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: April 25, 2013

TEST FACILITY: CeeTox, Inc.
4717 Campus Drive
Kalamazoo, MI 49008
USA

LABORATORY PROJECT ID: Study Number: 9070-100794ERTA
Human and Health Sciences No.
HHSN273200900005C
NIEHS Contract No. N01ES00005

SPONSOR(S): National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709 USA

STUDY MONITOR: [redacted]
(ILS, Inc, Durham, NC)

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

This page is intentionally left blank.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study Number: 9070-100794ERTA

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

I, the undersigned, hereby declare that this study was conducted in compliance with the U.S. Environmental Protection Agency Good Laboratory Practice regulations Title 40, Part 160 with the exception of section 160.113. Dose concentrations of test substance and control substances were not verified using 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

25 April 2013
Date

FLAGGING STATEMENT

This page is intentionally left blank.

QUALITY ASSURANCE STATEMENT

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

Study Number: 9070-100794ERTA

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
20Dec2012	Draft Protocol	20Dec2012	20Dec2012
28Jan2013	Test Substance Prep and Dosing	31Jan2013	31Jan2013
29Jan2013	PI and Luciferase Assays	31Jan2013	31Jan2013
27Feb2013	Draft Report	01Mar2013	01Mar2013
28Feb2013	Draft Report	01Mar2013	01Mar2013
22Apr13	Data Binder	22Apr13	22Apr13

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

[redacted] 25Apr2013

Date

Quality Assurance Auditor
4717 Campus Drive
Kalamazoo, MI 49008

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
[redacted]	Director of Project Management Senior Scientist Scientist Laboratory Manager Cell Culture Scientist II Study Director

Study Dates

Study initiation date: January 16, 2013
Experimental start date: January 21, 2013
Experimental termination date: February 15, 2013
Study completion date: April 25, 2013

Deviations from the Protocol

There were no deviations during the conduct of this study.

Other

All original data [including the original signed study protocol and all amendments (if any), test substance information, observations, etc.] and the original final report will be transferred to the National Toxicology Program Archives following finalization of the study report to the address below:

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

TABLE OF CONTENTS

1.0 EXECUTIVE SUMMARY

1.1 Study Design

The objective of this study was to evaluate the ability of ensulizole, avobenzone, homosalate and padimate-O to act as an agonists of human estrogen receptor alpha (hERα) using the hERα-HeLa-9903 cell line.

Preliminary range finding assessments of cytotoxicity and precipitation were conducted in order to identify soluble and cytotoxic concentrations of ensulizole, avobenzone, homosalate and padimate-O in the transcriptional activation assays.

Three independent runs were conducted. Due to calibration failure of the plate reader, the second run (28-January-2013) was invalid. The final concentrations of ensulizole, avobenzone, homosalate and padimate-O 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 ensulizole and avobenzone, and 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M for homosalate and padimate-O in both runs (24-January-2013 and 12-February-2013).

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 rangefinder assay, the highest soluble concentration for use in the transcriptional activation assays was determined to be 10^{-5} M for ensulizole and avobenzone, and 10^{-4} M homosalate and padimate-O. 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 ensulizole and avobenzone, and 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M for homosalate and padimate-O in both runs. In the rangefinder, there was cytotoxicity observed at 10^{-4} M avobenzone (though 10^{-3} M avobenzone exhibited no cytotoxicity), and 10^{-3} M homosalate. No cytotoxicity was observed for any test substance at any concentration during the two valid runs of the assay.

In the two valid runs of the transcriptional activation assay, exposure to any of the four test substances did not result in an increase in luciferase activity at any of the viable concentrations tested (RPC_{max}<10%).

1.3 Conclusion

Ensulizole, avobenzone, homosalate and padimate-O are not agonists of human estrogen receptor alpha (hERα) in the estrogen receptor transcriptional activation (Human Cell Line (HeLa-9903)) model system.

2.0 INTRODUCTION

2.1 Purpose

The objective of this study was to evaluate the ability of ensulizole, avobenzone, homosalate and padimate-O to act as agonists 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-Phenyl-5-benzimidazolesulfonic Acid (Ensulizole)
Test Substance Supplier:	Aldrich
CAS Number:	27503-81-7
Description:	White to off white powder
Solvent Used:	Dimethyl sulfoxide
Batch Number:	05117JE
Expiry Date:	Not provided
Purity:	99.6%
Molecular Formula:	$C_{13}H_{10}N_2O_3S$
Molecular Weight:	274.30 g/mol
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 2.

Test Substance Name:	Butyl-methoxydibenzoylmethane (Avobenzone)
Test Substance Supplier:	Universal-Preserv-A-Chem, Inc.
CAS Number:	70356-09-1
Description:	Off white to yellowish crystalline powder
Solvent Used:	Dimethyl sulfoxide
Batch Number:	L802809
Expiry Date:	Not provided
Purity:	98.5%
Molecular Formula:	$C_{20}H_{22}O_3$
Molecular Weight:	310.39 g/mol
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 2.

Test Substance Name:	3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)
Test Substance Supplier:	Spectrum
CAS Number:	118-56-9
Description:	Colorless to light yellow liquid
Solvent Used:	Dimethyl sulfoxide
Batch Number:	YT0976
Expiry Date:	Not provided
Purity:	99.3%
Molecular Formula:	262.34 g/mol
Molecular Weight:	$C_{16}H_{22}O_3$
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 2.

Test Substance Name:	2-Ethylhexyl-P-Dimethyl-Aminobenzoate (Padimate-O)
Test Substance Supplier:	Aldrich
CAS Number:	21245-02-3
Description:	Yellowish liquid
Solvent Used:	Dimethyl sulfoxide
Batch Number:	MKBF0590V
Expiry Date:	Not provided
Purity:	98.1%
Molecular Formula:	277.40 g/mol
Molecular Weight:	$C_{17}H_{27}NO_2$
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 2.

3.1.2 Vehicle selection

Dimethyl sulfoxide (DMSO) was selected as a suitable vehicle for ensulizole, avobenzone, homosalate and padimate-O. Ensulizole and avobenzone solutions up to 10^{-5} M (the limit concentration for the assay) and homosalate and padimate-O solutions up to 10^{-4} M (the limit concentration for the assay) were 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 January 20, 2013 for use in this study. Ensulizole, avobenzone, homosalate, and padimate-O were prepared in DMSO on January 24, 2013 for the first valid run, and on February 12, 2013 for use in the second valid run of the assay. Based upon historical data for reference compounds 17α-estradiol, corticosterone, 17β-estradiol and 17α-methyltestosterone, and the 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 3). The cells used in this study were passage 29 (rangefinder, run on 21-January-2013). New cells were brought up from cryopreservation and maintained for the two valid runs (24-January-2013 and 12-February-2013) and were passages 31 and 35, 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, ensulizole, avobenzone, homosalate and padimate-O 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 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 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 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 allow 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 substances 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 Procedure 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 ensulizole, avobenzone, homosalate and padimate-O. 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^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M for ensulizole and 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M for avobenzone, homosalate and padimate-O.

3.6 Transcriptional Activation Assay Data Analysis and Interpretation

For each of the valid independent runs of the transcriptional activation assays, the luciferase assay and PI assay data were added to a locked data spreadsheet (Microsoft EXCEL 2010 Version 14.0.6123.5001; Redmond, WA). The following statistics were assessed for transcriptional activation; mean percent of maximal induction control (%), standard deviation (SD), standard error of the mean (SEM). For viability assessments, the mean percent viability compared to negative control, standard deviation (SD), standard error of the mean (SEM) and percent coefficient of variation (% CV) were calculated using XLfit (Version 5.2.0.0; Guildford, Surrey, UK). XLfit was also used for graphing the results and determining the EC_{50} (if applicable) for each curve generated.

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^{-5} M for ensulizole and avobenzone, and 10^{-4} M for homosalate and padimate-O. There was cytotoxicity observed at 10^{-4} M avobenzone (though 10^{-3} M avobenzone exhibited no cytotoxicity), and 10^{-3} M homosalate. No cytotoxicity was observed with ensulizole and padimate-O.

The final concentrations of ensulizole, avobenzone, homosalate and padimate-O 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 ensulizole and avobenzone, and 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M for homosalate and padimate-O in both runs.

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 both valid runs of the assay, the $LogPC_{10}$ value for 17α-methyltestosterone was marginally greater than the specified range ($LogPC_{10}$ = -5.8 for both runs; compared to the specified range of -8.0 ~ -6.2)
- In both valid runs of the assay, the $LogPC_{50}$ value for 17α-methyltestosterone was not reached ($LogPC_{50}$ = -6.0 ~ -5.1) as the curve never reached 50% of maximal induction of control and therefore could not be calculated.

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

Three independent runs were conducted. Due to calibration failure of the plate reader, the second run (28-January-2013) was invalid. The data is located in the study binder but is not included in the analysis of this report.

In the two valid runs of the transcriptional activation assay, exposure to all four test substances 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 rangefinder assay, the highest soluble concentration for use in the transcriptional activation assays was determined to be 10^{-5} M for ensulizole and avobenzone, and 10^{-4} M homosalate and padimate-O. 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 ensulizole and avobenzone, and 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M for homosalate and padimate-O in both runs. In the rangefinder, there was cytotoxicity observed at 10^{-4} M avobenzone (though 10^{-3} M avobenzone exhibited no cytotoxicity), and 10^{-3} M homosalate. No cytotoxicity was observed for any test substances at any concentration during the two valid runs of the assay.

In the two valid runs of the transcriptional activation assay, exposure to all four test substances did not result in an increase in luciferase activity at any of the viable concentrations tested (RPC_{max}<10%).

5.0 CONCLUSIONS

Ensulizole, avobenzone, homosalate and padimate-O are not agonists of human estrogen receptor alpha (hERα) in the estrogen receptor transcriptional activation (Human Cell Line (HeLa-9903)) model system.

6.0 REFERENCES

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

OECD Guideline for 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 Rangefinder - Results of Preliminary Cytotoxicity and Precipitation Assays

Test Substance	Concentration (M)	Cell Viability (% of VC)		Precipitation
		Mean	SD	
Ensulizole	10^{-12}	103.8	8.0	-
	10^{-11}	102.7	6.8	-
	10^{-10}	101.6	6.0	-
	10^{-9}	99.4	7.1	-
	10^{-8}	103.1	9.5	-
	10^{-7}	104.8	5.1	-
	10^{-6}	110.9	12.8	-
	10^{-5}	108.7	15.0	-
Avobenzone	10^{-10}	104.2	2.2	-
	10^{-9}	99.8	10.4	-
	10^{-8}	98.8	12.3	-
	10^{-7}	101.1	10.7	-
	10^{-6}	100.1	8.6	-
	10^{-5}	99.0	8.0	-
	10^{-4}	62.2	14.9	+
	10^{-3}	80.6	12.0	+
Homosalate	10^{-10}	102.7	16.3	-
	10^{-9}	95.4	20.2	-
	10^{-8}	105.3	9.3	-
	10^{-7}	100.9	12.9	-
	10^{-6}	89.4	14.0	-
	10^{-5}	89.7	25.3	-
	10^{-4}	85.1	15.4	-
	10^{-3}	78.1	23.1	-
Padimate-O	10^{-10}	103.1	19.2	-
	10^{-9}	100.5	11.1	-
	10^{-8}	99.9	10.0	-
	10^{-7}	98.7	10.9	-
	10^{-6}	102.2	7.2	-
	10^{-5}	105.7	7.0	-
	10^{-4}	108.6	9.7	-
	10^{-3}	101.9	14.1	+

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^{-14}	0.3	0.7	1.3	-0.5	93.5	11.7	-
	10^{-13}	0.4	1.0	1.7	-0.7	95.7	11.1	-
	10^{-12}	1.8	1.4	1.2	-0.3	95.2	10.4	-
	10^{-11}	10.8	4.5	1.2	-0.5	94.5	9.4	-
	10^{-10}	81.3	20.5	1.7	0.1	95.9	10.2	-
	10^{-9}	121.8	18.4	1.9	0.1	96.1	8.0	-
	10^{-8}	125.1	26.8	2.5	0.3	97.2	6.1	-
	10^{-7}	93.6	28.4	6.2	3.8	96.7	14.2	-
17α-Estradiol	10^{-13}	0.1	0.5	1.1	-0.9	103.4	11.5	-
	10^{-12}	0.0	0.6	0.7	-0.8	106.0	10.2	-
	10^{-11}	1.0	0.9	1.4	-0.8	108.1	8.4	-
	10^{-10}	0.9	0.8	1.1	-0.9	105.2	5.6	-
	10^{-9}	18.0	5.4	1.8	-0.2	104.8	8.5	-
	10^{-8}	76.7	16.6	1.8	-0.1	105.3	4.8	-
	10^{-7}	113.3	22.9	2.0	-0.1	109.5	5.1	-
	10^{-6}	97.7	28.0	1.9	0.1	113.7	10.7	-
Corticosterone	10^{-11}	0.0	0.4	0.8	-0.6	105.0	8.6	-
	10^{-10}	0.0	0.4	0.7	-0.6	105.2	7.0	-
	10^{-9}	1.3	0.7	1.6	-0.6	103.3	6.0	-
	10^{-8}	0.2	0.7	0.8	-0.6	102.4	7.0	-
	10^{-7}	0.9	0.5	1.4	-0.1	103.0	6.0	-
	10^{-6}	0.5	0.6	0.9	0.0	105.1	8.1	-
	10^{-5}	-0.5	0.6	0.0	-0.5	102.9	10.3	-
	10^{-4}	-2.1	0.3	-1.2	-1.6	98.2	10.1	-
17α-Methyltestosterone	10^{-12}	0.2	0.7	0.8	-0.6	100.2	10.4	-
	10^{-11}	0.4	0.7	0.9	-0.7	102.4	10.7	-
	10^{-10}	1.2	0.7	1.2	-0.2	100.3	12.6	-
	10^{-9}	0.5	0.6	0.8	-0.6	99.5	8.6	-
	10^{-8}	1.7	0.6	1.7	0.0	96.9	9.1	-
	10^{-7}	1.6	0.7	1.1	0.5	101.8	9.4	-
	10^{-6}	4.1	1.3	1.3	-0.2	101.2	3.3	-
	10^{-5}	36.3	10.4	0.0	-0.6	98.9	12.0	-

RTA = Relative Transcriptional Activation
PC = Positive Control (1 nM 17β-Estradiol)
VC = Vehicle Control
SD = Standard Deviation
+ = Precipitation observed
- = No precipitation observed

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	
Ensulizole	10^{-12}	0.7	0.5	0.7	-0.7	101.0	15.2	-
	10^{-11}	0.3	0.4	0.8	-0.8	103.7	11.3	-
	10^{-10}	0.9	0.7	1.3	-0.6	106.9	12.1	-
	10^{-9}	0.6	1.0	1.1	-0.8	102.9	9.3	-
	10^{-8}	1.7	1.0	1.4	0.1	99.9	11.7	-
	10^{-7}	1.3	0.4	1.9	0.2	103.3	11.6	-
	10^{-6}	1.5	1.2	2.0	0.1	105.5	11.7	-
	10^{-5}	1.2	1.1	1.1	-0.3	102.2	13.3	-
Avobenzone	10^{-12}	0.3	0.6	0.4	-0.2	100.4	16.3	-
	10^{-11}	0.4	0.4	0.5	-0.4	103.8	12.4	-
	10^{-10}	1.4	0.7	0.4	-0.3	101.6	11.6	-
	10^{-9}	0.6	0.4	0.2	-0.4	99.5	10.1	-
	10^{-8}	1.9	0.8	1.3	0.1	100.0	10.3	-
	10^{-7}	1.4	1.1	1.3	0.0	97.0	9.5	-
	10^{-6}	2.2	0.9	1.0	-0.1	97.2	6.8	-
	10^{-5}	1.7	0.7	2.2	0.2	102.6	13.3	-
Homosalate	10^{-11}	0.4	0.9	0.5	-0.7	99.5	5.9	-
	10^{-10}	-0.1	0.6	0.8	-0.7	100.2	8.5	-
	10^{-9}	0.6	0.8	1.8	-0.6	103.1	3.7	-
	10^{-8}	0.4	1.0	1.2	-0.7	102.3	9.9	-
	10^{-7}	1.7	0.9	1.7	0.0	98.1	7.5	-
	10^{-6}	0.8	0.5	1.6	0.6	105.0	8.3	-
	10^{-5}	2.1	1.1	1.9	-0.8	105.0	10.9	-
	10^{-4}	6.2	2.9	-0.1	-0.3	100.3	20.9	-
Padimate-O	10^{-11}	0.1	0.3	0.4	-0.8	99.9	14.4	-
	10^{-10}	0.0	0.2	0.9	-0.8	99.4	12.8	-
	10^{-9}	0.8	0.7	0.9	-0.6	99.4	12.1	-
	10^{-8}	0.2	0.5	0.6	-0.9	101.8	13.1	-
	10^{-7}	1.2	0.5	1.3	-0.4	102.1	7.6	-
	10^{-6}	1.2	0.5	1.5	0.6	102.5	7.9	-
	10^{-5}	1.7	0.7	1.0	0.0	105.3	14.1	-
	10^{-4}	3.0	1.6	1.0	-0.1	98.2	12.3	-

RTA = Relative Transcriptional Activation
PC = Positive Control (1 nM 17β-Estradiol)
VC = Vehicle Control
SD = Standard Deviation
+ = Precipitation observed
- = No precipitation observed

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^{-14}	0.3	1.3	1.0	-0.8	107.9	18.3	-
	10^{-13}	0.2	1.3	0.9	-0.6	106.4	19.7	-
	10^{-12}	2.2	1.8	2.1	0.3	108.1	15.5	-
	10^{-11}	21.8	9.4	1.7	-0.4	109.0	17.7	-
	10^{-10}	112.5	36.2	1.6	0.1	100.1	11.0	-
	10^{-9}	162.3	38.9	1.1	0.2	113.9	15.7	-
	10^{-8}	181.6	51.6	2.2	0.7	108.2	11.0	-
	10^{-7}	163.7	16.6	14.5	5.8	102.2	8.5	-
17α-Estradiol	10^{-13}	0.2	0.8	0.9	0.1	103.2	16.6	-
	10^{-12}	-0.1	1.1	1.2	-0.2	106.1	14.4	-
	10^{-11}	1.0	1.4	1.7	0.2	106.0	14.4	-
	10^{-10}	2.0	1.4	1.4	-0.1	105.9	16.7	-
	10^{-9}	31.0	8.9	1.5	0.8	102.3	15.8	-
	10^{-8}	107.3	31.9	1.0	1.1	117.1	17.4	-
	10^{-7}	149.7	15.7	1.4	1.1	111.4	10.4	-
	10^{-6}	149.7	21.3	4.0	0.2	110.7	11.4	-
Corticosterone	10^{-11}	-0.3	0.6	0.3	-0.3	103.3	14.3	-
	10^{-10}	-0.6	0.7	0.4	-1.2	104.7	7.9	-
	10^{-9}	0.7	1.2	1.0	-1.2	107.6	5.7	-
	10^{-8}	0.0	1.0	0.5	-0.7	110.0	10.6	-
	10^{-7}	-0.8	0.6	0.0	-0.4	103.9	7.1	-
	10^{-6}	-0.6	0.9	-0.3	-1.0	115.3	13.6	-
	10^{-5}	-2.0	0.3	-1.9	-1.9	108.7	19.1	-
	10^{-4}	-3.3	0.1	-2.8	-3.0	85.3	9.3	-
17α-Methyltestosterone	10^{-12}	0.7	0.8	0.6	-0.5	107.2	19.1	-
	10^{-11}	-0.2	0.7	0.5	-0.5	104.9	13.9	-
	10^{-10}	1.0	1.2	1.0	-0.1	101.9	14.7	-
	10^{-9}	0.2	0.9	0.6	0.0	106.2	19.5	-
	10^{-8}	0.5	0.9	0.9	0.2	104.4	16.6	-
	10^{-7}	0.6	0.3	0.9	1.0	108.0	11.5	-
	10^{-6}	5.0	2.2	0.6	0.3	110.7	17.8	-
	10^{-5}	32.1	2.9	-1.6	-2.2	100.9	15.1	-

RTA = Relative Transcriptional Activation
PC = Positive Control (1 nM 17β-Estradiol)
VC = Vehicle Control
SD = Standard Deviation
+ = Precipitation observed
- = No precipitation observed

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	
Ensulizole	10^{-12}	0.1	1.1	0.1	-0.5	103.3	15.8	-
	10^{-11}	0.0	0.9	0.4	-0.3	104.6	9.6	-
	10^{-10}	0.8	1.0	1.9	0.6	104.7	7.1	-
	10^{-9}	0.7	0.9	1.9	0.0	106.7	9.4	-
	10^{-8}	0.8	0.6	1.1	0.1	104.4	9.2	-
	10^{-7}	0.7	0.7	0.8	0.1	111.5	10.9	-
	10^{-6}	1.3	0.8	0.7	0.8	119.4	24.6	-
	10^{-5}	1.3	0.5	0.7	-0.4	109.5	24.2	-
Avobenzone	10^{-12}	-0.3	0.9	0.5	-0.1	98.3	16.7	-
	10^{-11}	-0.2	1.0	0.6	-1.2	101.3	12.4	-
	10^{-10}	0.8	1.1	1.4	0.2	99.9	12.8	-
	10^{-9}	0.1	0.8	0.9	-0.2	99.4	10.0	-
	10^{-8}	1.1	1.1	1.7	0.1	99.6	7.4	-
	10^{-7}	0.3	0.5	1.2	0.8	105.2	8.9	-
	10^{-6}	0.7	0.7	1.3	0.8	102.2	9.9	-
	10^{-5}	2.4	1.2	1.9	0.4	102.2	19.2	-
Homosalate	10^{-11}	0.1	0.6	0.3	-0.5	108.8	10.2	-
	10^{-10}	0.2	1.0	1.4	-0.4	112.9	8.8	-
	10^{-9}	1.1	0.8	2.1	0.2	107.6	5.6	-
	10^{-8}	0.5	1.1	1.9	-0.7	109.4	8.1	-
	10^{-7}	0.6	1.0	1.7	0.3	113.4	7.8	-
	10^{-6}	0.7	0.7	1.2	0.2	119.3	15.9	-
	10^{-5}	2.5	1.5	1.1	-0.1	126.2	24.8	-
	10^{-4}	6.5	2.5	-0.4	-1.3	86.1	21.3	-
Padimate-O	10^{-11}	0.2	0.8	0.5	-0.9	96.5	9.5	-
	10^{-10}	0.3	0.7	0.4	-0.7	99.0	7.6	-
	10^{-9}	0.9	1.2	1.2	-0.4	92.3	6.6	-
	10^{-8}	0.4	0.9	1.3	0.1	90.6	10.8	-
	10^{-7}	1.2	0.7	1.2	0.4	93.7	8.2	-
	10^{-6}	0.6	0.3	1.6	0.8	95.0	6.5	-
	10^{-5}	2.3	0.7	1.2	1.4	98.6	15.8	-
	10^{-4}	2.2	1.6	0.5	-0.8	96.7	31.9	-

