using analytical methods.

7.2 Test Substance: 2-Hydroxy-4-Methoxybenzophenone (Oxybenzone)

CAS No.	131-57-7
Source:	Ivy Fine Chemicals Corporation
Lot/Batch No.:	20080801
ILS Repository No.:	11-29
Formula:	$C_{14}H_{12}O_3$
Description:	Light yellow powder
Storage	Room Temperature

7.3 Test Substance: 2-Ethylhexyl p-methoxycinnamate (Octylmethoxycinnamate)

CAS No.	5466-77-3
Source:	Acros Organics
Lot/Batch No.:	A0293319
ILS Repository No.:	11-32
Formula:	$C_{18}H_{26}O_3$
Description:	Clear colorless liquid
Storage	Room Temperature

7.4 Test Substance: Octyl Salicylate (Octylsalate)

CAS No.	118-60-5
Source:	Sigma-Aldrich
Lot/Batch No.:	44698PJ
ILS Repository No.:	11-30
Formula:	$C_{15}H_{22}O_3$
Description:	Colorless liquid
Storage	Room Temperature

7.5 Test Substance: 2-Ethylhexyl 2-Cyano-3,3-Diphenylacrylate (Octocrylene)

CAS No.	6197-30-4
Source:	Sigma-Aldrich
Lot/Batch No.:	01697MJ
ILS Repository No.:	11-31
Formula:	$C_{24}H_{27}NO_2$
Description:	Yellow viscous liquid
Storage	Room Temperature

7.6 Positive and Negative Reference Substances (Table 1)

17β-Estradiol (E2): CAS No: 50-28-2
17α-Estradiol: CAS No: 57-91-0
Corticosterone: CAS No: 50-22-6
17α-Methyltestosterone: CAS No: 58-18-4

Reference substances will be prepared as a stock in DMSO (final concentration in media of 0.1% (v/v)), or appropriate vehicle and serially diluted in the same solvent to prepare solutions for dilutions with media. Dilutions of the stock solutions will be prepared on the day of use in the assay.

Note: A certificate of analysis for each test substance will be provided by the sponsor and will be stored in the study data and appended to the study report. Confirmation of the identity of the test substance, characterization and stability will be verified by the sponsor. Test substance will be either returned to the Sponsor or destroyed following finalization of the study report.

Certificates of analysis for the positive and negative reference substances will be provided by the vendor and stored in the study data and appended to the study report (Table 1).

8 Test System

8.1 Source

Stably transfected hERα-HeLa-9903 cell line will be used for the assay. The cell line was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, 7-6-8 Asagi Saito, Ibaraki-shi, Osaka 567-0085, Japan. Only hERα–HeLa-9903 cells that test negative for mycoplasma will be used and certification will be included in the final report as an appendix. The hERα–HeLa-9903 cells used will be ≤ 40 passages and ≤ 3 months in culture when used in the assays. The hERα–HeLa-9903 cells will be grown for more than one passage from the frozen stock before use.

8.2 Stability of the Cell Line

The stability of the cell line will be monitored by reference substances 17β-estradiol (E2), 17α-estradiol, 17α-methyltestosterone and corticosterone. Reference substance $LogPC_{50}$, $LogPC_{10}$, $LogEC_{50}$ and Hill slope values should fall into the acceptable range values as outlined in Table 1. Reference substances that do not fall within the acceptable range of values (Table 1) can be acceptable if differences are minimal. A

complete concentration response curve (see Test Range in Table 1) for each reference substance will be run each time the assay is performed.

