CeeTox
In vitro models to predict toxicity

Estrogen Receptor Binding (Rat Uterine Cytosol)

Final Report

DATA REQUIREMENT(S): OPPTS 890.1250 (2009)

AUTHOR(S): [redacted]

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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study Number: 9070-100794ERB

Study Title: Estrogen Receptor Binding (Rat Uterine Cytosol)

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

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

[redacted]

Study Director

25 April 2013

Date

FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Title: Estrogen Receptor Binding (Rat Uterine Cytosol)

Study Number: 9070-100794ERB

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
20Dec12	Draft Protocol	20Dec12	20Dec12
23Jan13	Test Substance Prep. & Day 1 ERB Assay	28Jan13	28Jan13
24Jan13	Day 2 ERB Assay	28Jan13	28Jan13
13Mar13	Data Binder	13Mar13	13Mar13
13Mar13	Draft Report	13Mar13	13Mar13

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
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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
	Study Director

Study Dates

Study initiation date: January 16, 2013
Experimental start date: January 23, 2013
Experimental termination date: February 21, 2013
Study termination date: April 25, 2013

Deviations from the Protocol

See Appendix 3. There was one deviation however it did not impact the integrity of the data in this report.

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

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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 interact with the estrogen receptors (ERs) isolated from rat uteri.

Assessments of precipitation were conducted during each run in order to identify a suitable top concentration of ensulizole, avobenzone, homosalate and padimate-O for use in the binding assays. The concentrations assessed were: 10^{-5}, 10^{-4} and 10^{-3} M.

The final concentrations of ensulizole assessed in the binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M for all three valid independent runs (26-January-2013, 30-January-2013 and 18-February-2013), while the final concentrations of homosalate and padimate-O assessed in all three valid binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} because of precipitation of the test substances at 10^{-3} M. Finally, the final concentrations of avobenzone assessed in the three valid binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} because of precipitation of the test substances at 10^{-4} and 10^{-3} M.

Four independent runs of the ER binding assay were conducted. One run was considered invalid because the control data was well outside the acceptance criteria. All concentrations were tested in replicates of 3. In addition, solvent control tubes (3 replicates) were prepared to assess total binding. These replicates included the radioligand, cytosol (containing the ERs) and solvent but without the competitor 17β-estradiol. The total binding tubes allowed for the identification of maximal binding of [^{3}H]-17β-estradiol. Non-specific binding (NSB) was also assessed in replicates of 3 by determining the [^{3}H]-17β-estradiol bound in the presence of 100-fold excess unlabeled 17β-estradiol. Data was NSB subtracted, normalized to total binding and presented as % specific binding. Finally, 50 µL of master mix (containing TEDG buffer+PMSF and [^{3}H]-17β-estradiol) was added to scintillation vials (n=6) in order to determine both total radioligand added and to calculate the percentage of total radioligand added to the tube that was bound to ERs. The duration of incubation at approximately 4°C was 16-20 hours. A complete concentration response curve for the positive control 17β-estradiol, negative control (NC) octyltriethoxysilane and weak positive control (wPC) 19-norethindrone, was run each time the binding assay was performed.

1.2 Results

The suitable top concentration of ensulizole was 10^{-3} M for use in all three valid independent runs (26-January-2013, 30-January-2013 and 18-February-2013), while the suitable top concentration of avobenzone was 10^{-5} M as precipitation was seen at 10^{-4} and 10^{-3} M. The suitable top concentration of homosalate and padimate-O for use in all three valid independent runs was 10^{-4} M as precipitation was seen at 10^{-3} M.

In all three valid independent runs, the mean specific binding was > 80% for all concentrations of the negative control octyltriethoxysilane. In the first valid independent run

(26-January-2013), the mean specific binding was > 75% at every soluble concentration tested for ensulizole, avobenzone, homosalate and padimate-O, classifying them as "non-interacting" for this run. The weak positive control 19-norethindrone had a $LogIC_{50}$ of -5.5 M while the $LogIC_{50}$ of 17β-estradiol was -10.0 M.

In the second valid independent run (30-January-2013), the mean specific binding was > 75% at every soluble concentration tested for ensulizole, avobenzone, homosalate and padimate-O, classifying them as "non-interacting" for this run. The weak positive control 19-norethindrone had a $LogIC_{50}$ of -5.6 M while the $LogIC_{50}$ of 17β-estradiol was -9.0 M.

Finally, in the third valid independent run (18-February-2013), the mean specific binding was > 75% every soluble concentration tested for ensulizole, avobenzone, homosalate and padimate-O, classifying them as "non-interacting" for this run. The weak positive control 19-norethindrone had a $LogIC_{50}$ of -4.9 M while the $LogIC_{50}$ of 17β-estradiol was -9.0 M.