RTA = Relative Transcriptional Activation
PC = Positive Control (1 nM 17β-Estradiol)
VC = Vehicle Control
SD = Standard Deviation
+ = Precipitation observed
- = No precipitation observed

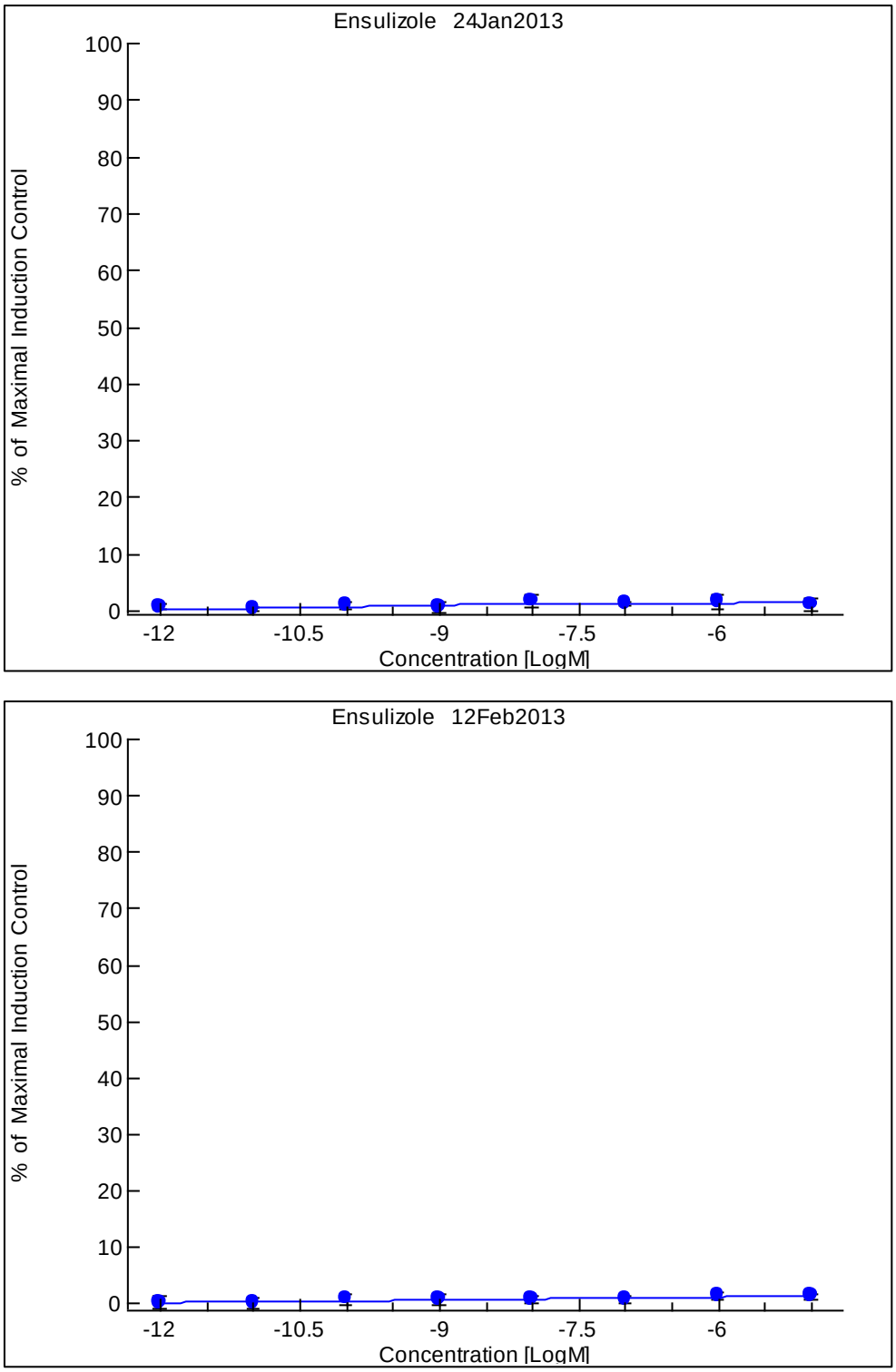
TABLE 6 $LogPC_{50}$, $LogPC_{10}$, $LogEC_{50}$ and Hill Slope Values for the Reference Chemicals

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	-10.4	-10.7	-11.1	-11.6	-10.3	-10.2	1.5	1.1	31.8	25.6
17α-Estradiol	-8.5	-8.8	-9.5	-9.7	-8.4	-8.4	1.2	1.0	33.5	27.5
Corticosterone	-	-	-	-	-	-	-	-	32.7	24.4
17α-Methyltestosterone	-	-	-5.8	-5.8	-	-	-	-	38.2	27.1

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

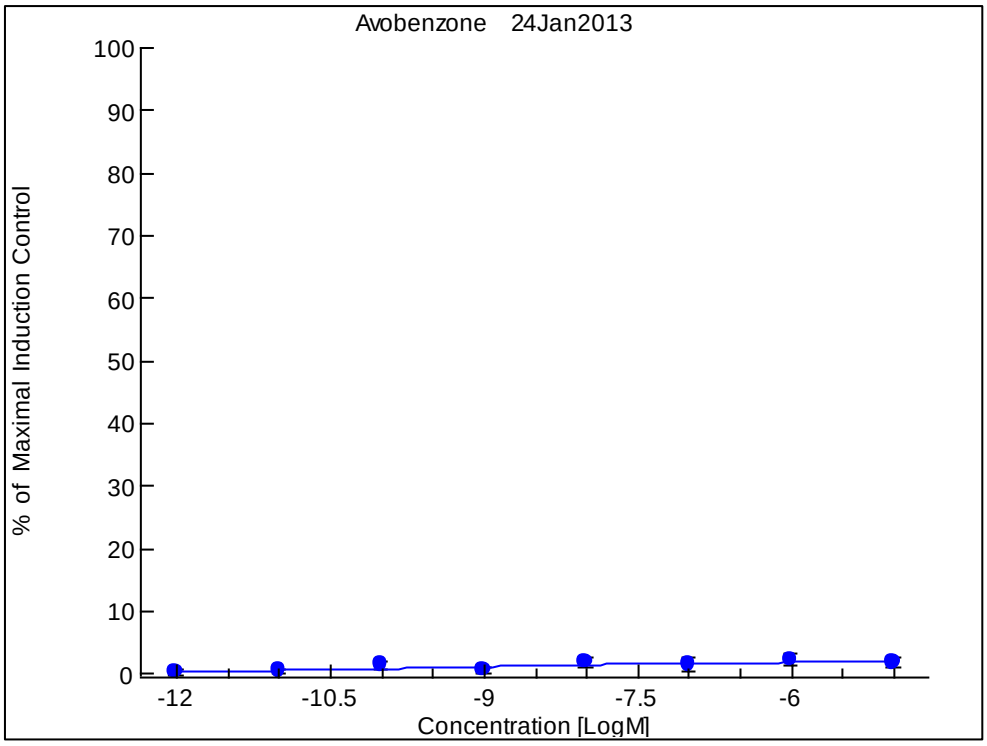
FIGURES SECTION

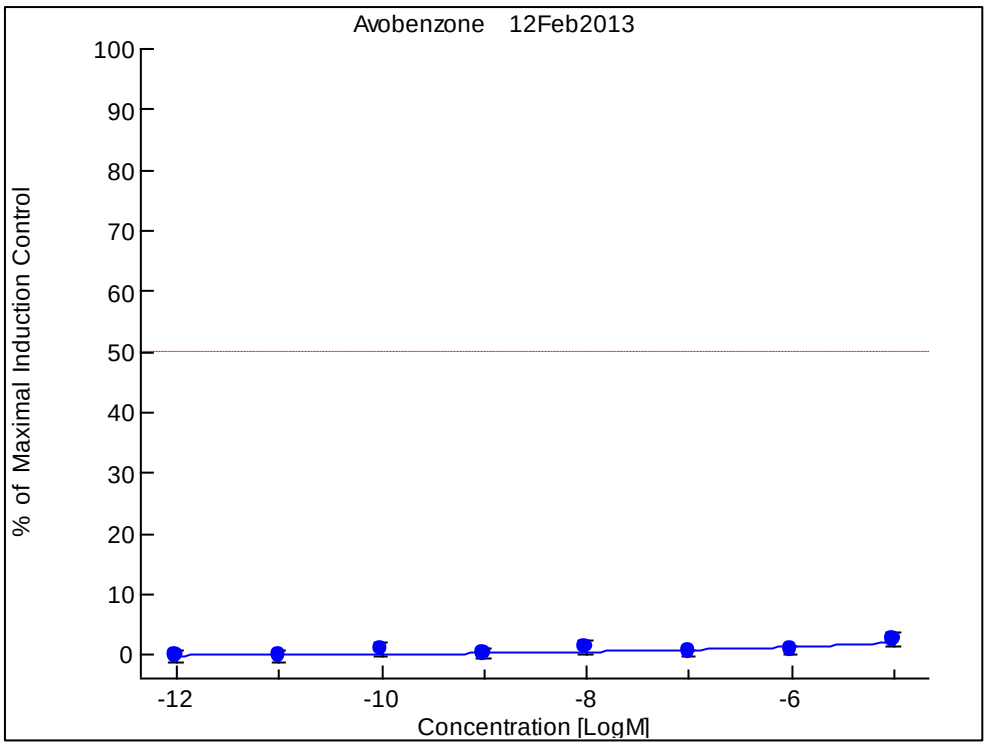
FIGURE 1 Ensulizole – Relative Transcriptional Activation



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).

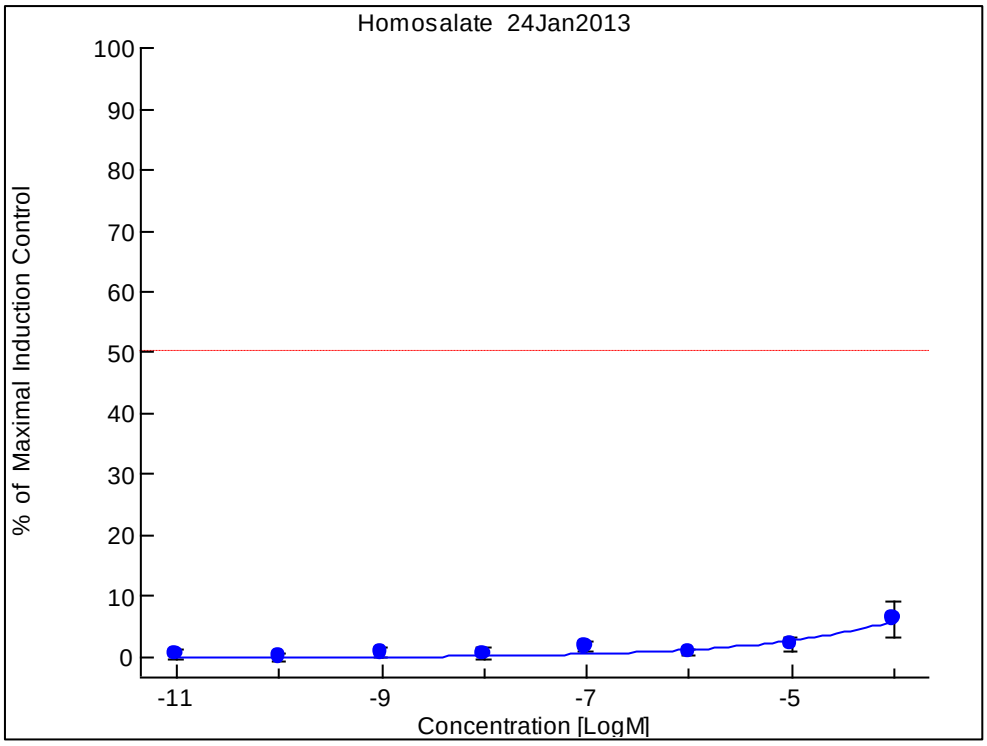
FIGURE 2 Avobenzone – Relative Transcriptional Activation

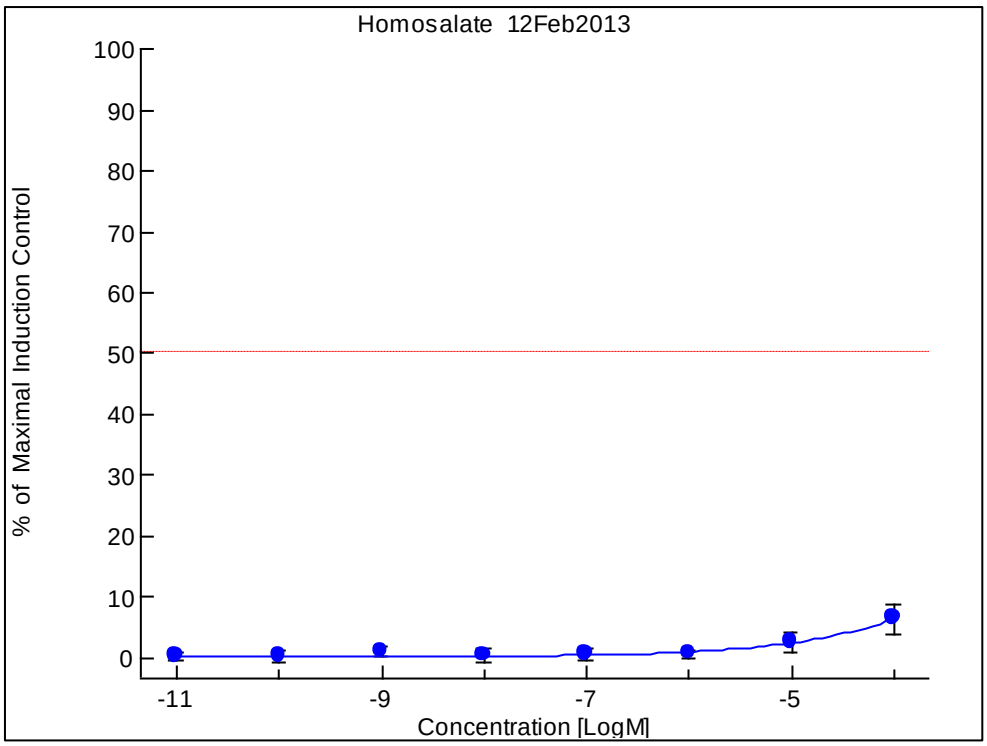




The two separate graphs represent the data (Means ± Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

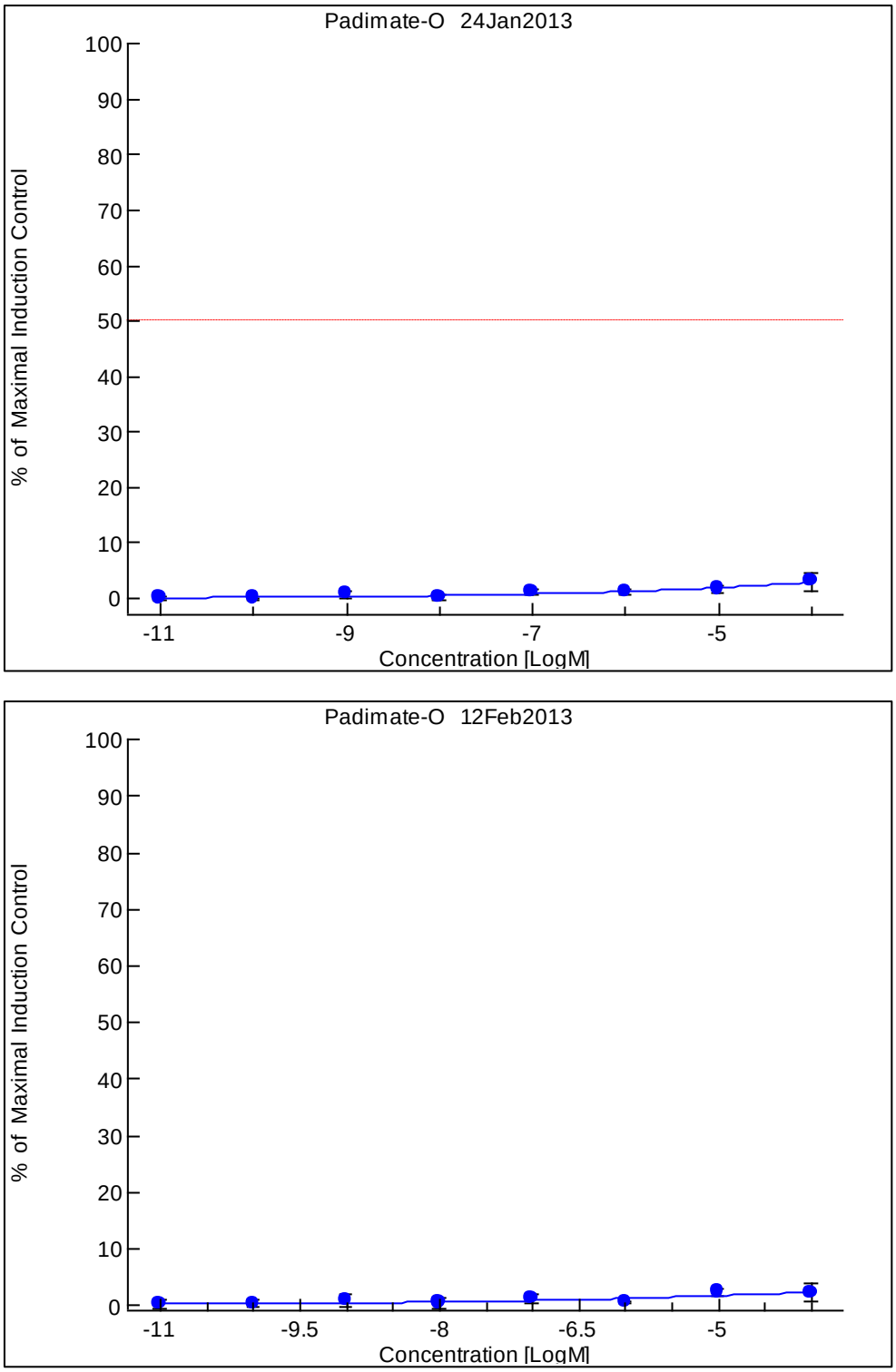
FIGURE 3 Homosalate – Relative Transcriptional Activation





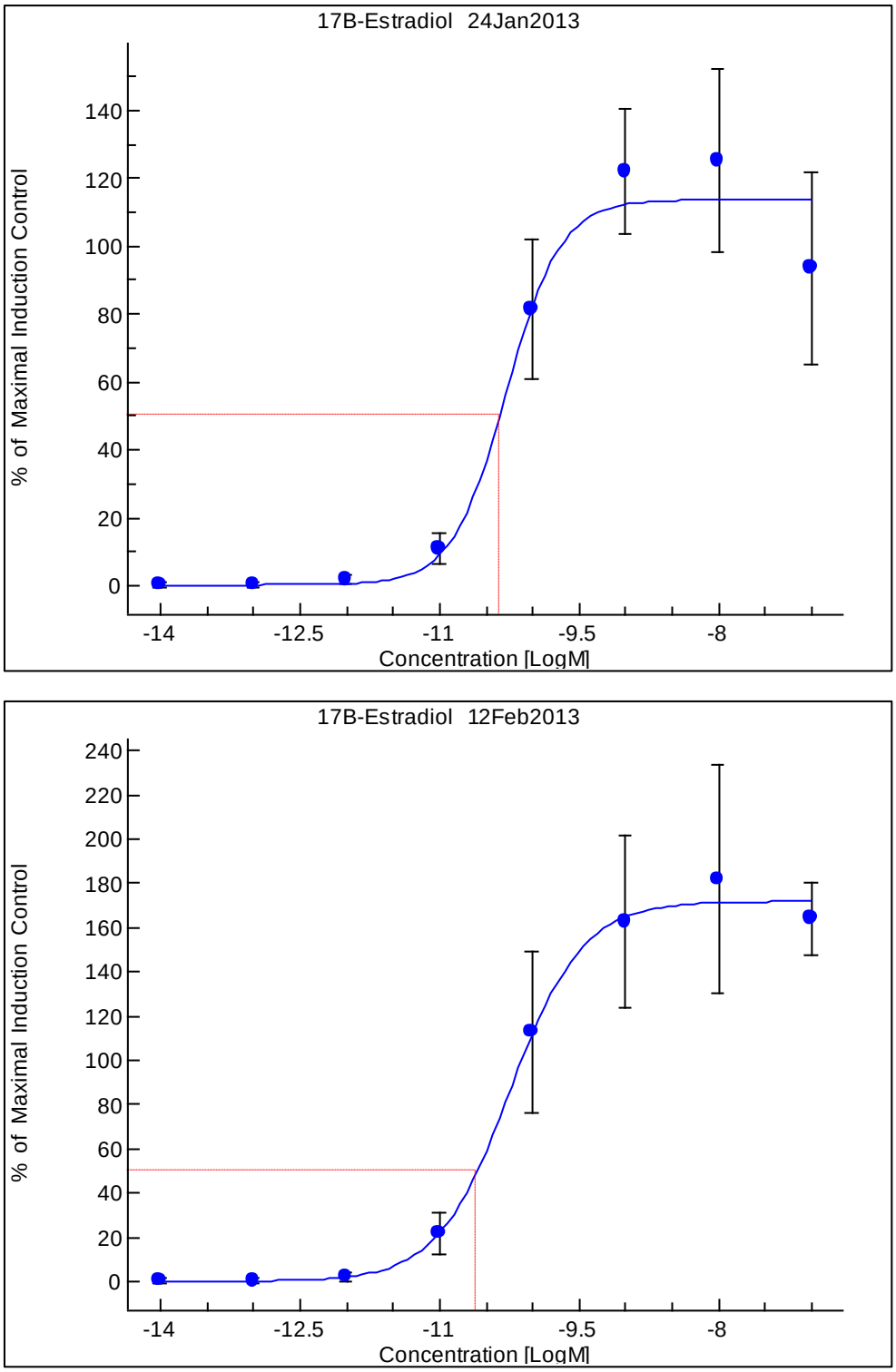
The two separate graphs represent the data (Means ± Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