Table 1. Acceptable Range Values of the 4 Reference Substances (means ± 2 standard deviations)

Name	$LogPC_{50}$	$LogPC_{10}$	$LogEC_{50}$	Hill slope	Test Range (M)
17β-Estradiol (E2) CAS No: 50-28-2	-11.4 ~ -10.1	<-11	-11.3 ~ -10.1	0.7 ~ 1.5	10^{-14} ~ 10^{-8}
17α-Estradiol CAS No: 57-91-0	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	10^{-12} ~ 10^{-6}
Corticosterone CAS No: 50-22-6	-	-	-	-	10^{-10} ~ 10^{-4}
17α-Methyltestosterone CAS No: 58-18-4	-6.0 ~ -5.1	-8.0 ~ -6.2	-	-	10^{-11} ~ 10^{-5}

8.3 Cell Culture and Plating Conditions

Cells will be maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with 60 mg/L of Kanamycin (antibiotic) and 10% dextran-coated-charcoal-treated fetal bovine serum (DCC-FBS), in a CO_2 incubator (5% CO_2) at approximately 37°C. When the cells reach 75-90% confluency, they will be subcultured at 10 mL of 0.4 X 10^5 – 1 X 10^5 cells/mL. The cells will be suspended with 10% DCC-FBS in EMEM and plated into wells of a 96 well plate at a density of ~1 X 10^4 cells/75 µL/well. The cells will then be placed into a 5% CO_2 incubator approximately 37°C for at least 3 hours prior to substance exposure.

9 Methods

9.1 Cytotoxicity Assay

Cell viability will be monitored by propidium iodide (PI) uptake. PI is a dye that cannot cross the plasma membrane of intact and viable cells. Cells that are dead or dying have weakened plasma membrane which allows PI to enter the cytosol of the damaged cells. Once inside the cell, it intercalates into DNA/RNA and yields a fluorescent signal. Fluorescence is directly proportional to cell viability. PI is a light sensitive compound; therefore all procedures will be conducted under low light conditions.

Cells will be seeded into a 96-well black sided culture plate at the same time cells are seeded for the ER transactivation assays described above. The PI working

solution will be prepared by adding PI powder to phosphate buffered saline (PBS) in an amount sufficient to yield a final concentration of 4.4 µM. Following an approximately 24 hour incubation with the test substance, the growth medium will be removed from the plate designated for cytotoxicity and 50 µL of the PI working solution will be added. Background fluorescence will be evaluated by reading fluorescence on a Packard Fusion fluorescent plate reader at an excitation wavelength of 544 nm and an emission wavelength of 612 nm. Following this determination 50 µL of a 2% triton X-100 solution prepared in water will be added and the plate incubated at room temperature for a minimum of 15 minutes and read at the same wavelengths. The total amount of fluorescence or cells present on the plate will be determined by subtracting the first read from the second read. The change in cell viability will be determined by comparing treated wells to the untreated or control wells. A 20% drop below vehicle treated controls will be considered cytotoxic.

9.2 Solubility/Precipitation Assay

The limit of solubility will be determined by visual inspection.

This technique is an effective means of determining changes in the cell culture and dosing matrices. However, it should be noted that changes in fluid turbidity can be affected by the test substance reaching saturation and precipitating out of the solution or by the substance causing the precipitation of components in the culture and dosing media such as protein or salts.

9.3 Range Finding

Before testing for hERα transcriptional activation activity using the HeLa-9903 cell line, a preliminary range finding assessment of cytotoxicity (as described in section 9.1) and solubility (as described in section 9.2) will be conducted to assist in determining the appropriate concentration range for the test substance. The maximum concentration of test substance to be tested in these preliminary assessments will be 1 mM.

Cytotoxicity and solubility will also be monitored in all definitive runs of the assay. Any concentrations of the test substance that are cytotoxic (as defined in section 9.1) or produce precipitation will be noted. Concentrations in subsequent runs will be adjusted as necessary.

9.4 Substance Exposure and Assay Plate Organization

The procedure for substance dilutions (steps 1 and 2) and exposure to cells (step 3) will be conducted as follows:

Step 1: Each test/reference substance will be diluted in DMSO (or appropriate solvent), serially diluted and added to wells of a 96-well microtiter plate to achieve final serial concentrations as determined by the range finding test (see section 9.3). All concentrations will be tested in replicates of at least 3. Several control groups will be included on each plate as follows: vehicle control, agonist maximal response control (17β-estradiol (E2), CAS No. 50-28-2), antagonist control (ICI 182,780, CAS No. 129-45361-8).

Step 2: Test substance will be diluted in media as appropriate (if DMSO is used it will not exceed 0.1%).