The mean relative binding affinity, or RBA (calculated by dividing the $LogIC_{50}$ of the control/test material by the $LogIC_{50}$ of the positive control 17β-estradiol) was 0.6 for 19-norethindrone.

1.3 Conclusion

Ensulizole, avobenzone, homosalate and padimate-O were classified as "non-interacting" with the ERs in all three independent runs and thus have a final classification of "non-interacting."

2.0 INTRODUCTION

2.1 Purpose

The objective of this study was to evaluate the ability of ensulizole, avobenzone, homosalate and padimate-O to interact with the estrogen receptors (ERs) isolated from rat uteri. The ER contains a highly specific hormone-binding domain (HBD) that is conserved across species. Upon binding endogenous estrogens to the HBD, the ER binds to specific sites in the genome controlling gene expression. Thus a testing a compound's ability to bind to ER constitutes a direct, simple evaluation of its estrogenic potential in thousands of vertebrate species.

This assay was used to provide information on the ability of a compound to interact with the estrogen receptors (ERs) isolated from rat uteri. This assay is not intended to be used to show that the interaction is, specifically, one-site competitive binding, or to precisely characterize the strength of the binding interaction. It therefore may not be appropriate to use in quantitative structure-activity relationship (SAR) model development for estrogen receptor binding without further refinement. This assay is intended to be used as one part of a screening program that includes other assays, to detect substances that can potentially interact with the estrogen hormonal system.

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 hormone systems. 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

OPPTS 890.1250: Estrogen receptor binding assay using rat uterine cytosol (ER-RUC). 2009.

3.0 MATERIALS AND METHODS

All materials and methods described in this report are in reference to the three valid independent runs (26-January-2013, 30-January-2013 and 18-February-2013) only.

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 4.

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 4.

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 4.

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 4.

The reference compound 17β-estradiol (CAS# 50-28-2) was purchased from Sigma-Aldrich (St. Louis, MO) and was 100% pure. The catalog number was E8875 and the lot number was SLBC5955V.

The negative control octyltriethoxysilane (CAS# 2943-75-1) was purchased from Sigma-Aldrich (Cheboygan Falls, WI) and was 99.34% pure. The catalog number was 440213 and the lot number was 24996KK.

The weak positive control 19-norethindrone (CAS# 68-22-4) was purchased from Sigma-Aldrich (St. Louis, MO) and was 99% pure. The catalog number was N4128 and the lot number was 030M1359V.

The radioligand [^{3}H]-17β-estradiol was purchased from Perkin-Elmer (Boston, MA) and had a specific activity (SA) of 140 Ci/mmol on the certification date (13-September-2012). The catalog number was NET517 and the lot number was 1664345. The $SA_{adjusted}$ was 137.1 Ci/mmol for the first independent run (26-January-2013), 137.0 Ci/mmol for the second independent run (30-January-2013) and 136.7 Ci/mmol for the third independent run (18-February-2013).

3.1.2 Vehicle selection

Dimethyl sulfoxide (DMSO) is one of the recommended solvents according to the US EPA guideline (OPPTS 890.1250) and was selected as a suitable vehicle for ensulizole, avobenzone, homosalate and padimate-O. Ensulizole solutions with a concentration of up to 10^{-3} M, and avobenzone, homosalate and padimate-O solutions with a concentration of 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 2% (v/v). 17β-estradiol, octyltriethoxysilane and 19-norethindrone were prepared on January 23, 2013 then frozen as aliquots and thawed on the day of the assay for use in the first, second and third valid independent runs. The test substances were prepared fresh on the day of the assay for all three valid independent runs. Based upon historical data for control compounds 17β-estradiol, octyltriethoxysilane and 19-norethindrone and OPPTS 890.1250 guideline criteria for these reference compounds, they are deemed stable over these times.

3.1.3 Test Substance Preparation

Vehicle (DMSO) was kept at the same concentration for the positive and negative controls and for the test substances. DMSO was tested with the reference chemical and control chemicals for the run as well. The maximum percent of DMSO allowed in assay tubes is 10%, however all concentrations of ensulizole, avobenzone, homosalate and padimate-O were kept at approximately 2% final concentration. The dose concentrations of ensulizole, avobenzone, homosalate and padimate-O were not verified using analytical methods.

Serial dilutions of test chemicals were prepared in DMSO to yield the final concentrations indicated below:

Example Dilution Procedure for Ensulizole, Avobenzone, Homosalate and Padimate-O.