FIGURE 4 Padimate-O – Relative Transcriptional Activation



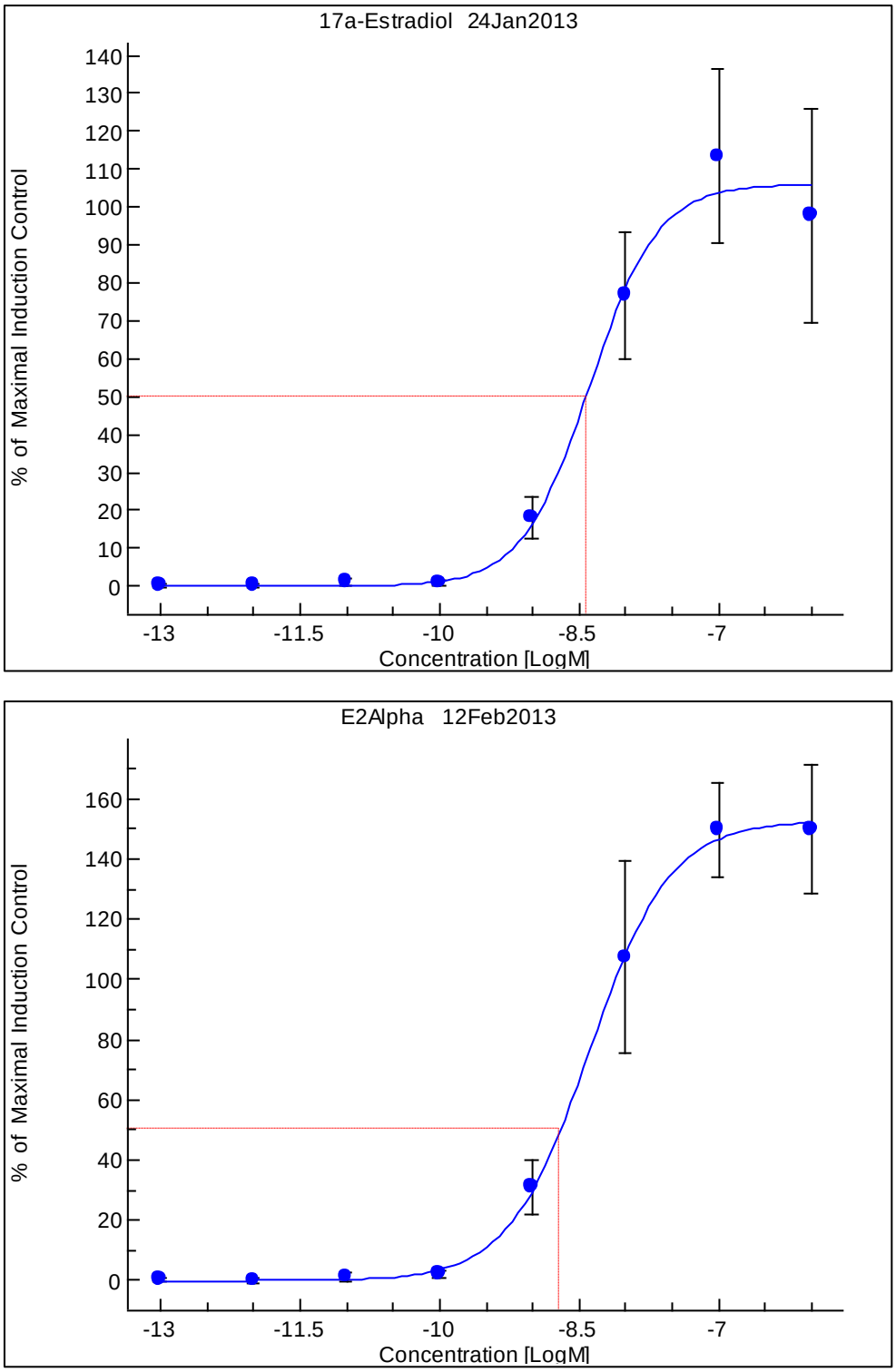
The two separate graphs represent the data (Means ± Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

FIGURE 5 17β-Estradiol – Relative Transcriptional Activation



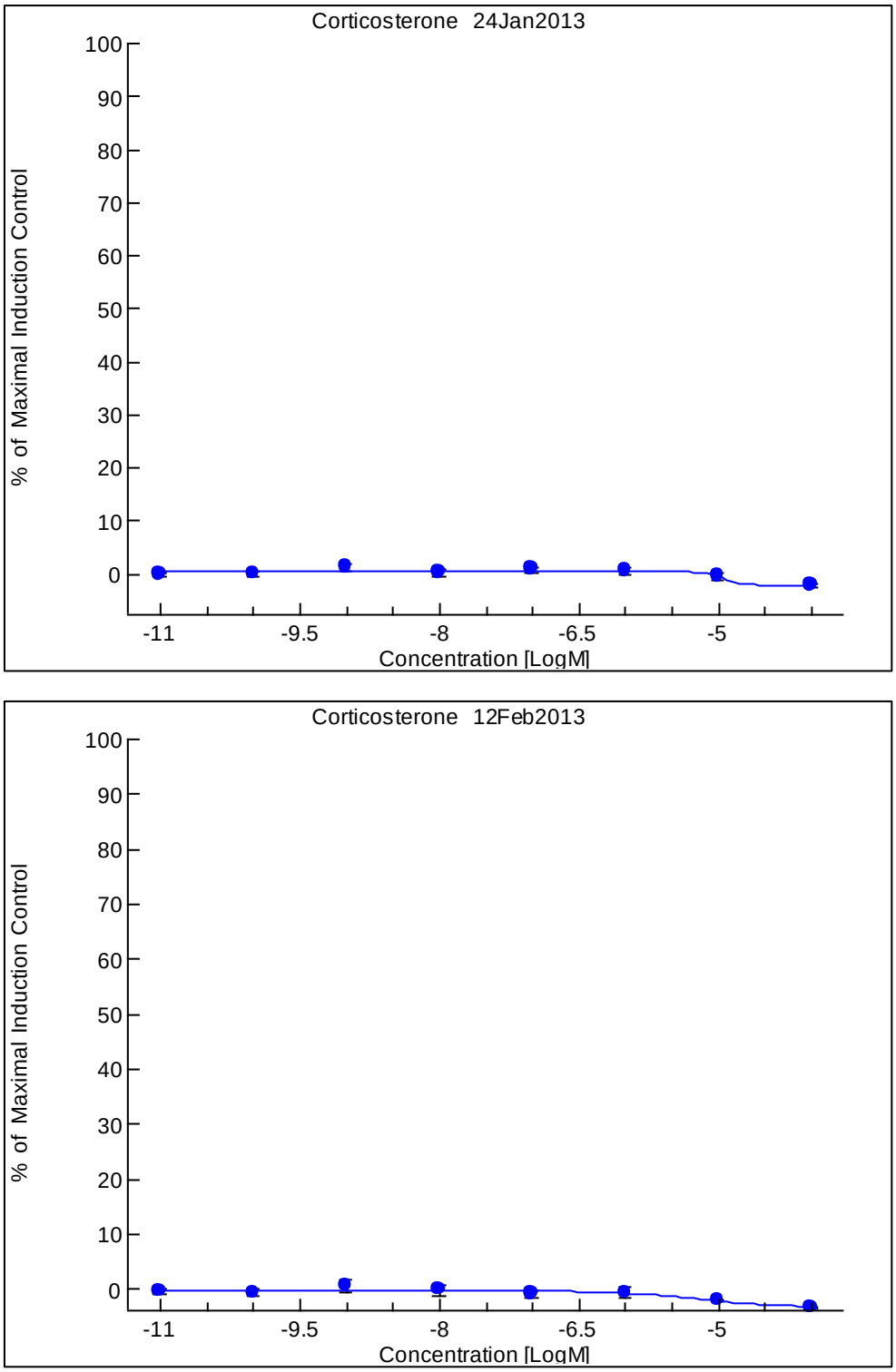
The two separate graphs represent the data (Means ± Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

FIGURE 6 17α-Estradiol – Relative Transcriptional Activation



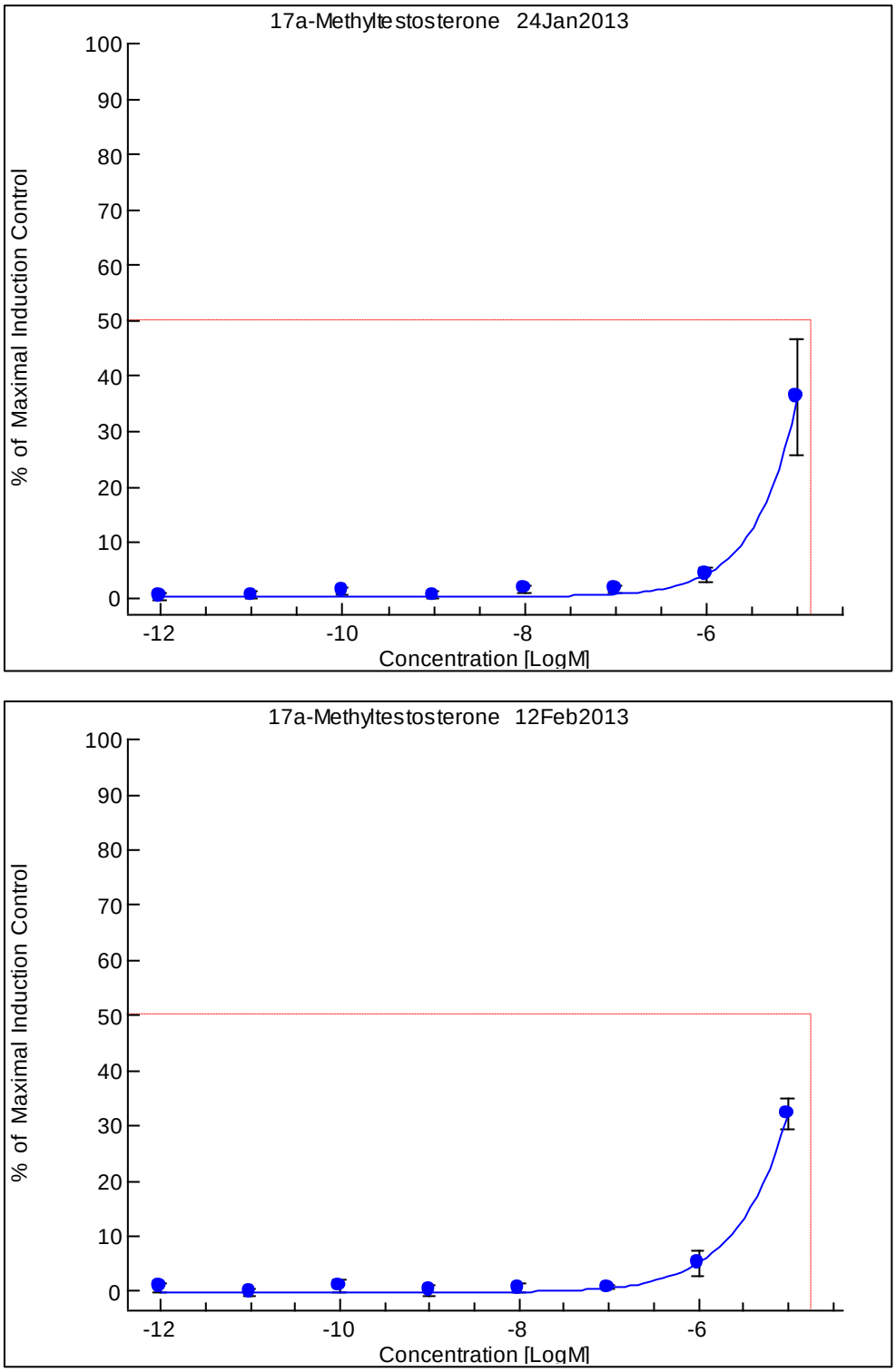
The two separate graphs represent the data (Means ± Standard Error of the Mean) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

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
January 24, 2013

					Concentration [LogM]							
Ensulizole (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	905850	20850	19700	31000	24750	23650	18000	27300	35300	25150	22150
B	0	902400	28800	28900	29150	23600	32850	24450	36650	35550	58900	32950
C	0	934450	28600	29250	32300	31000	34700	34450	49050	39950	37350	35250
D	0	986300	31400	26300	41300	30350	44200	38400	55650	38150	44600	53400
E	0	963200	26850	25100	32400	33450	38950	44900	44350	45000	36850	34350
F	0	1143250	24300	23300	28600	28550	34350	31200	41750	38550	41650	44300
G	0	20900	21450	20600	21250	22200	27250	25350	28250	33300	33900	25000
H	0	10450	8300	9700	8100	7550	9250	7350	15650	17300	15500	12200

Mean Vehicle Control (VC): 26113

Mean ICI 182,780 Control: 15013

					Concentration [LogM]							
Ensulizole (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	879738	-5263	-6413	4888	-1363	-2463	-8113	1188	9188	-963	-3963
B	0	876288	2688	2788	3038	-2513	6738	-1663	10538	9438	32788	6838
C	0	908338	2488	3138	6188	4888	8588	8338	22938	13838	11238	9138
D	0	960188	5288	188	15188	4238	18088	12288	29538	12038	18488	27288
E	0	937088	738	-1013	6288	7338	12838	18788	18238	18888	10738	8238
F	0	1117138	-1813	-2813	2488	2438	8238	5088	15638	12438	15538	18188
G	0	5888	6438	5588	6238	7188	12238	10338	13238	18288	18888	9988
H	0	-4563	-6713	-5313	-6913	-7463	-5763	-7663	638	2288	488	-2813

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 12, 2013

					Concentration [LogM]							
Ensulizole (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	100	698700	25950	23350	18800	19250	22950	24150	30500	35150	33200	37650
B	50	821500	32850	33400	27400	31800	33650	44750	32800	46200	37100	47600
C	0	984400	35000	29700	31450	29400	39200	41450	40400	35500	40050	43350
D	0	759750	26300	31150	30650	27600	42400	36950	37650	41450	49950	39700
E	100	888950	34400	36650	40900	36750	41350	33550	37500	30000	43700	41850
F	0	837500	34400	29600	43250	40850	44600	41450	44550	33350	45400	38350
G	0	29550	28750	27650	29900	32250	43950	44000	37700	35200	34300	34750
H	0	31950	27700	31050	24600	26050	33550	28400	29950	29600	34800	25900

Mean Vehicle Control (VC): 31063

Mean ICI Control: 182,780 28788

					Concentration [LogM]							
Ensulizole (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	100	667638	-5113	-7713	-12263	-11813	-8113	-6913	-563	4088	2138	6588
B	50	790438	1788	2338	-3663	738	2588	13688	1738	15138	6038	16538
C	0	953338	3938	-1363	388	-1663	8138	10388	9338	4438	8988	12288
D	0	728688	-4763	88	-413	-3463	11338	5888	6588	10388	18888	8638
E	100	857888	3338	5588	9838	5688	10288	2488	6438	-1063	12638	10788
F	0	806438	3338	-1463	12188	9788	13538	10388	13488	2288	14338	7288
G	0	763	-38	-1138	1113	3463	15163	15213	8913	6413	5513	5963
H	0	3163	-1088	2263	-4188	-2738	4763	-388	1163	813	6013	-2888

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
January 24, 2013

					Concentration [LogM]							
Avobenzone (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	709700	32200	18900	23750	31850	32550	31500	38150	30600	51200	41050
B	50	1143150	32300	27500	33500	29950	46300	35350	43650	35600	39000	41550
C	50	1015150	30900	24300	32500	39150	49850	36900	51900	62350	59150	52000
D	50	1147250	42400	32050	36800	40850	42800	44850	47400	51150	62500	50550
E	50	1013850	34650	30550	40750	37300	52750	36050	61550	46300	44550	60700
F	0	1208850	27050	37400	34150	32100	42650	36000	55000	46100	60350	44500
G	50	11050	13750	10900	14250	14550	14050	11400	22650	23350	20000	31650
H	0	5950	7650	6800	7600	6100	6950	6050	10700	9450	8300	11850

Mean Vehicle Control (VC): 30850

Mean ICI 182,780 Control: 9775

					Concentration [LogM]							
Avobenzone (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	678850	1350	-11950	-7100	1000	1700	650	7300	-250	20350	10200
B	50	1112300	1450	-3350	2650	-900	15450	4500	12800	4750	8150	10700
C	50	984300	50	-6550	1650	8300	19000	6050	21050	31500	28300	21150
D	50	1116400	11550	1200	5950	10000	11950	14000	16550	20300	31650	19700
E	50	983000	3800	-300	9900	6450	21900	5200	30700	15450	13700	29850
F	0	1178000	-3800	6550	3300	1250	11800	5150	24150	15250	29500	13650
G	50	1275	3975	1125	4475	4775	4275	1625	12875	13575	10225	21875
H	0	-3825	-2125	-2975	-2175	-3675	-2825	-3725	925	-325	-1475	2075

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 12, 2013

					Concentration [LogM]							
Avobenzone (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	467450	22550	27100	15400	19150	20100	21300	25850	30850	27900	41000
B	50	719400	28650	36400	26650	21750	41450	26950	40500	29950	39800	43550
C	0	686100	31750	28000	27450	28950	36950	32550	39250	36150	29600	45050
D	0	845700	24900	30000	28550	28150	36200	32800	30250	27800	36000	34100
E	0	777650	29450	28900	32950	31100	38050	35950	43500	28300	36100	53700
F	0	712700	36800	27500	31300	36950	36550	31300	42450	34800	35050	54600
G	0	27300	25200	24450	26800	27300	33250	29800	34700	31600	32450	36150
H	50	21950	24100	20350	22800	15400	25150	22500	24300	28800	28600	26400

Mean Vehicle Control (VC): 29333

Mean ICI 182,780 Control: 23525

					Concentration [LogM]							
Avobenzone (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	438117	-6783	-2233	-13933	-10183	-9233	-8033	-3483	1517	-1433	11667
B	50	690067	-683	7067	-2683	-7583	12117	-2383	11167	617	10467	14217
C	0	656767	2417	-1333	-1883	-383	7617	3217	9917	6817	267	15717
D	0	816367	-4433	667	-783	-1183	6867	3467	917	-1533	6667	4767
E	0	748317	117	-433	3617	1767	8717	6617	14167	-1033	6767	24367
F	0	683367	7467	-1833	1967	7617	7217	1967	13117	5467	5717	25267
G	0	3775	1675	925	3275	3775	9725	6275	11175	8075	8925	12625
H	50	-1575	575	-3175	-725	-8125	1625	-1025	775	5275	5075	2875

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
January 24, 2013

					Concentration [LogM]							
Homosalate (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	673150	22400	14700	26950	17100	22500	26650	27600	24200	26050	40150
B	0	800000	23000	23050	22400	26800	26200	26650	42500	30650	43800	82450
C	0	732550	40950	25250	28450	27200	31050	24050	35300	34650	45300	70350
D	50	812850	30500	26050	41550	30350	36800	31450	39600	34200	49550	108550
E	0	1004850	27100	28000	31800	27850	39050	44400	47300	34450	45450	66650
F	0	895650	24000	25850	23150	21500	29950	23550	41800	35400	45700	84000
G	0	20850	17900	16400	19900	21850	29900	25050	29450	28750	30750	15300
H	0	13000	14450	14500	10050	10000	11350	10500	16200	20300	9200	13650

Mean Vehicle Control (VC): 25904

Mean ICI 182,780 Control: 15813

					Concentration [LogM]							
Homosalate (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	647246	-3504	-11204	1046	-8804	-3404	746	1696	-1704	146	14246
B	0	774096	-2904	-2854	-3504	896	296	746	16596	4746	17896	56546
C	0	706646	15046	-654	2546	1296	5146	-1854	9396	8746	19396	44446
D	50	786946	4596	146	15646	4446	10896	5546	13696	8296	23646	82646
E	0	978946	1196	2096	5896	1946	13146	18496	21396	8546	19546	40746
F	0	869746	-1904	-54	-2754	-4404	4046	-2354	15896	9496	19796	58096
G	0	5038	2088	588	4088	6038	14088	9238	13638	12938	14938	-513
H	0	-2813	-1363	-1313	-5763	-5813	-4463	-5313	388	4488	-6613	-2163