Step 3: 75 µL of test substance dilution (2X) in media (prepared in step 2) will be added to wells containing ~1 X 10^4 cells/75 µL/well for a final volume of 150 µL/well.

Table 2. Example of Plate Concentration Assignment in the Assay Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank*	E2 (1 nM)	VC**	VC**	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6	Conc. 7	Conc. 8
B	↓***	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	----------As above + antagonist (1 µM ICI 182,780)----------											
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

*Blank wells contain only media + 0.1% (v/v) DMSO (no cells)
**Vehicle control (VC) wells contain cells and media + 0.1% (v/v) DMSO or appropriate vehicle
***↓ Indicates the composition of the well is identical to the well directly above it

After adding the test/reference substances, the plates will be incubated in a 5% CO_2 incubator at approximately 37°C for approximately 24 hours to allow for

induction of the reporter gene products. If test substances are thought to be volatile, plate sealers (breathe easy) will be used.

9.5 Luciferase Assay

A luciferase assay as described in CeeTox SOP 2041 will be performed. Luciferase assay reagent will be prepared as described in CeeTox SOP 2041 (proprietary).

10 Analysis of Data

All cytotoxic concentrations of test substance (as defined in section 9.1) will be excluded from data analysis.

To obtain the relative transcriptional activity to PC (positive control; 1 nM E2) the luminescence signals from the same plate will be analyzed according to the following steps:

Step 1: Mean values for the VC (vehicle control) will be calculated.

Step 2: The mean value of the VC will be subtracted from each well to normalize the data.

Step 3: The mean for the normalized PC will be calculated and outlier analysis performed. This consists of removing any data points that are outside of the mean $\pm$ 1.5 X standard deviation.

Step 4: The normalized value of each well will be divided by the mean value of the normalized PC (PC = 100%). The final value of each well is the relative transcriptional activity for that well compared to the PC response.

Step 5: The mean value of the relative transcriptional activity for each concentration of test substance will be calculated. There are 2 dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).

10.1 Statistics

For data generated at CeeTox, basic statistical analysis will be performed on the data, which will include means of replicates, standard error of the mean, standard deviations and %CV.

10.2 Considerations for Induction of EC_{50}, PC_{50} and PC_{10}

The full concentration response curve is required for the calculation of the EC_{50}. Calculating an EC_{50} might not always be achievable or practical due to the limitations of the test concentration range (for example due to cytoxicity and/or solubility issues). However, as the EC_{50} and maximum induction level (corresponding to the top value of the Hill-equation) are informative parameters, these parameters will be reported where possible. For the calculation of EC_{50} and maximum induction level, prism or xlfit will be used.

If the Hill's logistic equation is applicable to the concentration response data, the EC_{50} will be calculated by using the following equation:

$$Y = Bottom + (Top-Bottom)/ (1+10 EXP ((\log EC_{50} - X) \times Hill\ slope))$$

Where:

X is the logarithm of concentration; and,

Y is the response and Y starts at the Bottom and goes to the Top in a sigmoid curve.

Bottom is fixed at zero in the Hill's logistic equation

For each test substance the following data will be provided when possible:

- RPC_{Max} which is the maximum level of response induced by a test substance, expressed as a percentage of the response induced by 1 nM of E2 on the same plate, as well as the PC_{Max} (concentration associated with the RPC_{Max}).
- For positive substances, the concentrations that induce the PC_{10} and, if appropriate, the PC_{50} will be determined.

11 Data Interpretation Criteria

The results will be based on two or three independent runs, each of which will be conducted on separate days. If two runs give comparable data a third run will not be conducted. In order to be acceptable, the results should:

- Meet the performance standard requirements:
  - The mean luciferase activity of the PC (1 nM E2) should be at least 4-fold that of the mean VC on each plate.

- The results of the 4 reference substances should be within the acceptable ranges (see Table 1).

Data interpretation criteria are shown in Table 3. Positive results will be characterized by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (PC_{50}) or 10% (PC_{10}) of positive control values are reached accomplishes both of these goals. However, a test substance will be determined to be positive if the maximum response induced by the test substance (RPC_{Max}) is equal to or exceeds 10% of the response of the positive control in at least two of two or two of three runs. A test substance will be considered negative if the RPC_{Max} fails to achieve at least 10% of the response of the positive control in two of two or two of three definitive runs.