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted test chemical	Diluted test chemical concentration	*Final test chemical concentration in ER assay tube
TS1	Use 500 µl of stock test chemical (50 mM)	500 µl	1 ml	2.5×10^{-2} M	1×10^{-3} M
TS2	Use 100 µl of dilution TC1 (25 mM)	900 µl	1 ml	2.5×10^{-3} M	1×10^{-4} M
TS3	Use 100 µl of dilution TC2 (2.5 mM)	900 µl	1 ml	2.5×10^{-4} M	1×10^{-5} M
TS4	Use 100 µl of dilution TC3 (250 µM)	900 µl	1 ml	2.5×10^{-5} M	1×10^{-6} M
TS5	Use 100 µl of dilution TC4 (25 µM)	900 µl	1 ml	2.5×10^{-6} M	1×10^{-7} M
TS6	Use 100 µl of dilution TC5 (2.5 µM)	900 µl	1 ml	2.5×10^{-7} M	1×10^{-8} M
TS7	Use 100 µl of dilution TC6 (250 nM)	900 µl	1 ml	2.5×10^{-8} M	1×10^{-9} M
TS8	Use 100 µl of dilution TC7 (25 nM)	900 µl	1 ml	2.5×10^{-9} M	1×10^{-10} M

*Final concentration of test chemical in assay tube when 20 µl of diluted concentration is used in a total volume of 500 µl.

3.1.4 Positive and Negative Control Preparation

Octyltriethoxysilane was the negative control. A 100 mM stock was prepared in DMSO and serially diluted as described for the test chemicals. The concentration range for the negative control was 1×10^{-10} to 1×10^{-3} M with DMSO kept at approximately 2%.

The weak positive control was 19-norethindrone. A 10 mM stock was prepared in DMSO and serially diluted as described below. The concentration range tested for the weak positive control was from 3.16 x 10^{-9} to 1 x 10^{-4} M with DMSO kept at approximately 2%**.**

Example Dilution Procedure for 19-norethindrone

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted positive control	Positive Control Concentration	
				Diluted	*Final in ER assay tube
P1	Use 400 µl of stock positive control (5 mM)	400 µl	800 µl	2.5 x 10^{-3} M	1 x 10^{-4} M
P2	Use 150 µl of stock positive control (5 mM)	800 µl	950 µl	7.9 x 10^{-4} M	3.16 x 10^{-5} M
P3	Use 100 µl of P2 (790 µM)	900 µl	1 ml	7.9 x 10^{-5} M	3.16 x 10^{-6} M
Intermed	Use 100 µl of P1 (2.5 mM)	900 µl	1 ml	2.5 x 10^{-4} M	Not used
P4	Use 100 µl of Intermed (250 µM)	900 µl	1 ml	2.5 x 10^{-5} M	1 x 10^{-6} M
P5	Use 100 µl of P3 (79 µM)	900 µl	1 ml	7.9 x 10^{-6} M	3.16 x 10^{-7} M
P6	Use 100 µl of P4 (25 µM)	900 µl	1 ml	2.5 x 10^{-6} M	1 x 10^{-7} M
P7	Use 100 µl of P5 (7.9 µM)	900 µl	1 ml	7.9 x 10^{-7} M	3.16 x 10^{-8} M
P8	Use 100 µl of P7 (790 nM)	900 µl	1 ml	7.9 x 10^{-8} M	3.16 x 10^{-9} M

*Final concentration of control chemical in assay tube when 20 µl of diluted concentration is used in a total volume of 500 µl.

The positive control, 17β-estradiol, strongly binds ERs and was included to ensure that the run was properly performed and to allow an assessment of variability in the conduct of the assay across time. Final concentrations of unlabeled 17β-estradiol ranged from 1 x 10^{-7} to 1 x 10^{-11} M as described below. Fresh 50 µM 17β-estradiol stock was prepared and serial dilutions of the reference standard were performed in DMSO (final concentration of 2%).

Example Dilution Procedure for 17β-estradiol

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of 17β-estradiol	Diluted 17β-estradiol concentration	*Final 17β-estradiol concentration in ER assay tube
NSB1	Use 100 µl of stock 17β-estradiol (25 µM)	900 µl	1 ml	2.5 x 10^{-6} M	1 x 10^{-7} M
S2	Use 100 µl of dilution NSB1 (2.5 µM)	900 µl	1 ml	2.5 x 10^{-7} M	1 x 10^{-8} M
S3	Use 277 µl of dilution S2 (250 nM)	600 µl	877 µl	7.9 x 10^{-8} M	3.16 x 10^{-9} M
S4	Use 100 µl of dilution S2 (250 nM)	900 µl	1 ml	2.5 x 10^{-8} M	1 x 10^{-9} M
S5	Use 100 µl of dilution S3 (79 nM)	900 µl	1 ml	7.9 x 10^{-9} M	3.16 x 10^{-10} M
S6	Use 100 µl of dilution S4 (25 nM)	900 µl	1 ml	2.5 x 10^{-9} M	1 x 10^{-10} M
S7	Use 100 µl of dilution S6 (2.5 nM)	900 µl	1 ml	2.5 x 10^{-10} M	1 x 10^{-11} M

*Final concentration of control chemical in assay tube when 20 µl of diluted concentration is used in a total volume of 500 µl.