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 12, 2013

					Concentration [LogM]							
Homosalate (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	582950	18600	21350	19900	15100	20950	14500	17350	20600	25100	50500
B	0	641150	24550	20650	20600	21850	32400	24650	26100	28400	33300	57300
C	0	524450	27700	24000	26150	26500	33250	29000	27400	29250	36400	81400
D	0	563250	21950	26900	24700	27150	31050	30150	34400	26300	50600	56450
E	0	736750	24850	23000	27100	29450	34400	29850	27600	31350	39800	46500
F	100	612800	26750	24650	29200	30250	29050	30350	31200	30400	45600	77350
G	0	24750	20600	21550	21700	28050	32450	31350	30250	27350	26450	17650
H	0	18850	18500	19800	17200	17950	21250	15750	21600	21400	19650	12650

Mean Vehicle Control (VC): 23746

Mean ICI 182,780 Control: 20113

					Concentration [LogM]							
Homosalate (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	559204	-5146	-2396	-3846	-8646	-2796	-9246	-6396	-3146	1354	26754
B	0	617404	804	-3096	-3146	-1896	8654	904	2354	4654	9554	33554
C	0	500704	3954	254	2404	2754	9504	5254	3654	5504	12654	57654
D	0	539504	-1796	3154	954	3404	7304	6404	10654	2554	26854	32704
E	0	713004	1104	-746	3354	5704	10654	6104	3854	7604	16054	22754
F	100	589054	3004	904	5454	6504	5304	6604	7454	6654	21854	53604
G	0	4638	488	1438	1588	7938	12338	11238	10138	7238	6338	-2463
H	0	-1263	-1613	-313	-2913	-2163	1138	-4363	1488	1288	-463	-7463

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
January 24, 2013

					Concentration [LogM]							
Padimate-O (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	562150	19150	14000	18400	20150	18300	16750	24550	26100	27450	28150
B	0	599950	20850	27000	22350	22350	26250	23100	34350	34750	38300	48600
C	0	970650	32600	20250	23850	21050	28900	26750	35850	28350	40650	39900
D	50	762650	20400	17950	24150	22750	28750	23550	28750	31700	30700	36800
E	50	1032000	24250	24400	23600	20200	34650	28050	31200	29500	38300	60550
F	50	788100	19800	22800	21900	23450	29650	22350	32350	35800	35550	56700
G	0	21450	20500	15200	18000	21500	21500	19200	24600	26250	22600	22750
H	0	11250	14650	9550	8650	9000	10400	8350	12150	19450	14850	14000

Mean Vehicle Control (VC): 21954

Mean ICI Control: 182,780 14975

					Concentration [LogM]							
Padimate-O (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	540196	-2804	-7954	-3554	-1804	-3654	-5204	2596	4146	5496	6196
B	0	577996	-1104	5046	396	396	4296	1146	12396	12796	16346	26646
C	0	948696	10646	-1704	1896	-904	6946	4796	13896	6396	18696	17946
D	50	740696	-1554	-4004	2196	796	6796	1596	6796	9746	8746	14846
E	50	1010046	2296	2446	1646	-1754	12696	6096	9246	7546	16346	38596
F	50	766146	-2154	846	-54	1496	7696	396	10396	13846	13596	34746
G	0	6475	5525	225	3025	6525	6525	4225	9625	11275	7625	7775
H	0	-3725	-325	-5425	-6325	-5975	-4575	-6625	-2825	4475	-125	-975

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 12, 2013

					Concentration [LogM]							
Padimate-O (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	557400	21750	18850	17100	18400	16800	16250	21850	23600	31800	29900
B	50	708950	28800	19550	20800	24050	24150	23400	29350	25950	43350	39950
C	0	600050	21300	28650	28300	24100	27150	31400	33000	24800	38500	25650
D	50	586400	24750	20350	25700	23500	39800	24400	31700	27400	33700	28800
E	50	668050	23450	22800	30500	25700	30150	27150	33850	26550	37550	51800
F	0	730850	21050	21600	22600	31150	30650	30350	30750	28950	37900	41200
G	50	24000	23500	20300	24200	23100	28500	29200	28150	31000	28300	24200
H	0	24600	19800	19950	15250	16700	18200	21650	23350	25600	29700	16100

Mean Vehicle Control (VC): 22742

Mean ICI 182,780 Control: 20888

					Concentration [LogM]							
Padimate-O (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	534658	-992	-3892	-5642	-4342	-5942	-6492	-892	858	9058	7158
B	50	686208	6058	-3192	-1942	1308	1408	658	6608	3208	20608	17208
C	0	577308	-1442	5908	5558	1358	4408	8658	10258	2058	15758	2908
D	50	563658	2008	-2392	2958	758	17058	1658	8958	4658	10958	6058
E	50	645308	708	58	7758	2958	7408	4408	11108	3808	14808	29058
F	0	708108	-1692	-1142	-142	8408	7908	7608	8008	6208	15158	18458
G	50	3113	2613	-588	3313	2213	7613	8313	7263	10113	7413	3313
H	0	3713	-1088	-938	-5638	-4188	-2688	763	2463	4713	8813	-4788

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
January 24, 2013

					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	0	692150	34250	20650	25450	20700	28000	102450	615150	1065200	885950	508300
B	0	1085600	34800	31000	34150	38000	46150	109700	697450	1307550	1201650	1034150
C	0	1113900	34900	33850	42300	35900	63150	207500	1123100	1217800	1531200	924650
D	0	1255350	37800	34950	41200	52950	60800	208150	1034050	1612350	1548250	1271850
E	0	1278500	45800	42400	46800	47950	69300	173600	1147950	1477700	1627850	1244200
F	0	1307700	37300	35600	43550	39250	60300	116500	894650	1475100	1575650	1331050
G	0	29600	24700	26050	33300	37450	31850	31800	37500	39800	46800	86500
H	50	15800	15000	11250	14050	11750	15750	13400	20550	20650	22000	60700

Mean Vehicle Control (VC): 35275

Mean ICI Control: 182,780

19250

					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	0	656875	-1025	-14625	-9825	-14575	-7275	67175	579875	1029925	850675	473025
B	0	1050325	-475	-4275	-1125	2725	10875	74425	662175	1272275	1166375	998875
C	0	1078625	-375	-1425	7025	625	27875	172225	1087825	1182525	1495925	889375
D	0	1220075	2525	-325	5925	17675	25525	172875	998775	1577075	1512975	1236575
E	0	1243225	10525	7125	11525	12675	34025	138325	1112675	1442425	1592575	1208925
F	0	1272425	2025	325	8275	3975	25025	81225	859375	1439825	1540375	1295775
G	0	10350	5450	6800	14050	18200	12600	12550	18250	20550	27550	67250
H	50	-3450	-4250	-8000	-5200	-7500	-3500	-5850	1300	1400	2750	41450

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 12, 2013

					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	0	452650	14650	26750	12700	12600	22250	84650	335000	771050	716050	1242100
B	0	769200	36900	28200	31100	28350	45800	126000	812200	924900	1277300	1131050
C	0	758050	28350	26150	38600	34850	60650	199950	896700	1290000	1475950	986300
D	50	848150	31950	26750	33850	32200	40800	218150	1057300	1281150	1488150	1240850
E	50	741050	31200	33500	34250	38550	45400	267650	878300	1503450	1168350	1244950
F	0	875050	31100	31950	35150	33950	52050	209200	999750	1334800	1801550	1318300
G	0	26850	25250	24050	29950	29650	37900	35450	34350	30750	39000	126500
H	50	22350	21000	21950	17300	19050	25250	20550	23450	24500	28100	64450

Mean Vehicle Control (VC): 28954

Mean ICI 182,780 Control: 23063

					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	0	423696	-14304	-2204	-16254	-16354	-6704	55696	306046	742096	687096	1213146
B	0	740246	7946	-754	2146	-604	16846	97046	783246	895946	1248346	1102096
C	0	729096	-604	-2804	9646	5896	31696	170996	867746	1261046	1446996	957346
D	50	819196	2996	-2204	4896	3246	11846	189196	1028346	1252196	1459196	1211896
E	50	712096	2246	4546	5296	9596	16446	238696	849346	1474496	1139396	1215996
F	0	846096	2146	2996	6196	4996	23096	180246	970796	1305846	1772596	1289346
G	0	3788	2188	988	6888	6588	14838	12388	11288	7688	15938	103438
H	50	-713	-2063	-1113	-5763	-4013	2188	-2513	388	1438	5038	41388

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
January 24, 2013

					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	874450	22800	24950	23400	22550	27000	24850	124450	621050	771850	564200
B	50	1087600	31950	27800	30100	29450	34900	43000	193050	636100	1109050	1043950
C	50	1075750	35500	31750	33950	40050	44800	43550	241250	870300	1219250	1291200
D	0	984750	30200	33250	36600	34150	45150	45100	268450	927800	1383800	1131550
E	50	1299800	35200	37550	36600	33000	51800	45550	271100	1061250	1303700	1336800
F	0	1050000	41750	27900	35600	32900	49000	41950	205150	816500	1407000	864100
G	0	28600	28950	25700	31400	27100	34700	31350	38400	38750	40650	39350
H	0	13550	13150	11750	10400	11850	11150	10450	17400	18600	18350	21200

Mean Vehicle Control (VC): 31717

Mean ICI 182,780 Control: 19888

					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	842733	-8917	-6767	-8317	-9167	-4717	-6867	92733	589333	740133	532483
B	50	1055883	233	-3917	-1617	-2267	3183	11283	161333	604383	1077333	1012233
C	50	1044033	3783	33	2233	8333	13083	11833	209533	838583	1187533	1259483
D	0	953033	-1517	1533	4883	2433	13433	13383	236733	896083	1352083	1099833
E	50	1268083	3483	5833	4883	1283	20083	13833	239383	1029533	1271983	1305083
F	0	1018283	10033	-3817	3883	1183	17283	10233	173433	784783	1375283	832383
G	0	8713	9063	5813	11513	7213	14813	11463	18513	18863	20763	19463
H	0	-6338	-6738	-8138	-9488	-8038	-8738	-9438	-2488	-1288	-1538	1313

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 12, 2013

					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	50	716800	22600	20350	26350	13900	17650	31500	183400	560250	1107450	1700100
B	0	870950	36750	28100	26900	33000	43050	42400	241800	944100	1342200	1199700
C	0	1076000	31900	34400	35800	32050	42450	61400	315850	959050	1298850	1193500
D	0	794250	31950	35850	36450	35250	48700	53050	362250	879200	1472550	1356950
E	0	1093250	39250	39100	36850	43200	51250	63150	352100	1118600	1438300	1392000
F	0	938400	41750	37100	45500	36550	48100	52250	384500	1414150	1457750	1274900
G	0	28900	31550	27150	36400	38600	43250	40400	41200	37400	40250	63100
H	0	30050	26850	27050	29100	26000	30250	27700	34850	37600	38150	30350

Mean Vehicle Control (VC): 33258

Mean ICI 182,780 Control: 28150

					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	50	683542	-10658	-12908	-6908	-19358	-15608	-1758	150142	526992	1074192	1666842
B	0	837692	3492	-5158	-6358	-258	9792	9142	208542	910842	1308942	1166442
C	0	1042742	-1358	1142	2542	-1208	9192	28142	282592	925792	1265592	1160242
D	0	760992	-1308	2592	3192	1992	15442	19792	328992	845942	1439292	1323692
E	0	1059992	5992	5842	3592	9942	17992	29892	318842	1085342	1405042	1358742
F	0	905142	8492	3842	12242	3292	14842	18992	351242	1380892	1424492	1241642
G	0	750	3400	-1000	8250	10450	15100	12250	13050	9250	12100	34950
H	0	1900	-1300	-1100	950	-2150	2100	-450	6700	9450	10000	2200

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
January 24, 2013

					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	701250	21500	14600	26800	25850	35050	20250	28200	22750	14650	4750
B	0	904900	32400	28450	26050	24300	33650	26800	36850	31500	24850	9200
C	0	1128350	28850	37150	24850	27750	40150	33800	36450	33300	29950	8300
D	50	855900	28000	25200	27700	30050	40850	30200	37450	39350	22750	11650
E	0	1039100	36250	37150	34300	35050	50000	38750	42050	35900	28950	12050
F	0	953700	24250	27600	28800	30350	43050	32650	37000	36400	23600	9000
G	0	21750	21050	22800	25400	24050	32600	25600	30450	26300	18050	6800
H	50	13350	14550	13650	12900	12350	12300	12300	17400	18350	13150	4000

Mean Vehicle Control (VC): 28450

Mean ICI 182,780 Control: 18013

					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	672800	-6950	-13850	-1650	-2600	6600	-8200	-250	-5700	-13800	-23700
B	0	876450	3950	0	-2400	-4150	5200	-1650	8400	3050	-3600	-19250
C	0	1099900	400	8700	-3600	-700	11700	5350	8000	4850	1500	-20150
D	50	827450	-450	-3250	-750	1600	12400	1750	9000	10900	-5700	-16800
E	0	1010650	7800	8700	5850	6600	21550	10300	13600	7450	500	-16400
F	0	925250	-4200	-850	350	1900	14600	4200	8550	7950	-4850	-19450
G	0	3738	3038	4788	7388	6038	14588	7588	12438	8288	38	-11213
H	50	-4663	-3463	-4363	-5113	-5663	-5713	-5713	-613	338	-4863	-14013

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 12, 2013

					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	741200	25450	32850	22750	20300	20600	21600	19300	20500	16250	5850
B	0	852950	29550	32100	31900	25000	44850	28500	26500	23650	14400	8000
C	50	817850	35000	39250	36600	31400	41350	33150	28400	39850	17700	8100
D	50	802950	27600	49200	31850	30450	41550	43950	31300	24700	21100	9300
E	0	883450	30850	37700	36050	34700	48150	37800	31750	30700	19800	7300
F	0	895250	32650	36600	29700	33300	41300	38200	30700	35800	19950	8450
G	50	29650	29700	29200	29000	29900	35100	30900	26750	24700	12050	4300
H	50	26050	26250	22500	24900	17400	17250	21000	23500	19100	11850	2600

Mean Vehicle Control (VC): 34067

Mean ICI 182,780 Control: 26913

					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	707133	-8617	-1217	-11317	-13767	-13467	-12467	-14767	-13567	-17817	-28217
B	0	818883	-4517	-1967	-2167	-9067	10783	-5567	-7567	-10417	-19667	-26067
C	50	783783	933	5183	2533	-2667	7283	-917	-5667	5783	-16367	-25967
D	50	768883	-6467	15133	-2217	-3617	7483	9883	-2767	-9367	-12967	-24767
E	0	849383	-3217	3633	1983	633	14083	3733	-2317	-3367	-14267	-26767
F	0	861183	-1417	2533	-4367	-767	7233	4133	-3367	1733	-14117	-25617
G	50	2738	2788	2288	2088	2988	8188	3988	-163	-2213	-14863	-22613
H	50	-863	-663	-4413	-2013	-9513	-9663	-5913	-3413	-7813	-15063	-24313

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
January 24, 2013

					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	655450	20750	14050	17100	22300	24400	19050	31500	28050	39650	192000
B	0	958900	21350	22350	22650	24200	32700	25250	34050	39100	59050	332400
C	0	1103650	27950	21350	34000	26050	35600	28150	42800	48450	66500	402700
D	0	960250	29600	27750	28450	31000	40200	34000	40100	37600	70350	476800
E	0	1054900	30450	31550	30550	39050	39750	32950	44700	42600	66500	344550
F	0	902250	23950	23650	27050	29000	42050	32750	45450	40350	68650	391300
G	0	24950	20650	19450	23450	24600	27100	24150	32050	26150	28650	16000
H	0	15300	13450	12000	10950	10050	14550	11250	16650	20650	14600	10800

Mean Vehicle Control (VC): 24563

Mean ICI 182,780 Control: 16388

					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	630888	-3813	-10513	-7463	-2263	-163	-5513	6938	3488	15088	167438
B	0	934338	-3213	-2213	-1913	-363	8138	688	9488	14538	34488	307838
C	0	1079088	3388	-3213	9438	1488	11038	3588	18238	23888	41938	378138
D	0	935688	5038	3188	3888	6438	15638	9438	15538	13038	45788	452238
E	0	1030338	5888	6988	5988	14488	15188	8388	20138	18038	41938	319988
F	0	877688	-613	-913	2488	4438	17488	8188	20888	15788	44088	366738
G	0	8563	4263	3063	7063	8213	10713	7763	15663	9763	12263	-388
H	0	-1088	-2938	-4388	-5438	-6338	-1838	-5138	263	4263	-1788	-5588

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 12, 2013

					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	630750	25850	23300	23750	19500	20550	17250	24150	34550	52150	311000
B	50	751950	31400	32050	44200	26600	37500	38250	32400	36800	53350	280500
C	0	864950	33700	34450	38150	31950	43950	34950	31300	36800	98500	306850
D	0	876600	24450	31400	36050	32100	47900	33550	41000	39800	73750	249750
E	0	1000600	37350	31800	38500	33100	43750	34550	41050	32550	80550	279850
F	0	863700	31750	30450	37000	31550	36200	33300	39800	33850	65600	300400
G	50	30950	29500	26550	31850	31250	35100	31800	34100	34600	32200	14450
H	0	19000	27950	24100	22750	23050	26350	26850	28900	34700	29250	9250

Mean Vehicle Control (VC): 30663

Mean ICI 182,780 Control: 27025

					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	600088	-4813	-7363	-6913	-11163	-10113	-13413	-6513	3888	21488	280338
B	50	721288	738	1388	13538	-4063	6838	7588	1738	6138	22688	249838
C	0	834288	3038	3788	7488	1288	13288	4288	638	6138	67838	276188
D	0	845938	-6213	738	5388	1438	17238	2888	10338	9138	43088	219088
E	0	969938	6688	1138	7838	2438	13088	3888	10388	1888	49888	249188
F	0	833038	1088	-213	6338	888	5538	2638	9138	3188	34938	269738
G	50	3925	2475	-475	4825	4225	8075	4775	7075	7575	5175	-12575
H	0	-8025	925	-2925	-4275	-3975	-675	-175	1875	7675	2225	-17775

APPENDIX 1 Raw Propidium Iodide Data

VALID RUN 1
January 24, 2013
READ 1

					Concentration [LogM]							
Ensulizole (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	170	239	173	199	246	258	219	223	224	213	219	239
B	201	232	213	221	269	242	218	229	249	229	236	215
C	201	252	265	248	239	222	222	229	240	227	238	238
D	182	210	217	223	234	206	215	228	214	193	230	226
E	215	227	242	264	211	239	226	227	229	209	231	221
F	176	197	235	213	228	200	230	240	249	210	211	211
G	264	583	633	543	540	572	529	542	581	525	562	513
H	212	514	533	508	477	465	472	457	517	503	500	500

READ 2

					Concentration [LogM]							
Ensulizole (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	445	1845	1436	2190	2014	2111	2124	2191	2039	2146	2227	2300
B	443	2537	2462	2567	2362	2633	2448	2399	2304	2343	2463	2051
C	422	2651	2454	2405	2330	2438	2657	2450	2516	2533	2523	2633
D	398	2638	2793	2484	2668	2643	2710	2601	2620	2709	2882	2759
E	451	2814	2888	2791	2807	2660	2753	2692	2503	2455	2448	2660
F	364	2292	2565	2467	2613	2571	2710	2637	2666	2734	2728	2463
G	473	825	801	762	742	718	858	758	737	713	672	593
H	421	552	573	753	594	754	675	580	582	639	571	529

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 12, 2013
READ 1

					Concentration [LogM]							
Ensulizole (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	205	241	185	192	181	208	194	182	178	145	97	76
B	241	235	248	241	247	217	211	246	240	230	266	301
C	270	236	251	217	229	245	233	240	212	273	204	233
D	222	225	272	228	221	244	189	238	231	239	234	272
E	245	226	229	241	229	217	228	249	241	232	208	167
F	244	237	251	197	225	205	218	194	249	223	197	188
G	225	633	692	712	700	741	753	676	624	724	667	620
H	224	630	633	738	621	617	654	619	580	637	670	695

READ 2

					Concentration [LogM]							
Ensulizole (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	453	1638	1914	2050	1886	2223	2389	2327	2385	2718	3467	2548
B	496	2442	2359	2613	2375	2408	2350	2318	2202	2263	2210	2167
C	482	2610	2528	2785	2698	2490	2443	2726	2578	2632	3056	3038
D	409	2619	2538	2622	2658	2579	2618	2755	2594	2807	2847	3098
E	479	2853	2860	2901	2778	2770	2717	2685	2697	2687	2542	2819
F	467	2215	2466	2518	2555	2640	2543	2557	2651	2827	2618	1920
G	453	861	939	1042	929	660	775	794	754	689	634	1203
H	481	628	700	731	711	651	658	646	791	680	774	857

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
January 24, 2013
READ 1

					Concentration [LogM]							
Avobenzone (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	179	219	184	199	218	214	189	201	207	223	212	235
B	252	217	319	254	212	200	222	241	222	241	233	186
C	147	219	195	250	229	229	227	243	220	241	239	219
D	247	236	275	240	224	224	235	240	223	217	227	246
E	249	246	244	240	237	216	254	237	244	236	225	260
F	203	265	233	220	252	237	215	240	224	225	228	237
G	214	646	686	669	677	650	644	680	677	761	755	613
H	225	627	691	674	710	705	777	654	744	615	783	573