Table 3. Positive and Negative Decision Criteria

Positive	If the RPC_{Max} obtained is equal to or exceeds the 10% response of the positive control in at least two of two or two of three definitive runs.
Negative	If the RPC_{Max} obtained fails to achieve at least 10% of the response of the positive control in two of two or two of three definitive runs.

12 Study Reports

The data to be reported in the draft report and final report will be determined per Standard Operating Procedure (SOP) and will include (but will not be limited to) the following information: assay date and run number, laboratory personnel involved in the study, chemical/test substance information (including but not limited to substance name, code, molecular weight, concentrations tested, notes regarding solubility).

13 Alterations of the Study Design

Alterations of this protocol may be made as the study progresses. No changes in the protocol will be made without the specific written request or consent of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, CeeTox will honor such a change. However, written authorization will be obtained thereafter. All protocol amendments and justifications will be documented, signed and dated by the Study Director, Study Monitor and Sponsor and added to the report. A copy of the protocol and all amendments will be issued to the Sponsor as well as CeeTox and placed into the study binder.

14 Data Retention and Archiving

All raw data, documentation, records, protocol, and the final report generated as a result of this study will be retained at CeeTox for 15 years. Retention of the materials after 15 years will be subjected to a future contractual agreement between the Sponsor and CeeTox.

Study Records to be maintained:

- All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and substances used.
- Protocol and protocol Amendments
- List of any Protocol Deviations
- Final Report

CeeTox

Protocol Amendment

Study Number: 9070-100107ERTA

Title of Study to be Amended: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903))

Reason for Amendment to Protocol: Current Study Director's work load and number of studies directing has reached a maximal capacity deemed acceptable by test facility management.

Change: [redacted] will be designated the Study Director for this study.

Signature

CeeTox, Inc.

[redacted] _____ 06 Oct 2011
Study Director (Project Manager) Date

[redacted] _____ 06 OCT 2011
President Date

CeeTox

Protocol Amendment

Study Number: 9070-100107ERTA

Title of Study to be Amended: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903))

Reason for Amendment to Protocol: The Two-Read Propidium Iodide SOP has been revised and the protocol is being amended to reflect these changes. First, a typo was identified in both CeeTox SOP and the protocol which stated the final concentration of propidium iodide was 4.4 µM. The correct concentration, and the concentration prepared and used in the study, was 44 µM.

Secondly, the Propidium Iodide (PI) working solution was stated as being prepared in phosphate buffered saline (PBS), however the recent SOP revisions states that the PI working solution may be prepared in either PBS or cell culture media

Change:
Section 9.1 Cytotoxicity Assay, Paragraph 2, the first two sentences state:

"Cells will be seeded into a 96-well black sided culture plate at the same time cells are seeded for the ER transactivation assays described above. The PI working solution will be prepared by adding PI powder to phosphate buffered saline (PBS) in an amount sufficient to yield a final concentration of 4.4 µM."

Section 9.1 Cytotoxicity Assay, Paragraph 2 will now state:

"Cells will be seeded into a 96-well black sided culture plate at the same time cells are seeded for the ER transactivation assays described above. The PI working solution will be prepared by adding PI powder to either the cell culture media used or phosphate buffered saline (PBS) in an amount sufficient to yield a final concentration of 44 µM."

Signature

CeeTox, Inc.

[redacted]

18 Oct 2011
Date

Study Director (Project Manager)

CeeTox

Protocol Amendment

Study Number: 9070-100107ERTA

Title of Study to be Amended: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903))

Reason for Amendment to Protocol: Client requested amendment

Change:
Section Data Retention and Archiving will now state:

At the study closure, all study records including all original raw data and original final report, will be shipped to the sponsor at the following address:

NTP Archives
[redacted]
615 Davis Drive, Suite 300
Durham, NC 27713

Signature

CeeTox, Inc.

[redacted]
Study Monitor

12-6-11
Date

[redacted]
Study Director (Project Manager)

06 Dec 11
Date