READ 2

					Concentration [LogM]							
Avobenzone (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	354	2077	2013	2098	2092	2283	2256	2275	2391	2361	2479	2568
B	485	2786	2582	2654	2714	2726	2593	2619	2459	2524	2584	2408
C	338	2880	3050	2602	2539	2724	2705	2662	2669	2514	2448	3125
D	499	2972	2752	2857	2910	2920	2966	2809	2710	2610	2832	3122
E	442	3035	3160	3049	3206	3133	3022	2982	3003	2871	2712	2796
F	399	2788	2704	2811	2776	2855	2829	2803	2923	2903	2737	2522
G	425	894	992	885	880	1015	843	924	910	883	779	785
H	454	701	595	673	647	870	859	1053	884	796	907	768

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 12, 2013
READ 1

					Concentration [LogM]							
Avobenzone (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	239	253	268	213	240	221	235	229	225	257	255	255
B	217	229	243	224	246	197	223	247	251	228	212	226
C	250	235	287	199	233	223	210	236	242	205	210	233
D	216	247	266	211	200	214	206	235	225	221	228	261
E	218	253	213	242	244	226	233	231	257	247	218	246
F	232	221	238	201	219	222	232	244	215	227	203	195
G	248	630	654	690	667	690	668	627	660	668	492	611
H	239	636	579	622	587	636	606	527	586	551	518	552

READ 2

					Concentration [LogM]							
Avobenzone (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	475	1790	1971	2048	1866	2046	1992	2079	2252	2405	2289	2936
B	476	2677	2551	2418	2414	2569	2551	2530	2499	2401	2602	2091
C	481	2827	2587	2667	2537	2715	2687	2628	2531	2627	2736	2967
D	454	2704	2726	2476	2572	2469	2617	2560	2399	2592	2519	2781
E	445	2866	2818	2713	2898	2748	2586	2625	2602	2815	2338	2128
F	442	2238	2459	2339	2486	2512	2496	2524	2685	2776	2708	2382
G	451	821	874	1112	906	819	824	777	847	766	679	676
H	443	638	667	592	571	612	600	695	555	762	659	742

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
January 24, 2013
READ 1

					Concentration [LogM]							
Homosalate (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	163	210	203	182	198	186	187	180	194	171	185	267
B	210	262	228	211	232	205	230	228	209	223	277	234
C	228	214	228	217	221	235	212	203	207	194	245	331
D	197	204	245	236	218	218	191	206	233	195	287	245
E	220	202	221	215	201	209	242	202	240	238	200	232
F	206	231	223	219	226	206	253	224	220	269	243	309
G	200	661	616	579	551	564	519	581	548	515	545	479
H	211	556	585	582	617	575	609	574	608	606	558	577

READ 2

					Concentration [LogM]							
Homosalate (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	347	2084	1975	2149	2336	2259	2481	2326	2267	2459	2694	3307
B	459	2867	2562	2598	2611	2657	2572	2506	2454	2408	2280	2514
C	413	2853	2627	2563	2492	2603	2685	2730	2599	2695	2640	2543
D	422	2816	2594	2484	2517	2496	2561	2386	2404	2764	2915	2892
E	438	3023	2975	2707	2717	2814	2685	2761	2653	2889	2808	2352
F	428	2683	2639	2534	2585	2474	2744	2857	2707	2738	2766	2070
G	415	738	682	787	782	772	736	778	712	711	797	535
H	416	670	581	632	667	771	686	638	783	704	724	554

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 12, 2013
READ 1

					Concentration [LogM]							
Homosalate (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	203	219	209	189	199	185	192	146	158	134	139	202
B	231	239	234	246	260	252	241	262	214	238	188	358
C	206	228	231	247	222	217	229	230	218	229	236	457
D	209	220	232	208	244	221	216	231	228	251	205	281
E	260	220	256	204	232	229	221	220	239	224	325	300
F	233	227	245	238	240	237	221	238	235	234	184	275
G	208	530	576	531	560	561	567	570	496	571	406	604
H	233	450	487	440	478	474	480	460	536	539	555	418

READ 2

					Concentration [LogM]							
Homosalate (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	437	1297	1416	1766	2004	2344	2272	2493	2581	2949	2943	1756
B	485	2262	2175	2142	2288	2341	2246	2263	2251	2278	2759	1904
C	478	2641	2331	2549	2407	2461	2226	2321	2338	2441	2657	2358
D	435	2230	2406	2573	2466	2286	2297	261	2406	2420	2857	2592
E	454	2457	2500	2669	2519	2656	2477	2242	2344	2394	1921	1544
F	426	1710	2246	2251	2287	2249	2250	2302	2415	2479	2509	1960
G	466	739	736	897	860	757	831	827	599	691	630	660
H	475	706	619	636	659	614	662	715	819	664	934	525

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
January 24, 2013
READ 1

					Concentration [LogM]							
Padimate-O (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	221	229	251	232	235	224	232	234	226	199	213	193
B	223	227	270	222	240	193	222	249	210	221	210	232
C	204	220	225	249	200	238	219	206	222	225	213	240
D	213	207	235	236	220	206	188	240	215	214	239	261
E	222	229	228	222	245	214	211	233	218	234	236	222
F	245	203	191	216	190	230	229	219	211	209	222	222
G	219	718	595	657	622	633	645	600	631	643	666	524
H	207	702	678	611	629	602	688	600	593	607	586	860

READ 2

					Concentration [LogM]							
Padimate-O (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	447	1934	1961	2010	2171	2156	2180	2202	2392	2374	2283	2621
B	433	3008	2794	2830	2743	2705	2716	2746	2815	2712	2604	2535
C	438	2862	2673	2634	2503	2665	2664	2616	2753	2925	3026	2924
D	399	2994	3067	2955	3112	3004	2917	3081	2862	2795	3205	2990
E	436	3203	2990	2915	2878	2790	2835	2843	2757	2802	2792	2553
F	418	2223	2612	2531	2610	2606	2600	2832	2701	2726	2834	2202
G	403	839	828	804	791	845	879	767	787	890	786	732
H	405	637	557	651	687	600	646	684	668	632	676	671

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 12, 2013
READ 1

					Concentration [LogM]							
Padimate-O (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	233	231	236	226	264	228	223	227	220	203	209	312
B	222	225	236	222	234	213	206	253	215	223	180	160
C	222	218	248	254	241	234	204	229	232	246	309	264
D	226	251	211	227	244	205	245	250	270	264	229	244
E	208	215	261	206	222	217	235	244	255	229	195	226
F	229	241	226	244	237	223	231	226	206	227	213	243
G	255	661	655	739	797	714	705	718	708	741	740	590
H	215	703	599	671	659	638	681	611	604	492	566	638

READ 2

					Concentration [LogM]							
Padimate-O (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	595	2000	2102	1986	1932	1925	1896	1737	1916	1935	1835	1167
B	589	2083	2032	1934	1888	1915	1837	1826	1874	1911	1705	2123
C	607	2028	2017	1931	1966	2097	1854	1940	1857	1919	2120	2219
D	584	2118	2037	2188	2063	2036	2014	1922	2119	2018	2025	2225
E	557	2097	2040	2158	2080	2131	2010	2067	1993	2090	2288	2414
F	542	2324	2262	2268	2268	2199	2119	2173	2151	2141	2312	2082
G	588	785	771	751	711	793	846	752	791	758	726	830
H	531	829	729	728	758	756	762	876	730	891	856	870

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
January 24, 2013
READ 1

					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	217	272	235	214	220	201	232	211	209	183	220	221
B	227	205	235	244	233	207	250	220	230	226	236	238
C	200	215	238	252	249	215	219	215	212	213	207	248
D	274	218	234	240	235	240	236	212	250	225	235	245
E	255	218	216	249	213	248	237	244	208	237	205	203
F	222	211	210	205	234	203	240	246	208	233	207	202
G	211	637	642	748	563	601	583	580	618	631	654	624
H	201	545	660	595	558	524	543	528	575	601	575	533

READ 2

					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	427	1916	2154	1877	1939	1959	2099	2004	2007	2081	2211	1949
B	465	2361	2374	2287	2357	2369	2272	2348	2320	2350	2408	2191
C	434	2432	2569	2347	2298	2345	2319	2388	2488	2392	2518	2567
D	430	2589	2675	2418	2536	2613	2631	2573	2639	2505	2520	2725
E	427	2581	2724	2630	2638	2629	2652	2502	2465	2501	2379	2503
F	395	2542	2475	2216	2409	2465	2452	2450	2495	2608	2532	2611
G	378	765	722	632	648	646	694	655	630	679	680	564
H	432	534	543	578	604	548	559	555	547	520	523	597

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 12, 2013
READ 1

					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	250	254	281	241	219	255	217	206	228	220	218	225
B	201	213	241	288	264	214	258	244	249	240	234	235
C	235	255	235	213	246	230	241	244	213	249	200	230
D	207	224	247	239	236	228	211	232	230	236	255	249
E	255	241	242	245	221	234	237	221	247	225	238	232
F	261	238	239	223	248	229	252	246	247	269	235	230
G	246	485	535	556	571	562	586	532	482	501	495	350
H	264	430	421	403	465	412	453	472	387	366	359	391

READ 2

					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	486	1448	1465	1668	1665	1577	1739	1718	1767	1919	1980	2182
B	455	1885	2009	1994	2073	2095	2107	1995	1958	1992	1926	2122
C	503	1982	2051	2240	2204	2191	2229	2205	2023	2427	2226	2194
D	427	1953	2242	2328	2327	2293	2315	2433	2150	2517	2310	2192
E	478	2165	2508	2478	2509	2398	2474	2427	2162	2341	2323	1953
F	464	1998	2125	2328	2319	2361	2240	2386	2274	2476	2313	1883
G	458	759	728	791	698	671	688	724	685	568	599	544
H	483	637	662	722	716	661	719	638	860	724	709	818

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
January 24, 2013
READ 1

					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	210	197	193	183	178	198	183	174	199	157	194	175
B	230	213	221	225	233	211	211	218	182	230	211	246
C	243	229	250	239	246	214	209	220	226	215	231	216
D	202	214	228	246	247	206	224	250	231	194	214	246
E	224	218	255	251	234	240	227	223	225	230	222	229
F	239	210	220	236	239	237	217	216	211	192	244	218
G	188	572	554	563	517	511	541	498	557	532	512	508
H	214	455	526	543	563	572	541	515	496	512	519	507

READ 2

					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	402	1782	1896	1944	1989	2019	2188	2153	2127	2287	2374	2417
B	462	2368	2393	2326	2245	2423	2274	2445	2343	2405	2462	2182
C	448	2596	2374	2254	2220	2299	2309	2360	2184	2175	2286	2629
D	418	2496	2403	2405	2537	2406	2465	2298	2353	2355	2534	2589
E	465	2561	2222	2631	2607	2621	2634	2360	2526	2394	2493	2502
F	429	2261	2294	2327	2361	2410	2505	2464	2479	2400	2421	2721
G	415	699	695	758	624	709	686	647	660	746	783	678
H	470	577	570	724	790	704	822	788	792	765	833	740

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 12, 2013
READ 1

					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	221	267	248	264	264	230	249	238	225	212	163	204
B	204	205	253	261	248	235	188	240	224	205	211	238
C	242	226	257	289	244	260	234	241	221	233	220	236
D	220	234	258	214	247	254	280	203	230	225	260	187
E	238	232	224	242	249	216	223	226	219	230	206	262
F	220	248	260	254	235	232	212	211	233	254	231	210
G	240	587	627	614	642	603	651	560	577	620	609	533
H	215	516	594	587	555	559	546	514	578	565	468	426

READ 2

					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	512	1759	1703	1721	1838	1926	1926	1913	1903	1987	2117	2311
B	487	2189	2224	2247	2243	2211	2176	2169	2294	2396	2300	2219
C	463	2277	2285	2244	2157	2286	2318	2180	2130	2484	2389	2518
D	452	2471	2415	2356	2547	2493	2441	2552	2531	2888	2758	2652
E	487	2565	2437	2621	2604	2546	2578	2539	2079	2674	2450	2568
F	424	2230	2411	2402	2412	2576	2550	2596	2635	2697	2452	2171
G	461	761	957	879	856	820	886	814	658	577	886	886
H	460	589	569	528	575	696	545	665	569	600	541	527

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
January 24, 2013
READ 1

					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	223	233	224	221	199	209	219	214	215	212	200	227
B	192	221	212	238	203	210	215	211	226	234	210	236
C	221	237	216	226	221	228	245	219	235	219	229	209
D	220	228	224	202	209	223	204	232	238	222	198	216
E	203	212	228	243	231	197	218	204	251	223	217	219
F	206	245	230	208	216	209	199	234	245	219	224	236
G	221	606	561	494	492	513	506	493	541	533	573	519
H	224	517	519	428	447	510	496	497	532	505	474	480

READ 2

					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	436	1921	1872	2060	2113	2186	2161	2240	2211	2325	2223	2179
B	456	2388	2271	2296	2303	2453	2351	2163	2335	2202	2151	2012
C	447	2409	2316	2246	2416	2458	2390	2327	2294	2310	2333	2444
D	429	2628	2562	2478	2505	2307	2412	2396	2393	2604	2698	2490
E	418	2698	2542	2469	2602	2535	2480	2499	2523	2481	2357	2194
F	414	2391	2297	2256	2429	2450	2403	2487	2515	2502	2371	2351
G	427	674	726	617	623	708	775	706	693	697	714	583
H	393	569	548	526	615	573	559	646	594	642	533	567

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 12, 2013
READ 1

					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	209	202	222	179	180	186	159	158	195	184	136	152
B	214	202	222	226	216	201	245	242	270	212	275	269
C	220	235	219	230	250	241	227	217	220	218	216	231
D	219	252	253	235	234	257	224	237	242	253	219	249
E	252	260	255	223	247	229	244	237	224	216	262	220
F	214	216	240	228	241	216	217	237	228	216	227	251
G	233	489	531	596	622	558	616	517	514	596	562	521
H	277	505	526	525	481	581	550	463	510	487	510	507

READ 2

					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	455	1544	1768	1719	1859	2068	2322	2541	2265	2719	2750	2006
B	452	2091	2083	2089	2105	2156	2197	2155	2104	2035	1999	1671
C	475	2153	2229	2295	2259	2254	2351	2328	2272	2450	2435	2026
D	495	2334	2329	2245	2406	2362	2259	2269	2222	2510	2441	1999
E	489	2453	2526	2704	2656	2500	2473	2583	2396	2580	2036	1991
F	420	2089	2320	2228	2270	2324	2346	2335	2372	2440	2424	1997
G	424	815	687	761	818	710	854	902	586	755	845	829
H	491	636	698	733	724	655	690	669	787	591	604	607

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
January 24, 2013
READ 1

					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	203	206	237	219	189	218	183	199	211	195	207	195
B	233	222	270	241	206	233	212	230	188	245	239	253
C	216	238	236	250	222	211	218	241	238	229	208	239
D	202	246	216	220	219	219	197	241	226	239	241	258
E	261	226	245	243	241	252	214	239	247	221	220	271
F	254	221	234	250	227	260	242	253	247	227	221	229
G	244	562	521	518	464	515	480	499	475	467	535	476
H	245	517	522	498	501	601	540	517	539	518	521	543

READ 2

					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	413	1960	2088	1999	2158	2180	2083	2292	2120	2334	2398	2313
B	460	2464	2576	2477	2342	2481	2412	2351	2448	2397	2539	2116
C	426	2700	2421	2424	2429	2479	2336	2355	2347	2359	2531	2834
D	442	2691	2604	2646	2695	2642	2757	2644	2431	2741	2525	2628
E	455	2886	2911	2828	2755	2759	2760	2658	2660	2707	2616	2529
F	412	2484	2523	2643	2638	2833	2640	2727	2645	2724	2565	2575
G	414	768	728	886	653	700	723	704	718	677	728	762
H	472	649	614	661	680	720	722	699	746	651	621	702

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 12, 2013
READ 1

					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	247	238	240	246	256	226	233	257	223	239	220	266
B	231	251	228	224	184	218	218	212	217	186	196	225
C	224	239	252	245	246	261	271	245	238	254	268	221
D	232	226	247	224	208	238	205	238	220	251	233	189
E	208	211	230	258	200	266	253	258	256	240	231	247
F	227	230	261	211	247	224	221	240	212	238	286	285
G	205	606	597	645	614	618	662	622	550	580	614	536
H	222	448	515	509	472	522	514	549	520	524	490	476

READ 2

					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	495	1660	1764	1649	1808	1904	1841	1762	1882	2072	2029	2276
B	464	2268	2223	2326	2325	2334	2331	2221	2368	2252	2222	1770
C	471	2319	2265	2517	2384	2401	2287	2646	2106	2546	2773	2548
D	449	2452	2394	2546	2663	2685	2342	2645	2598	2546	2901	2308
E	448	2483	2775	2686	2736	2495	2662	2534	2599	2512	2313	2451
F	406	2028	2330	2247	2510	2442	2439	2616	2585	2650	2654	2470
G	432	807	919	808	800	834	864	880	801	822	793	794
H	418	570	541	560	561	525	557	738	573	606	628	635

APPENDIX 1 Solubility Data

Valid Run 1 – January 24, 2013

	1	2	3	4	5	6	7	8	9	10	11	12	
A					-15	-14	-13	-12	-11	-10	-9	-8	17β-Estradiol
B					-15	-14	-13	-12	-11	-10	-9	-8	
C					-13	-12	-11	-10	-9	-8	-7	-6	17β-Estradiol
D					-13	-12	-11	-10	-9	-8	-7	-6	
E					-11	-10	-9	-8	-7	-6	-5	-4	Corticosterone
F					-11	-10	-9	-8	-7	-6	-5	-4	
G					-12	-11	-10	-9	-8	-7	-6	-5	17α-Methyltestosterone
H					-12	-11	-10	-9	-8	-7	-6	-5	

	1	2	3	4	5	6	7	8	9	10	11	12	
A					99	90	82	91	97	109	105	109	17β-Estradiol
B					108	116	112	122	119	125	124	124	
C					120	120	119	129	130	158	131	3189	17β-Estradiol
D					125	140	131	148	6223	123	130	3242	
E					121	128	130	143	131	128	137	359	Corticosterone
F					120	128	117	153	122	129	126	130	
G					159	132	121	125	128	128	122	124	17α-Methyltestosterone
H					124	106	103	111	113	127	134	128	

APPENDIX 1 Solubility Data (Continued)

Valid Run 1 – January 24, 2013

	1	2	3	4	5	6	7	8	9	10	11	12	
A					-12	-11	-10	-9	-8	-7	-6	-5	Ensulizole
B					-12	-11	-10	-9	-8	-7	-6	-5	
C					-12	-11	-10	-9	-8	-7	-6	-5	Avobenzone
D					-12	-11	-10	-9	-8	-7	-6	-5	
E					-11	-10	-9	-8	-7	-6	-5	-4	Homosalate
F					-11	-10	-9	-8	-7	-6	-5	-4	
G					-11	-10	-9	-8	-7	-6	-5	-4	Padimate-O
H					-11	-10	-9	-8	-7	-6	-5	-4	

	1	2	3	4	5	6	7	8	9	10	11	12	
A					97	95	90	88	685	114	113	111	Ensulizole
B					110	118	109	120	121	148	109	112	
C					125	145	119	132	5996	115	115	157	Avobenzone
D					120	119	130	156	146	151	123	165	
E					115	128	132	136	138	119	111	132	Homosalate
F					134	119	128	140	133	116	117	124	
G					113	129	123	119	120	131	125	119	Padimate-O
H					130	142	134	112	122	123	138	131	

APPENDIX 1 Solubility Data (Continued)

Valid Run 2 – February 12, 2013

	1	2	3	4	5	6	7	8	9	10	11	12	
A					-15	-14	-13	-12	-11	-10	-9	-8	17β-Estradiol
B					-15	-14	-13	-12	-11	-10	-9	-8	
C					-13	-12	-11	-10	-9	-8	-7	-6	17β-Estradiol
D					-13	-12	-11	-10	-9	-8	-7	-6	
E					-11	-10	-9	-8	-7	-6	-5	-4	Corticosterone
F					-11	-10	-9	-8	-7	-6	-5	-4	
G					-12	-11	-10	-9	-8	-7	-6	-5	17α-Methyltestosterone
H					-12	-11	-10	-9	-8	-7	-6	-5	

	1	2	3	4	5	6	7	8	9	10	11	12	
A					95	114	119	146	110	110	128	124	17β-Estradiol
B					119	125	121	117	116	112	117	127	
C					119	137	125	132	161	130	123	115	17β-Estradiol
D					111	121	131	148	161	126	91	103	
E					118	168	115	145	112	111	99	101	Corticosterone
F					115	100	132	99	105	124	97	80	
G					155	106	166	97	93	113	86	80	17α-Methyltestosterone
H					120	95	87	292	78	76	83	111	

APPENDIX 1 Solubility Data (Continued)

Valid Run 2 – February 12, 2013

	1	2	3	4	5	6	7	8	9	10	11	12	
A					-12	-11	-10	-9	-8	-7	-6	-5	Ensulizole
B					-12	-11	-10	-9	-8	-7	-6	-5	
C					-12	-11	-10	-9	-8	-7	-6	-5	Avobenzone
D					-12	-11	-10	-9	-8	-7	-6	-5	
E					-11	-10	-9	-8	-7	-6	-5	-4	Homosalate
F					-11	-10	-9	-8	-7	-6	-5	-4	
G					-11	-10	-9	-8	-7	-6	-5	-4	Padimate-O
H					-11	-10	-9	-8	-7	-6	-5	-4	

	1	2	3	4	5	6	7	8	9	10	11	12	
A					819	328	172	140	101	125	171	191	Ensulizole
B					1250	360	272	123	132	401	366	138	
C					855	192	210	110	106	118	96	138	Avobenzone
D					147	112	129	127	111	118	122	133	
E					265	169	144	102	116	117	117	278	Homosalate
F					122	139	107	113	119	100	167	116	
G					216	121	134	116	112	87	136	104	Padimate-O
H					115	100	128	102	81	84	93	89	

APPENDIX 2 Certificates of Analysis

SIGMA-ALDRICH®

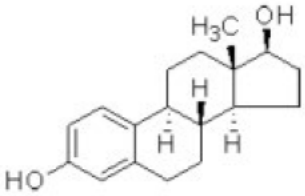
sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
β-Estradiol – ≥98%

Product Number:	**E8875**
Lot Number:	**SLBC5955V**
Brand:	SIGMA
CAS Number:	50-28-2
MDL Number:	MFCD00003693
Formula:	C18H24O2
Formula Weight:	272.38 g/mol
Quality Release Date:	15 MAY 2012
Recommended Retest Date:	APR 2015



Test	Specification	Result
Appearance (Color)	White to Off-White	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless	Colorless
Solubility (Turbidity) 50 mg/ml, EtOH	Clear	Clear
Purity (HPLC)	≥ 98 %	100 %
Recommended Retest Period 3 Years	----------	----------

[redacted]
[redacted] Manager
Analytical Services
St. Louis, Missouri US

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Version Number: 1

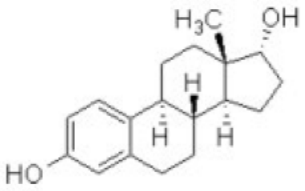
Page 1 of 1

SIGMA-ALDRICH®
sigma-aldrich.com
3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
α-Estradiol - powder, ≥98% (TLC)

Product Number:	**E8750**
Lot Number:	**041M4065V**
Brand:	SIGMA
CAS Number:	57-91-0
Formula:	C18H24O2
Formula Weight:	272.38 g/mol
Quality Release Date:	17 MAR 2011
Recommended Retest Date:	MAR 2014



Test	Specification	Result
Appearance (Form)	Powder	Powder
Appearance (Colour)	White to White w/ Yellow Cast	White
Solubility (Solvent)	Ethanol	Ethanol
Solubility (Conc)	49.00 - 51.00 mg/ml	50.00 mg/ml
Solubility (Heating)	Yes	Yes
Solubility (Turbidity)	Clear	Clear
Solubility (Color)	Colorless	Colorless
Water (by Karl Fischer)	≥ 0.00 %	0.67 %
Elemental Anal. (%C anhydrous)	78.87 - 79.87 %	79.74 %
NMR (Solvent)	DMSO-d6	DMSO-d6
Identity by NMR	Consistent	Consistent with Structure
Purity (TLC)	≥ 98.00 %	99.00 %
Purity (HPLC)	≥ 98.00 %	99.72 %
Specific Rotation (Solvent)	Ethanol	Ethanol
Specific Rotation (Alpha D)	52.00 - 56.00 deg	55.06 deg
UV (Solvent)	Ethanol	Ethanol
UV (EmM at Lambda max)	1.80 - 2.20	2.13

[redacted]
Manager, Quality and Regulatory Affairs
Jerusalem, Israel IL

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Version Number: 1

SIGMA-ALDRICH
Industriestrasse 25, CH-9471 Buchs (SG), Switzerland
Tel: +41 81 755 2511 Fax: +41 81 756 5449

Certificate of Analysis

Product Name:	CORTICOSTERONE >= 98.5 % HPLC
Product Number:	27840
Product Brand:	Sigma
Molecular Formula:	$C_{21}H_{30}O_4$
Molecular Mass:	346.46
CAS Number:	50-22-6

TEST	SPECIFICATION	LOT BCBD9137V RESULTS
APPEARANCE (COLOR)	WHITE TO ALMOST WHITE	WHITE
APPEARANCE (FORM)	POWDER TO FINE CRYSTALS WITH LUMPS	FINE CRYSTALS
PURITY (HPLC AREA %)	≥ 98.5 %	98.8 %
SPECIFIC ROTATION (20/D)	223.0 ± 3.0 DEGREES	225.8 DEGREES
CONCENTRATION	C=1 IN ETOH	C=1 IN ETOH
MELTING POINT	179 - 183 C	182 C
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS
QC RELEASE DATE	18/JAN/11	

[redacted]
[redacted]Manager
Quality Control
Buchs, Switzerland

Certificate of Analysis

SIGMA-ALDRICH

Product Name	17α-Methyltestosterone, solid (photosensitive)
Product Number	M7252
Product Brand	SIGMA
CAS Number	58-18-4
Molecular Formula	$C_{20}H_{30}O_2$
Molecular Weight	302.45

TEST	SPECIFICATION	LOT 060m1543v RESULTS
Appearance (Color)	White to Off-White	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless to Faint Yellow	Colorless
Solubility (Turbidity)	Clear 50 mg/mL, CHCl3	Clear
Infrared spectrum	Conforms to Structure	Conforms
Carbon	77.8 - 81.0 %	78.0 %
Specific Rotation	69 - 75 ° (C = 1 in Dioxane at 25 deg C)	71 °
Purity (HPLC)	≥98 %	99 %
Specification Date:		JUL 2010
Date of QC Release:		JUL 2010
Print Date:		JUL 20 2010

[redacted]
[redacted] Manager
Quality Control
St. Louis, Missouri USA

SIGMA-ALDRICH®

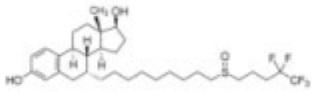
sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Fulvestrant – >98% (HPLC), solid

Product Number:	**I4409**
Lot Number:	**051M4757V**
Brand:	SIGMA
CAS Number:	129453-61-8
MDL Number:	MFCD00903953
Formula:	C32H47F5O3S
Formula Weight:	606.77 g/mol
Storage Temperature:	Store at 2 - 8 °C
Quality Release Date:	31 MAY 2011



Test	Specification	Result
Appearance (Color)	White to Tan	White
Appearance (Form)	Powder	Powder
Elemental Composition C32H47F5O3S	Pass	Pass
Purity (HPLC)	≥ 98.0 %	99.5 %
HPLC Identity Coelution with the standard	Conforms	Conforms
Specific Rotation (dry basis) C= 1, methanol)	27.0 - 39.0 °	30.6 °

[redacted]
[redacted] QC Team Leader
Quality Control
Natick, Massachusetts US

RTI INTERNATIONAL
turning knowledge into practice

NTP Analytical Chemistry Services

3040 Cornwallis Road • PO Box 12194 • Research Triangle Park, NC 27709-2194 • USA
Telephone 919.541.6730 or 919.541.5975 • Fax 919.485.2650 • www.rti.org

Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.080
ChemTask No. CHEM11786
CAS No. 27503-81-7

This pdf is an exact duplicate of the original approved report

[redacted]
Program Information Coordinator

ENSULIZOLE

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted] 09.05-12
Task Leader Date

Approved by:

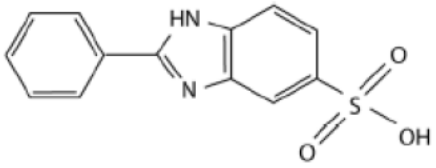
[redacted] 09/05/12
Reshan Fernando, Ph.D. Date
Principal Investigator

Submitted to:

[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

ENSULIZOLE

CAS No.: 27503-81-7	Study Lab: (Investigator): ILS ([redacted]
RTI Chemical ID Code: N60	Lot No. (Vendor): 05117JE(Aldrich)
ChemTask No.: CHEM11786	Vendor Purity: 99.9% (by HPLC, Aldrich COA)
RTI Log Nos. (Amt. Received): Analytical: 082010-C-15 (~50 g) Reference: 082010-C-05 (~5 g)	Receipt Date: Aug 20, 2010 (Bulk receipt and reference)
Program Supported: TOX	Receipt Condition: No damage noted
Analysis Dates: May 11, 15 and 24, 2012	Submitter: [redacted](RTI)
Interim Results Date: May 29, 2012	Shipping Container: NA (in-house transfer)
	Storage Conditions: Bulk: Room temperature Reference: Freezer (~ -20 °C)

STRUCTURE	MOL. WT.	MOL. FORMULA
	274.30	$C_{13}H_{10}N_2O_3S$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, an aliquot of ensulizole was submitted for bulk chemical reanalysis. Chemical purity of the bulk sample was determined relative to a reference standard of the same lot/batch number which had been stored at RTI under freezer conditions. Analytical results obtained by LC chromatographic method indicated that the sample had a percent relative purity of 99.6% when compared to the frozen reference standard. The FTIR spectrum of the bulk sample matched the spectrum of the frozen reference and was consistent with the structure for ensulizole.

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: **Ensulizole**

Task Type: **Chemical Reanalysis**

Chem Task Number: **CHEM11786**

This study/task was audited by the Regulatory and Quality Assurance (RQA) – Quality Assurance Unit and the results of the inspections and audits were reported to the task leader/study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Sample Preparation Inspection for HPLC Analysis	05/15/12	05/22/12
Data & Report Audit	08/24/12 & 08/28/12	08/28/12

Prepared by:

[redacted]
Quality Assurance Specialist

9-5-12
Date

Reviewed by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

turning knowledge into practice

TABLE OF CONTENTS

Figures

ENSULIZOLE

1.0 INTRODUCTION

The objective of this work was to determine the purity and verify the identity of ensulizole to the current studies being conducted at RTI International. To accomplish this objective, a bulk chemical reanalysis was performed. The identity of the chemical was confirmed by FTIR and its purity assessed by LC.

2.0 CHEMICAL ANALYSIS

An aliquot of the bulk sample of ensulizole was received at the analytical laboratory on March 27, 2012 for chemical reanalysis (RTI log 082010-C-15). The aliquot was stored at room temperature. A frozen reference (RTI log 082010-C-05) sample was received at the analytical laboratory on May 10, 2012 and was stored at freezer temperature.

3.0 CONFIRMATION OF IDENTITY - INFRARED SPECTROMETRY (IR)

3.1 IR Parameters

System	Thermo Nicolet 6700 FTIR
Software	Omnic, Ver. 7.3
Method	KBr pellet, scan 4000 - 400 cm^{-1}

3.2 Results

Bulk Sample Frequency (1/cm)	Frozen Reference Sample Frequency (1/cm)	Assignment
3367	3372	N-H stretch
3059-2725	3059-2725	O-H, N-H, C-H stretch
1633, 1568	1630, 1567	C=C, C=N stretch
1368	1368	C-N stretch
1176	1176	C-C, SO_2 stretch
1028	1028	N-H bend
780	777	C-H, N-H bend
631	630	S-O stretch

The observed spectrum for the bulk sample matched the spectrum of the frozen reference sample, and is consistent with the structure of ensulizole (as reported in the characterization protocols development task CHEM11291). Figure 1 shows the IR spectra for the bulk and frozen samples.

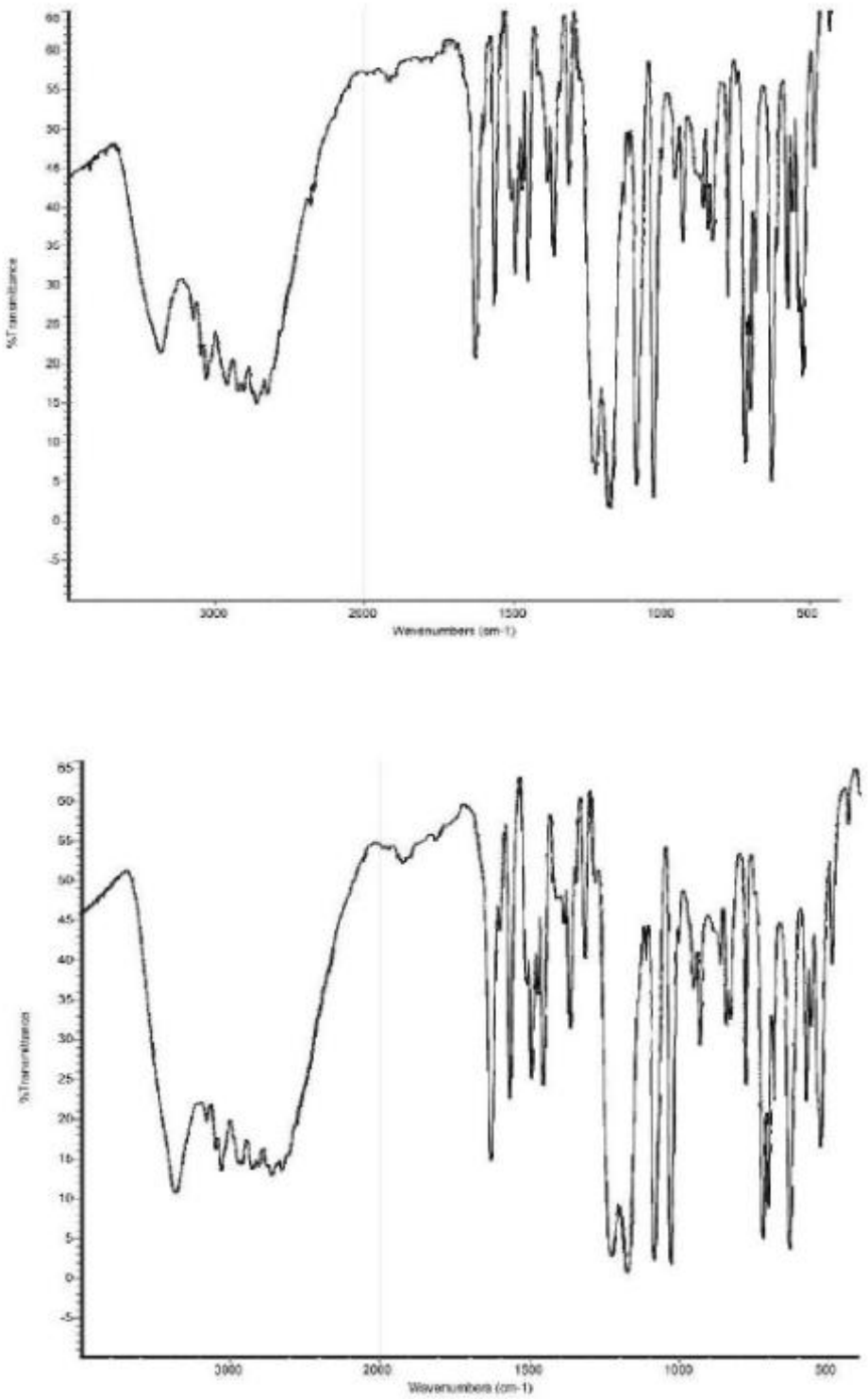


Figure 1: Infrared Spectrum of Ensulizole Frozen Reference (top spectrum) and Bulk Sample (bottom spectrum)

2

4.0 DETERMINATION OF PURITY - LIQUID CHROMATOGRAPHY

This section describes the liquid chromatographic method used to estimate sample purity.

4.1 Preparation of Internal Standard (IS) Solution

A stock solution of IS was prepared by weighing 500 mg of padimate O and transferring it into a 10-mL volumetric flask. The IS was diluted to volume with mobile phase B (methanol with 0.1% formic acid). The flask was mixed by inversion. A working IS solution (WIS) was prepared as a 1 mL to 1 L dilution with mobile phase B and mixing by inversion, yielding 0.050 mg/mL working IS.

4.2 Bulk Sample and Frozen Reference Standard Solution Preparation

Triplicate solutions of the reference standard and bulk samples were prepared by transferring approximately 25 mg of compound to individual 100-mL volumetric flasks and diluting to volume with WIS and mixing by inversion. All samples were transferred to autosampler vials and analyzed by liquid chromatography.

4.3 Analysis

LC Parameters

System	Waters Alliance 2695
Software	Empower 2; Build 2154
Column	Waters XBridge C18 3.5 µm, 100 x 2.1 mm, guard column, 5 µm 2.1 x 10 mm
Column Temp	40 °C
Mobile Phases	A: 0.1% formic acid in water B: 0.1% formic acid in methanol
Flow Rate	0.25 mL/min
Gradient	Hold 90 % A for 0.67 min., 90% A to 90% B in 10 min., hold 90% B for 10 min., 90% B to 90% A in 5 min., hold 90% A for 5 min.
Injection Volume - Solvent	2 µL – Mobile Phase B
Retention Time (min)	Ensulizole – 5.73 min Padimate O (IS) – 16.59 min
Detector	Waters 2996 PDA, 312 nm

The suitability of the system was evaluated, and the results are shown below.

Parameter	Result	Criteria	Pass/Fail
Capacity Factor, k	2.8	$2 \geq k \leq 12$	Pass
Tailing Factor, T	1.2	$0.5 \geq T \leq 2.0$	Pass
Column Efficiency, N	29,000	$N \geq 6{,}000$ plates	Pass

4.4 Results

Calculations based on a major peak comparison technique gave the results shown in the following table.

RTI Log No.	Chemical	RRF[a]	Mean RRF[a] (%RSD)	Percent Relative Purity[b]
082010-C-15	Analytical Replicate #1	3.072	3.046 (0.82)	99.6
	Analytical Replicate #2	3.022		
	Analytical Replicate #3	3.045		
082010-C-05	Reference Replicate #1	3.034	3.057 (0.81)	--
	Reference Replicate #2	3.083		
	Reference Replicate #3	3.054		

[a] RRF = Relative Response Factor; normalized to sample concentration.

[b] Relative Purity = (Mean RRF, bulk/Mean RRF, ref.) x 100.

Based on the chromatographic results, the bulk sample had not significantly changed as compared to the frozen reference, and no significant impurities were observed. Typical chromatograms are shown in Figure 2.

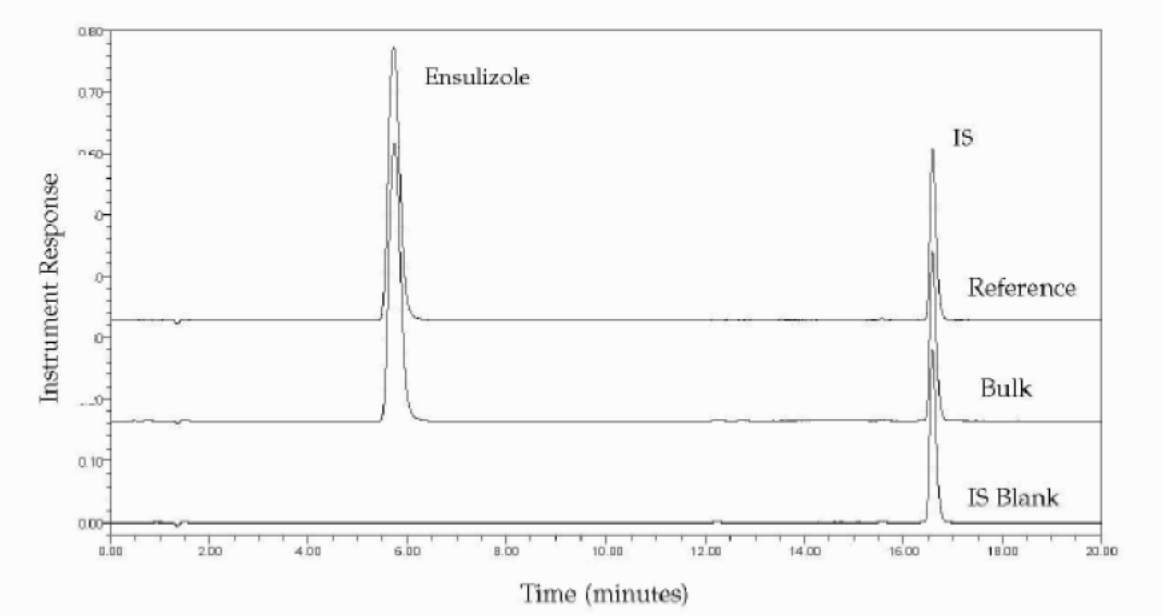


Figure 2: Example Liquid Chromatograms of Ensulizole Reference and Bulk Sample, and a Blank

5.0 REFERENCE

RTI International report "Ensulizole, Characterization Protocols Development, (CHEM11291), January 9, 2012.

6.0 ACKNOWLEDGMENTS

Personnel contributing to this task: [redacted]

This PDF File is an Exact Copy of the Report
Signature: [redacted]
Date: 2-16-12

Analytical Chemistry Services for the NTP
NIEHS Contract No. HHSN273201100001C
MRI Project No.: 110730
NTP ChemTask No.: CHEM10985

Chemical Comprehensive Analysis Final Report

Avobenzone

Chemical Comprehensive Analysis of Avobenzone

MRI Assignment No.: 2003

February 16, 2012

Prepared by:

[redacted]
Study Director

Approved by:

[redacted]
Joseph W. Algaier, Ph.D.
Principal Investigator

Reviewed by:

[redacted]
Group Leader

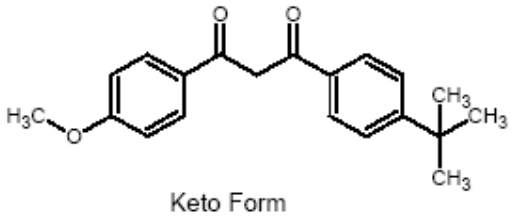
Submitted to:

[redacted]
National Institute of Environmental Health Sciences
111 T. W. Alexander Drive, MD K2-07
P.O. Box 12233
Research Triangle Park, NC 27709-2233

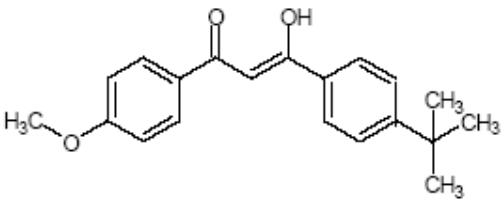
Chemical Comprehensive Analysis of Avobenzone

Chemical Information

CAS No.: 70356-09-1 MRI Assignment No.: 2003 ChemTask No. CHEM10985 Program Supported: TOX Analysis Dates: 2/11/11 to 12/14/11 Interim Result Date(s): 2/25/11, 4/7/11, 5/17/11	Lot No.: L802809 MRI Assigned Batch No.: 01 Amount Received: 20 Kg Sample Receipt Date: 1/5/11 Appearance: Off white to yellowish crystalline powder per CoA; confirmed by visual observation Supplier: Universal Preserv-A-Chem Inc. Supplier Purity: 98.30% per CoA Storage conditions (at Analytical Lab): Ambient, protected from light



Keto Form



Enol Form (predominant)

Mol. Wt.	Mol. Formula
310.39	$C_{20}H_{22}O_3$

Executive Summary

The purpose of this assignment was to perform a chemical comprehensive analysis for avobenzone, Lot No. L802809, received from Universal Preserv-A-Chem Inc. Based on the results, the identity of the test article was confirmed to be avobenzone, with a purity of approximately 98.5%. Evaluation by gas chromatography with flame ionization detection of samples stored at various temperatures indicated avobenzone is stable when stored for 2 weeks, protected from light, at temperatures up to approximately 60°C. Nuclear magnetic resonance spectroscopic analysis of these samples, as well as samples exposed to light for 1 week, detected some conversion of enol to keto form under elevated temperature and light exposure.

The chemical comprehensive analysis included identity confirmation using infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy, residual solvent analysis for volatile content using gas chromatography (GC)/headspace analysis, ultraviolet/visible (UV/Vis) spectroscopy, water content using Karl Fischer titration, elemental analysis, determination of melting point, and log P, differential scanning calorimetry (DSC), and chromatographic profiling using gas chromatography (GC) with flame ionization detection (FID). Additionally, gas chromatography/mass spectrometry (GC/MS) was performed to confirm identity of the test article.

Spectra obtained for the test article using IR and NMR spectroscopy techniques were consistent with reference spectra and the proposed structure for the enol form of the test article. One absorbance maximum was observed using ultraviolet/visible spectroscopy: 358 nm, ϵ_{max} = 36241 ± 186(s). Analysis using GC/MS with electron capture ionization provided confirmation of identity based on the molecular ion (310 Da) observed, as well as comparison to a reference spectrum.

Water content determined by Karl Fischer was 0.223 ± 0.008(s) %. Elemental analysis determined 77.36% carbon, 7.39% hydrogen, and 0.02% nitrogen compared to expected values of 77.39 carbon, 7.15% hydrogen, and no nitrogen. The observed melting point range was 83.0° to 85.5°C (literature values of 83.5°C and 81° to 86°C). The determined log P was 3.10.

Differential scanning calorimetry was performed, and the observed melting point range was consistent with the melting point range from the MSDS. The results indicated a purity of 98.8 ± 0.5(d) %. Chromatographic profiling, using GC with a DB-5 column and FID, indicated 98.7% purity, with seven reportable impurities totaling 1.26% relative to the total peak area. GC/headspace analysis indicated residual solvent peak responses for methanol and cis-1,2-dichloroethene, but they were not present at levels greater than the Class 2 Mixture A Standard. There were no other Class 1 or Class 2 solvents observed to be present in the test article.

Accelerated stability was performed using GC with FID to evaluate possible degradation of the test article. The test variability limit (TVL), which is statistically determined, established that in order to be statistically significant at the 95% confidence level, the loss or gain under ambient, refrigerated, or elevated storage conditions must be greater than 3.8% relative to the sample under the frozen storage condition. The maximum variance from the frozen storage condition was +0.7%, observed for the sample stored at approximately 60°C. Using the TVL criteria,

avobenzone is stable when stored for 2 weeks as the bulk chemical, protected from light, at temperatures up to approximately 60°C. An additional evaluation using ^{1}H-NMR spectroscopy of the accelerated stability samples and stability samples exposed to light exhibited decreased enol/keto ratios of the –OH and $-CH_2$ functional groups for the samples stored at 60°C, as well as samples exposed to fluorescent or mercury/xenon lighting. This indicates some conversion of the enol to the keto form.

Quality Assurance Statement

Chemical Comprehensive Analysis of Avobenzone

ChemTask No. CHEM10985
MRI Project No. 110730
MRI Assignment No. 2003

This study was inspected by the Quality Assurance Unit of MRI (QAU) and the findings reported to the Study Director and Management as follows:

Phase inspected	Date inspected	Date reported
Protocol Audit	3/1/11	3/1/11
In-life Audit; Stability analysis	3/1/11	3/1/11
Protocol Amendment No. 1 Audit	2/8/12	2/10/12
Protocol Amendment No. 2 Audit	2/8/12	2/10/12
Protocol Amendment No. 3 Audit	2/8/12	2/10/12
Data Audit	2/9/12	2/10/12
Draft Final Report Audit	2/9/12	2/10/12

In addition to the study-specific audits/inspections cited above, inspection of applicable facilities and equipment was performed by the QAU and reports were submitted to management as follows:

Facility/equipment	Inspection date	Management submitted date
285N laboratory complex	7/13/11	7/14/11
GC facility	7/14/11	7/15/11

MIDWEST RESEARCH INSTITUTE

[redacted]

Senior Quality Assurance Officer

Approved:

[redacted]

Director, Quality and Regulatory Systems

February 16, 2012

Good Laboratory Practice Compliance Statement

Chemical Comprehensive Analysis of Avobenzone

ChemTask No. CHEM10985
MRI Project No. 110730
MRI Assignment No. 2003

All work performed at Midwest Research Institute for this assignment was conducted in compliance with the Good Laboratory Practice regulations of the U.S. Food and Drug Administration (21 *CFR* Part 58). Elemental analysis was performed by ICON Developmental Solutions, LLC, in compliance with FDA current Good Laboratory Practices (21 *CFR* Part 58).

The raw data and report will be stored in the MRI Archives.

[redacted]
Study Director

2/16/12
Date:

RTI INTERNATIONAL
turning knowledge into practice

NTP Analytical Chemistry Services

3040 Cornwallis Road • PO Box 12194 • Research Triangle Park, NC 27709-2194 • USA
Telephone 919.541.6730 or 919.541.5975 • Fax 919.485.2650 • www.rti.org

Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.082
ChemTask No. CHEM11788
CAS No. 118-56-9

This pdf is an exact duplicate of the original approved report.

[redacted]
Program Information Coordinator

HOMOSALATE

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted]
Task Leader

09-05-12
Date

Approved by:

[redacted]
Reshan Fernando, Ph.D.
Principal Investigator

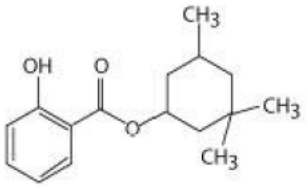
09/05/12
Date

Submitted to:

[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

HOMOSALATE

CAS No.: 118-56-9	Study Lab: (Investigator): ILS [redacted]
RTI Chemical ID Code: N67	Lot No. (Vendor): YT0976 (Spectrum)
ChemTask No.: CHEM11788	Vendor Purity: 99.88% (Spectrum COA)
RTI Log Nos. (Amt. Received): Analytical: 091410-A-14 (~50 g) Reference: 091410-A-05 (~5 g)	Receipt Date: Sep 14, 2010 (Bulk) Receipt Condition: No damage noted
Program Supported: TOX	Submitter: [redacted] (RTI)
Analysis Date: May 11, 21-23, 2012	Shipping Container: NA (in-house transfer)
Interim Results Date: May 29, 2012	Storage Conditions: Bulk: Room temperature Reference: Freezer (~ -20 °C)

STRUCTURE	MOL. WT.	MOL. FORMULA
	262.34	$C_{16}H_{22}O_3$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, an aliquot of homosalate was submitted for bulk chemical reanalysis. Chemical purity of the bulk sample was determined relative to a reference standard of the same lot/batch number which had been stored at RTI under freezer conditions. Analytical results obtained by a GC/FID chromatographic method indicated that the sample had a percent relative purity of 99.3% when compared to the frozen reference standard. The FTIR spectrum of the bulk sample matched the spectrum of the frozen reference and was consistent with an identity of homosalate.

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: **Homosalate**

Task Type: **Chemical Reanalysis**

Chem Task Number: **CHEM11788**

This study/task was audited by the Regulatory and Quality Assurance (RQA) – Quality Assurance Unit and the results of the inspections and audits were reported to the task leader/study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Sample Preparation Inspection	05/21/12	05/21/12
Data & Report Audit	08/16/12	08/16/12

Prepared by:

[redacted] 9/5/12

Quality Assurance Specialist Date

Reviewed by:

[redacted] 9/5/12

Quality Assurance Specialist Date

turning knowledge into practice

TABLE OF CONTENTS

Figures

HOMOSALATE

1.0 INTRODUCTION

The objective of this work was to determine the purity and verify the identity of homosalate in support of studies being conducted at ILS. To accomplish this objective, a chemical reanalysis was performed. The identity of the chemical was confirmed by FTIR and its purity assessed by GC.

2.0 CHEMICAL ANALYSIS

An aliquot of the bulk sample of homosalate was received on March 27, 2012 for chemical reanalysis (RTI log 091410-A-14). The aliquot was stored at room temperature. A frozen reference (RTI log 091410-A-05) sample was received May 10, 2012 and was stored at freezer temperature.

3.0 CONFIRMATION OF IDENTITY - INFRARED SPECTROMETRY (IR)

3.1 IR Parameters

System	Thermo Nicolet 6700 FTIR
Software	Omnic, Ver. 7.3
Method	NaCl disks, scan 4000 - 400 cm^{-1}

3.2 Results

Bulk Sample Frequency (1/cm)	Frozen Reference Sample Frequency (1/cm)	Assignment
3150	3150	O-H stretch
2953-2869	2953-2869	C-H stretch
1672	1672	C=C, C=0 stretch
1614	1614	C=C stretch
1585	1585	C=C stretch
1089	1089	C-C, C-O stretch
757	757	C-H bend

The observed spectrum for the bulk sample matched the spectrum of the frozen reference sample, and is consistent with the structure of homosalate (as reported in the bulk chemical comprehensive task CHEM11090). Figure 1 shows the bulk and frozen reference IR spectra.

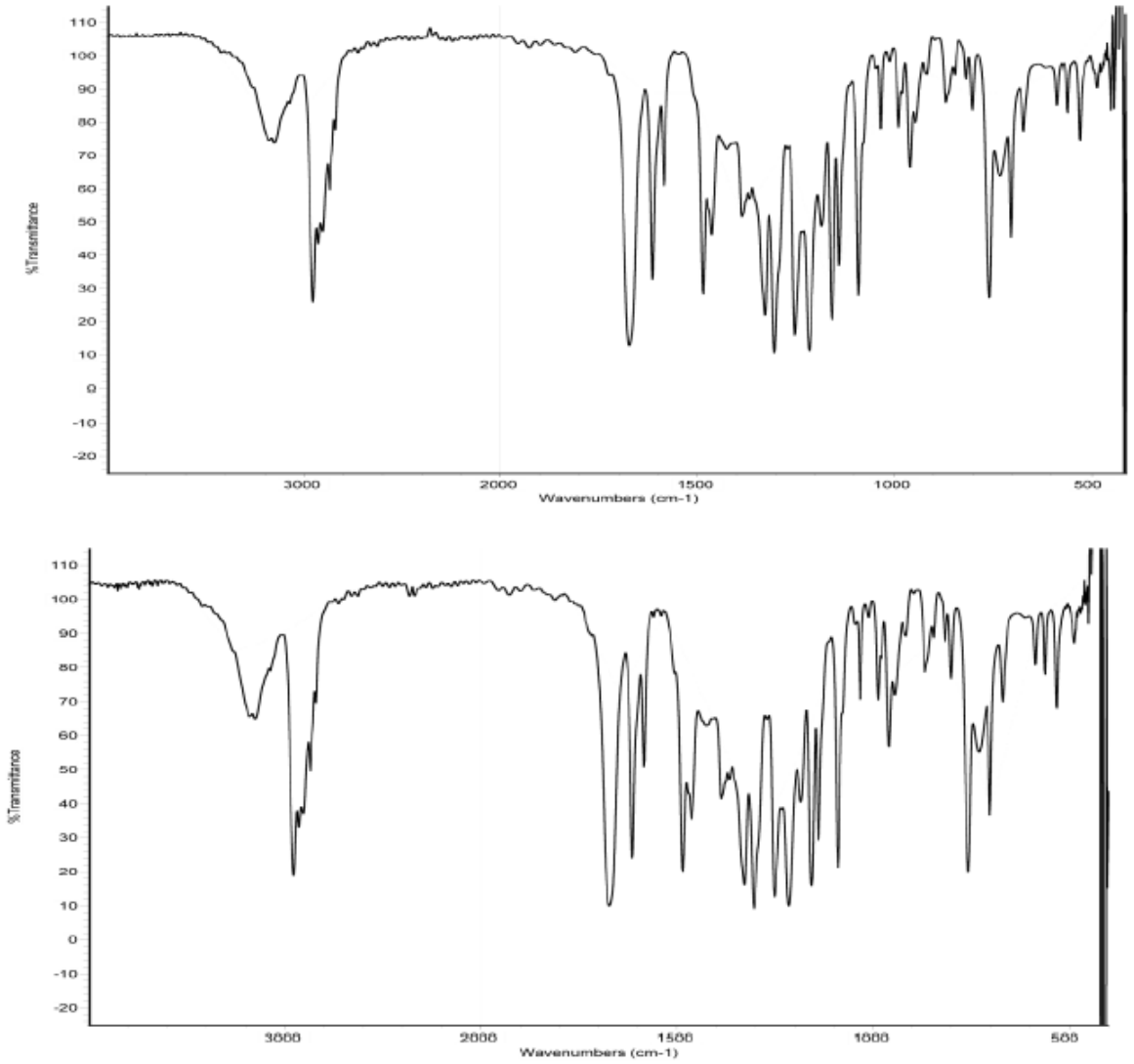


Figure 1: Infrared Spectrum of Homosalate Bulk (top spectrum)
and Frozen Reference (bottom spectrum)

4.0 DETERMINATION OF PURITY - GAS CHROMATOGRAPHY

This section describes the gas chromatographic method used to estimate sample purity.

4.1 Preparation of Internal Standard (IS) Solution

A solution of IS was prepared by weighing 115.49 mg of octanophenone and transferring it into a 200-mL volumetric flask. The IS was diluted to volume with dichloromethane. The flask was mixed by inversion. The IS solution had a concentration of 0.577 mg/mL.

4.2 Bulk Sample and Frozen Reference Standard Solution Preparation

Triplicate solutions of the reference standard and bulk samples were prepared by transferring approximately 25 mg of compound to individual 25-mL volumetric flasks and diluting to volume with IS solution and mixing by inversion. An aliquot of the bulk and reference solutions were transferred to GC vials for analysis. The samples were analyzed by gas chromatography.

4.3 Analysis

GC Parameters

Instrument	Agilent 6890N GC
Data System	Empower 2; Build 2154
Column	Phenomenex ZB-5MS (30 m x 0.25 mm ID, 0.5 µm film) with 5 m pre-guard
Carrier Gas	Helium
Flow Rate	1.5 mL/min
Oven Temperature	70 °C for 1 min., ramp to 270 °C at 20 °C/min with a 7 min hold
Retention Times	Homosalate: ~11.1 min. and 11.2 min (two peaks – cis/trans isomers) Octanophenone (IS): ~9.9 min.
Injector Type and Volume	Split (20:1), 1 µL
Injector Temperature	250 °C
Detector-Temperature	FID at 290 °C

The suitability of the system was evaluated, and the results are shown below.

Parameter	Criteria	Result	Pass/Fail
Tailing Factor, T	$0.5 \geq T \leq 2.0$	1.0	Pass
Column Efficiency, N	≥ 250,000 plates	2,460,486	Pass
Precision (%RSD)	≤ 5% (n=6)	0.2	Pass
Resolution	≥ 40	41	Pass

4.4 Results

Calculations based on a major peak comparison technique gave the results shown in the following table. Typical chromatograms are shown in Figure 2.

RTI Log No.	Chemical	RRF[a]	Mean RRF[a] (%RSD)	Percent Relative Purity[b]
091410-A-14	Analytical Replicate #1	1.443	1.414 (2.0)	99.3
	Analytical Replicate #2	1.412		
	Analytical Replicate #3	1.388		
091410-A-05	Reference Replicate #1	1.430	1.424 (0.69)	--
	Reference Replicate #2	1.430		
	Reference Replicate #3	1.413		

[a] RRF = Relative Response Factor; normalized to sample concentration.
[b] Relative Purity = (Mean RRF, bulk/Mean RRF, ref.) x 100.

Based on the chromatographic results, the bulk sample had not significantly changed as compared to the frozen reference, and no significant impurities were observed.

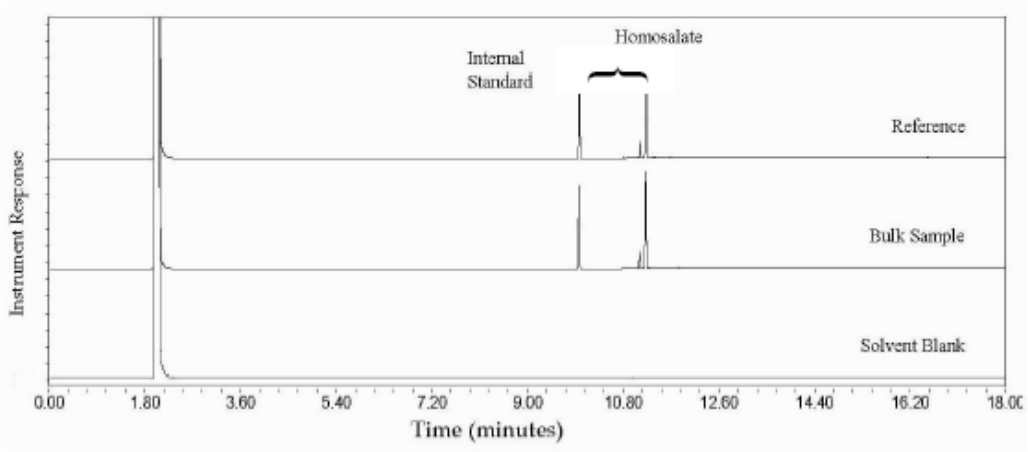


Figure 2: Example Gas Chromatograms of Homosalate Reference and Bulk Sample, and a Blank

5.0 REFERENCE

RTI International report "Homosalate, Characterization Protocols Development, (CHEM11293), January 6, 2012.

6.0 ACKNOWLEDGMENTS

Personnel contributing to this task: [redacted]

RTI INTERNATIONAL
turning knowledge into practice

NTP Analytical Chemistry Services

3040 Cornwallis Road • PO Box 12194 • Research Triangle Park, NC 27709-2194 • USA
Telephone 919.541.6730 or 919.541.5975 • Fax 919.485.2650 • www.rti.org

Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.081
ChemTask No. CHEM11787
CAS No. 21245-02-3

This pdf is an exact duplicate of the original approved report.

[redacted]
Program Information Coordinator

2-ETHYLHEXYL-P-DIMETHYL-AMINOBENZOATE (PADIMATE O)

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted] 09-05-12
Date
Task Leader

Approved by:

[redacted] 09/05/12
Reshan Fernando, Ph.D. Date
Principal Investigator

Submitted to:

[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

2-ETHYLHEXYL-P-DIMETHYL-AMINOBENZOATE (PADIMATE O)

CAS No.: 21245-02-3

RTI Chemical ID Code: L98

ChemTask No.: CHEM11787

RTI Log Nos. (Amt. Received):
Bulk Analytical: 082010-B-14 (~50 g)
Reference: 082010-B-05 (~5 g)

Program Supported: TOX

Analysis Dates: May 21-22, 24, 2012

Interim Results Date: May 30, 2012

Study Lab: (Investigator): ILS ([redacted])

Lot No. (Vendor): MKBF0590V (Aldrich)

Vendor Purity: 98.3% (Aldrich COA)

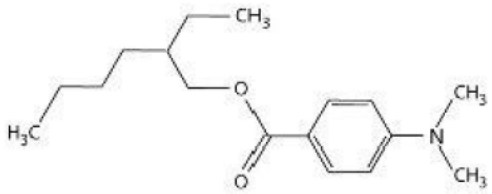
Receipt Date: Aug 20, 2010 (Bulk)

Bulk Receipt Condition: Good, room temperature

Submitter: [redacted] (RTI)

Shipping Container: NA (in-house transfer)

Storage Conditions:
Bulk: Room temperature
Reference: Freezer (~ -20 °C)

STRUCTURE	MOL. WT.	MOL. FORMULA
	277.40	$C_{17}H_{27}NO_2$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, an aliquot of padimate O was submitted for bulk chemical reanalysis. Chemical purity of the bulk sample was determined relative to a reference standard of the same lot/batch number which had been stored at RTI under freezer conditions. Analytical results obtained by a GC/FID chromatographic method indicated that the sample had a percent relative purity of 98.1% when compared to the frozen reference standard. The FTIR spectrum of the bulk sample matched the spectrum of the frozen reference and was consistent with an identity of padimate O.

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: 2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O)

Task Type: Chemical Reanalysis

RTI Task Number: 0212839.200.003.051

Chem Task Number: CHEM11787

This study/task was audited by the Regulatory and Quality Assurance (RQA) – Quality Assurance Unit and the results of the inspections and audits were reported to the task leader/study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Sample Analysis Inspection	05/15/12	05/22/12
Data & Report Audit	08/20/12	08/20/12

Prepared by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

Reviewed by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

turning knowledge into practice

TABLE OF CONTENTS

Figures

2-ETHYLHEXYL-P-DIMETHYL-AMINOBENZOATE (PADIMATE O)

1.0 INTRODUCTION

The objective of this work was to determine the purity and verify the identity of 2-Ethylhexyl-p-dimethyl-aminobenzoate (padimate O) in support of studies being conducted at ILS. To accomplish this objective, a chemical reanalysis was performed. The identity of the chemical was confirmed by FTIR and its purity assessed by GC.

2.0 CHEMICAL ANALYSIS

An aliquot of the bulk sample of padimate O was received on March 27, 2012 for chemical reanalysis (RTI log 082010-B-14). The aliquot was stored at room temperature. A frozen reference (RTI log 082010-B-05) sample was received May 10, 2012 and was stored at freezer temperature.

3.0 CONFIRMATION OF IDENTITY - INFRARED SPECTROMETRY (IR)

3.1 IR Parameters

System	Thermo Nicolet 6700 FTIR
Software	Omnic, Ver. 7.3
Method	NaCl disks, scan 4000 - 400 cm^{-1}

3.2 Results

Bulk Sample Frequency (1/cm)	Frozen Reference Sample Frequency (1/cm)	Assignment
2958-2860	2958-2860	C-H Stretch
2819	2820	N-CH_3 stretch
1703	1703	C = O stretch
1609, 1527	1609, 1527	C=C Stretch
1317	1317	C-N (tertiary amine stretch)
1183	1184	C = O Stretch
1107	1107	C-O-C Stretch

The observed spectrum for the bulk sample matched the spectrum of the frozen reference sample, and is consistent with the structure of padimate O (as reported in the bulk chemical comprehensive task CHEM11089). Figure 1 shows the bulk and frozen reference IR spectra.

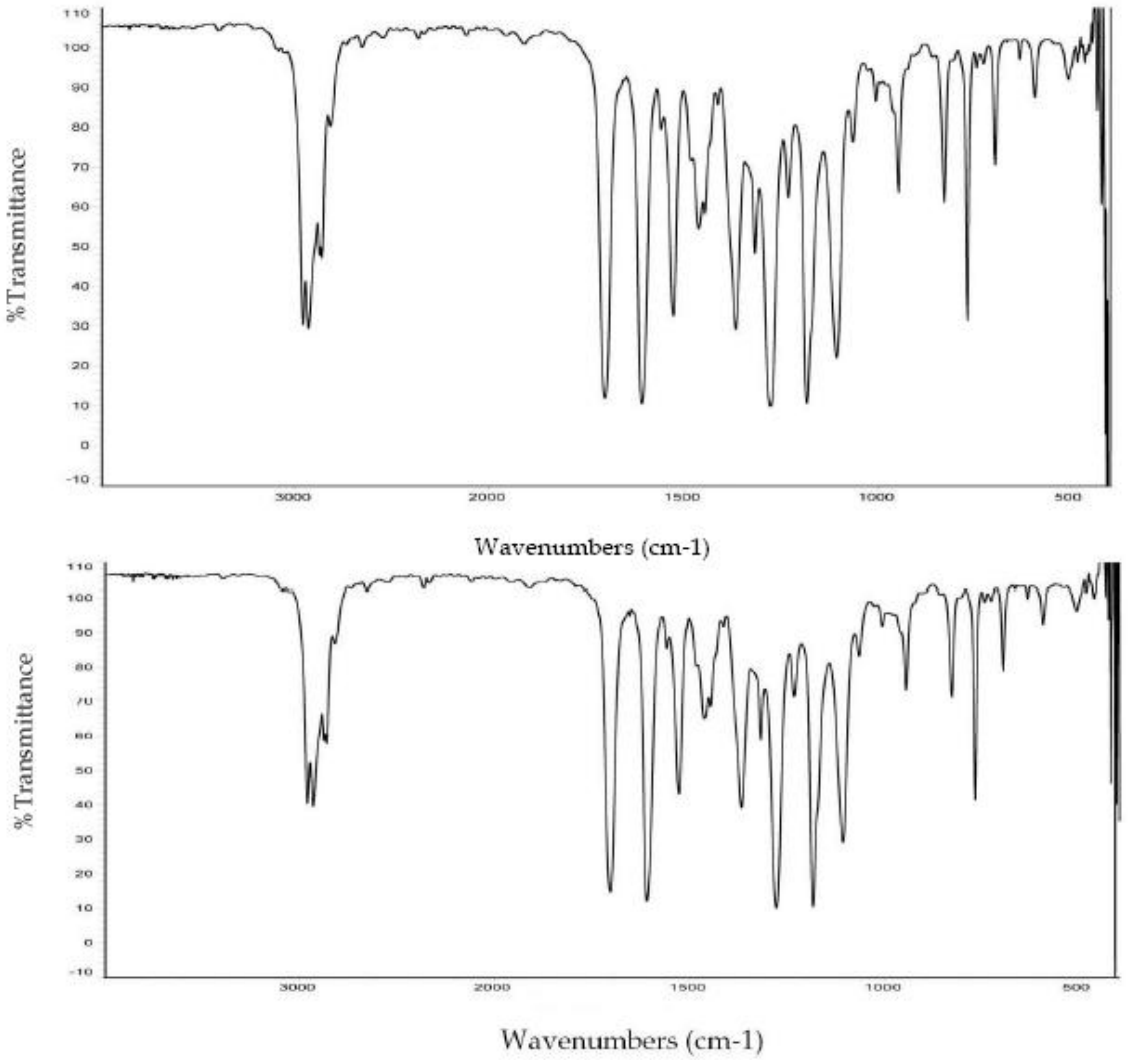


Figure 1: Infrared Spectrum of Padimate O Bulk (top spectrum)
and Frozen Reference (bottom spectrum)

4.0 DETERMINATION OF PURITY - GAS CHROMATOGRAPHY

This section describes the gas chromatographic method used to estimate sample purity.

4.1 Preparation of Internal Standard (IS) Solution

A solution of IS was prepared by weighing 103.4 mg of octanophenone and transferring it into a 200-mL volumetric flask. The IS was diluted to volume with dichloromethane. The flask was mixed by inversion. The IS solution had a concentration of 0.517 mg/mL.

4.2 Bulk Sample and Frozen Reference Standard Solution Preparation

Triplicate solutions of the reference standard and bulk samples were prepared by transferring approximately 25 mg of compound to individual 25-mL volumetric flasks and diluting to volume with IS solution and mixing by inversion. An aliquot of the bulk and reference solutions were transferred to GC vials for analysis. The samples and an IS blank was analyzed by gas chromatography.

4.3 Analysis

GC Parameters

Instrument	Agilent 6890N GC
Data System	Empower 2; Build 2154
Column	Phenomenex ZB-5MS (30 m x 0.25 mm ID, 0.5 µm film) with 5 m pre-guard
Carrier Gas	Helium
Flow Rate	1.5 mL/min
Oven Temperature	70 °C for 1 min., ramp to 270°C at 20 °C/min with a 7 min hold;
Retention Times	Padimate O: ~13.6 min. ; Octanophenone (IS): ~9.9 min.
Injector Type (ratio)	Split (20:1); 1 µL
Injector Temperature	250 °C
Detector-Temperature	FID at 290 °C

The suitability of the system was evaluated, and the results are shown below.

Parameter	Criteria	Result	Pass/Fail
Tailing Factor, T	$0.5 \leq T \leq 2.0$	0.79	Pass
Column Efficiency, N	$\geq$ 250,000 plates	1,070,819	Pass
Precision (%RSD)	$\leq$ 5% (n=6)	0.6%	Pass
Resolution	$\geq$ 40	91.5	Pass

4.4 Results

Calculations based on a major peak comparison technique gave the results shown in the following table. Typical chromatograms are shown in Figure 2.

RTI Log No.	Chemical	RRF[a]	Mean RRF[a] (%RSD)	Percent Relative Purity[b]
082010-B-14	Analytical Replicate #1	1.637	1.640 (0.4)	98.1
	Analytical Replicate #2	1.647		
	Analytical Replicate #3	1.637		
082010-B-05	Reference Replicate #1	1.661	1.672 (2.1)	--
	Reference Replicate #2	1.645		
	Reference Replicate #3	1.711		

[a] RRF = Relative Response Factor; normalized to sample concentration.
[b] Relative Purity = (Mean RRF, bulk/Mean RRF, ref.) x 100.

Based on the chromatographic results, the bulk sample had not significantly changed as compared to the frozen reference, and no significant impurities were observed.

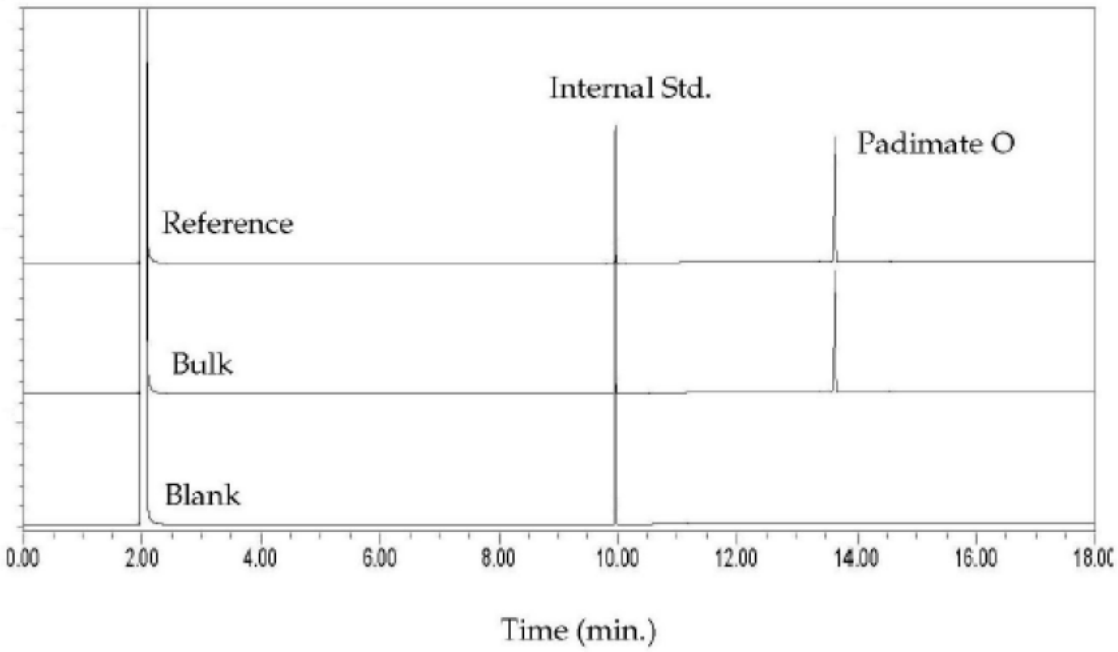


Figure 2: Example Gas Chromatograms of Padimate O Reference and Bulk Sample, and an IS Blank

5.0 REFERENCE

RTI International report "2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O), Characterization Protocols Development, (CHEM11292), January 6, 2012.

6.0 ACKNOWLEDGMENTS

Personnel contributing to this task: [redacted]

APPENDIX 3 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

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

Company/University Cectax, Inc.

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

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

Sample Designation or #: Hela0129 free

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 4 Protocol

4717 Campus Drive, Kalamazoo, MI 49008 (269) 353-5555 (office) www.ceetox.com

CeeTox®
In vitro models to predict toxicity

PROTOCOL

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

Data Requirements: *OPPTS 890.1300*

Study Number:
9070-100794ERTA

Sponsor:
National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709 USA

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
Research Triangle Park, NC **Phone:** [redacted]
Study Monitor: [redacted] **E-mail:** [redacted] **Phone:** [redacted]
CoStudy Monitor: N/A **Phone:** N/A
Sponsor Protocol/Project No: N/A
Test Substance Name(s): 2-Phenyl-5-benzimidazolesulfonic Acid (Ensulizole)
Purity: 99.6%
Batch or Lot#: 05117JE
Test Substance Name(s): Butyl-methoxydibenzoylmethane (Avobenzone)
Purity: 98.5%
Batch or Lot#: L802809
Test Substance Name(s): 3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)
Purity: 99.3%
Batch or Lot#: YT0976
Test Substance Name(s): 2-Ethylhexyl-P-Dimethyl-Aminobenzoate (Padimate-O)
Purity: 98.1%
Batch or Lot#: MKBF0590V
***Proposed Experimental Start Date:** January 23, 2013 (date subject to change; actual experimental start date to be provided in final report)
***Proposed Experimental Termination Date:** February 22, 2013 (date subject to change; actual experimental termination date to be provided in final report)

Sponsor
National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709

[redacted]
Contract Office Technical Representative
National Toxicology Program, National Institutes of Environmental Health

[redacted]
National Toxicology Program (NTP) Investigator
Telephone No.: [redacted]
Facsimile No.:
E-mail:

Study Monitor
[redacted]
Integrated Laboratory Systems, Inc.
Telephone No.: [redacted]
Facsimile No.:
E-mail:

Project Identification
ILS Project No.: N135
Study No.: 007
Human and Health Science Number: HHSN273200900005C
NIEHS contract number: N01ES00005

Table of Contents

Signatures

[redacted]
Chief, Toxicology Branch
National Toxicology Program, NIEHS
1/15/13
Date

[redacted]
Contract Office Technical Representative
National Toxicology Program, NIEHS
1/15/13
Date

[redacted]
Integrated Laboratory Systems, Inc
Study Monitor
15Jan2013
Date

[redacted]
Study Director
16 Jan 2013
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 four test substances for human estrogen receptor alpha transcriptional activation activity using the hERα–HeLa-9903 reporter cell line. The endpoints are cell viability (propidium iodide uptake) and estrogen receptor transcriptional activity, assessed via luminescence.

The results of this screen are intended to be used in conjunction with results from other Tier 1 in vitro and in vivo screening assays (OCSPP 890 test guideline series) that constitute the full screening battery under the Endocrine Disruptor Screening Program (EDSP). Results of the Tier 1 screening battery, along with other scientifically relevant information, are to be used in a weight-of-evidence assessment leading to the determination of a substance's potential to interact with the endocrine system. The Tier 1 battery is intended for screening purposes only and should not be used for endocrine classification or risk assessment.

3. Compliance Statement

This study will be conducted in compliance with the U.S. Environmental Protection Agency Good Laboratory Practice regulations Title 40, Part 160 with the exception of section 160.113. Dose concentrations of test substance and control substances will not be verified using analytical methods.

4. Quality Assurance

This study will be subjected to periodic inspections. The data and the draft final report will be reviewed by the Quality Assurance Unit of CeeTox in accordance with CeeTox standard operating procedures (SOPs).

5. Regulatory Citations / Guidelines

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.

6. Test Facility

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

7. Experimental Design

The estrogen receptor transactivation assay is to be used in conjunction with other guidelines in the OPPTS 890 series to identify substances that have the potential to

interact with the estrogen, androgen, or thyroid hormone pathways. This assay is intended to identify the ability of test compounds to activate estrogen receptors (ERs) present in the HeLa-9903 cell line. In this assay, the test materials and the controls are exposed to the HeLa-9903 cells at eight concentrations for approximately 24 hours. At the end of the exposure period, the medium is removed from the cells, cell viability is monitored using a propidium iodide (PI) uptake assay, and effects on estrogen receptor transactivation are determined using a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin estrogen-responsive element. The positive controls are 17β-estradiol and 17α-estradiol. The weak positive control is 17α-methyltestosterone and the negative control is corticosterone. All four controls are assessed every time the activation assay is performed.

8. Justification of the Test System

The HeLa-9903 human cervical cell line will be used in the study as required in the test guideline (OPPTS 890.1300). This cell line 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).

9. Identification of the Test System

The cells used for the estrogen receptor transcriptional activation assay will be the HeLa-9903 human cervical carcinoma cell line (JCRB No. JCRB1318).

The cells used in the study will be appropriately labeled and will be identified by cell type and passage number. The passage number used in the assay will be provided in the report. Bias is not a factor in this test system.

10. Test & Control Substance(s)

10.1 Test Substance

A certificate of analysis for the test substances 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 or sponsor's designee. Test substance will be either returned to the Sponsor or destroyed following finalization of the study report.

Test Substance: 2-Phenyl-5-benzimidazolesulfonic acid (Ensulizole)

CAS No. 27503-81-7

Source:	Sigma-Aldrich
Lot/Batch No.:	05117JE
Formula:	$C_{13}H_{10}N_2O_3S$
Description:	White powder
Purity:	99.6%
Test Substance:	Butyl-methoxydibenzoylmethane (Avobenzone)
CAS No.	70356-09-1
Source:	Universal Preserv-A-Chem Inc.
Lot/Batch No.:	L802809
Formula:	$C_{20}H_{22}O_3$
Description:	Off White to Yellowish Crystalline Powder
Purity:	~98.5%
Test Substance:	3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)
CAS No.	118-56-9
Source:	Spectrum Chemical Mfg. Corp
Lot/Batch No.:	YT0976
Formula:	$C_{16}H_{22}O_3$
Description:	Colorless to light yellow liquid
Purity:	99.3%
Test Substance:	2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O)
CAS No.	21245-02-3

Source:	Sigma-Aldrich
Lot/Batch No.:	MKBF0590V
Formula:	$(CH_3)_2NC_6H_4CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3$
Description:	Colorless liquid
Purity:	98.1%

10.2 Preparation of Test Substance

The test substances will be prepared in HeLa-9903 cell culture media (section 11.3). Each test material will be prepared as a stock in DMSO (final concentration in media of 0.1% (v/v)) and serially diluted in the same solvent to prepare solutions for dilutions with media. All dilutions of the stock solutions will be prepared on the day of use in the assay.

10.3 Control and Reference Substances

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

Each control substance will be prepared as a stock in DMSO (final concentration in media of 0.1% (v/v)) and serially diluted in the same solvent to prepare solutions for dilutions with media. 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.

11. Test System

11.1 Source

As per the guideline (OPPTS 890.1300), 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 have gone through ≤ 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.

11.2 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 2) can be acceptable if they fall within CeeTox historical data. A complete concentration response curve (see Test Range in Table 2) 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)

Reference Substance	$LogPC_{50}$	$LogPC_{10}$	$LogEC_{50}$	Hill slope	Test Range (M)
17β-Estradiol (E2)	-11.4 ~ -10.1	<-11	-11.3 ~ -10.1	0.7 ~ 1.5	10^{-14} ~ 10^{-8}
17α-Estradiol	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	10^{-12} ~ 10^{-6}
Corticosterone	-	-	-	-	10^{-10} ~ 10^{-4}
17α-Methyltestosterone	-6.0 ~ -5.1	-8.0 ~ -6.2	-	-	10^{-11} ~ 10^{-5}

11.3 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 approximately 1 X 10^4 cells/100 µL/well. The cells will then be placed into a ~5% CO_2 incubator approximately 37°C for approximately 3 hours prior to substance exposure.

12. Methods

12.1 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 membranes 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 for the cytotoxicity assay will be seeded into a 96-well black sided culture plate at the same time cells are seeded for the ER transactivation assays. 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.

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 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 approximately 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.

12.2 Solubility/Precipitation Assay

The limit of test substance solubility will be determined by laser based light scattering. The test substance will be prepared in the HeLa-9903 culture media at the final exposure concentrations and added to wells of a 96-well plate. The samples will be assessed using a NEPHELOstar nephelometer (BMG LabTech, Ortenberg, Germany).

12.3 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 12.1) and solubility (as described in section 12.2) 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. The final eight concentrations used will be noted in the final report, but are typically in the range of 1 X 10^{-13} to 1 X 10^{-3}.

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 12.1) or produce precipitation will be noted. Concentrations in subsequent runs will be adjusted as necessary.

12.4 Substance Exposure and Assay Plate Organization

The cell viability and luciferase assays described below will be performed a minimum of two times. 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 12.3). 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 (10% DCC-FBS in EMEM) as appropriate (if DMSO is used the final concentration of DMSO in media will not exceed 0.1%).

Step 3: 75 µL of test substance dilution (2X) in media (prepared in step 2) will be added by pipetting to wells containing approximately 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 an approximately 37°C incubator at approximately 5% CO_2 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.

12.5 Luciferase Assay

A luciferase assay as described in CeeTox SOP 2041 (proprietary) will be performed. Luciferase assay reagent will be prepared as described in CeeTox SOP 2041. Briefly, cells will be lysed and the cell lysate will be combined with CeeTox proprietary Luciferase reagent and read using a Packard aHT Fusion or equivalent plate reader.

13. Analysis of Data

All cytotoxic concentrations of test substance (as defined in section 12.1) 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 (an average of the raw luminescence reads from the VC wells).

Step 2: The mean value of the VC will be subtracted from each well to normalize the data (each wells' individual luminescence read – the mean value of the VC calculated in step 1).

Step 3: The mean for the normalized PC will be calculated (an average of the values of the positive control wells (calculated in step 2)) .

Step 4: The normalized value of each well will be divided by the mean value of the normalized PC (PC = 100%) (step 2 for each well ÷ the mean value of the normalized PC (calculated in step 3)). 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).

13.1 Proposed Statistical Methods

For data generated at CeeTox, basic statistical analysis will be performed on the data, which will include mean specific binding (%), standard deviation (SD), standard error of the mean (SEM), percent coefficient of variation (% CV) using XLfit (Guildford, Surrey, UK).

13.2 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. XLfit will be used or the calculation of EC_{50} and maximum induction level.

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

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

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.

14. 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 following 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 2).

Data interpretation criteria are shown in Table 4. 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.

15. Final Study Reports

The data to be reported in the draft report and final report will be determined per 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), data, data analysis and interpretation and classification of the test substances.

16. 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 to document this verbal request. All protocol amendments with justifications will be documented, signed and

dated by the Study Director and Sponsor's Representative. A copy of the protocol and all amendments will be issued to the Sponsor and the originals will be placed into the study binder.

17. Data Retention and Archiving

All original data [including the original signed study protocol and all amendments (if any), test substance information, observations, etc.] and the original final report will be transferred to the National Toxicology Program Archives following finalization of the study report to the address below:

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

18. Test Substance Disposition

Test substance will be either returned to the sponsor or destroyed following finalization of the study report.