3.2 Solubility/Precipitation Assay

The limit of test chemical solubility was determined by visual observation. Compound solubility was determined in solvent. In addition, the solutions were watched closely when added to the experiment tube (as the test compound may precipitate upon addition to the assay tube mixtures).

3.3 Rat Uterine Cytosol

Uteri from 106 female Sprague-Dawley rats (85 to 100 days of age at time of kill) ovariectomized seven days prior to being humanely killed were purchased from Harlan Labs and were used to prepare the cytosol. Cytosol was prepared and verified at CeeTox per OPPTS guideline and CeeTox SOP 2057 for use on this study. As the cytosol was prepared in large batches for use in multiple assays of different test substances, related data are maintained separate from this study and the pertinent information is available in Appendix 2.

3.4 Stock Solution Preparation

A 200 mM EDTA stock solution was prepared and stored at approximately 4°C. A 1 M Tris buffer was prepared and the pH was adjusted to 7.4. The buffer can be stored at

approximately 4°C for up to 12 months. These solutions were then used to prepare 2X TEG Buffer (20 mM Tris, 3 mM EDTA, 20% glycerol, pH 7.4 [cooled to approximately 4°C before adjusting to pH 7.4 and stored at approximately 4°C up to 3 months]).

The 60% hydroxyapatite (HAP) slurry was prepared one day before use. The HAP was gently mixed with ~3X volume of TEDG + PI buffer in a graduated cylinder, and refrigerated for approximately 2 hours at approximately 4°C. The HAP was then washed twice as follows. The supernatant was removed and the HAP was resuspended again in ~3X fresh TEDG + PI buffer (approximately 4°C). The slurry was mixed gently and allowed to settle for approximately 2 hours at approximately 4°C. After the second wash, the HAP slurry settled overnight (at least 8 to 10 hours at approximately 4°C).

The next day (day of use), the volume of HAP on the graduated cylinder was noted. The supernatant was removed and the HAP was resuspended to a final volume of 60% HAP and 40% cold TEDG + PI. The HAP slurry was well-suspended and ice-cold when used in the separation procedure.

3.5 Assays

3.5.1 Working Assay Buffer Preparation

Summary Table of Assay Conditions

		Competitive Binding Assay Protocol
Source of receptor		Rat uterine cytosol
Concentration of radioligand		1 nM
Concentration of receptor		Sufficient to bind 10-15% of radioligand
Concentration of test substance (as serial dilutions)		100 pM to 1 mM
Temperature		~4°C
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10% (v/v)
	Protease Inhibitor	0.5% (v/v)
	DTT	1 mM

On the day of assay, the Working Assay Buffer, or TEDG+PI buffer (10 mM Tris, 1.5 mM EDTA, 1 mM DTT, 0.5% Protease Inhibitor (v/v), 10% glycerol, pH 7.4) was prepared using the 2X TEG buffer.

3.5.2 [^{3}H]-17β-estradiol Preparation

[^{3}H]-17β-estradiol was prepared from the stock solution on the day of assay. The specific activity was adjusted for decay over time prior to performing dilutions. The specific activity was calculated on the day of the assay using the following equation:

$SA_{adjusted}$ (Fraction Isotope Remaining) = SA * $e^{-Kdecay*Time}$

SA is the specific activity on the packaging date.

Kdecay is the decay constant for tritium (equal to 1.54 x 10^{-4}/day).
Time = days since the date on the stock bottle from the manufacturer.

The [^{3}H]-17β-estradiol was diluted with TEDG + PI buffer so that each assay tube contained 1 nM final concentration of [^{3}H]-17β-estradiol using the following procedure:

The specific activity was converted from Ci/mmole to nM. If SA = X Ci/mmole, and Y = concentration of radiolabel, then X Ci/mmole was converted to nM and the SA activity adjusted for decay over time by the following conversion:

(Y mCi/ml / X Ci/mmole) * 1 Ci/1000 mCi * 10^6 nmole/mmole * 1000 ml/L = (Y/X) * 10^6 nM

A 50 nM diluted stock of the [^{3}H]-17β-estradiol was prepared so that 10 µl in a total volume of 500 µl per assay tube will give a final concentration of 1 nM. The 50 nM [^{3}H]-17β-estradiol was kept on ice until standards, test chemicals, and assay tubes were prepared.

3.5.3 Assay Preparations

Siliconized 12 x 75 mm glass tubes were used for the assay. A master mixture of radioligand and buffer was prepared. An example is 153 tubes are required for a run that includes the solvent control, three standards, and three unknowns. Trace tubes are also required. The following table describes the preparation of a master mixture for 155 tubes:

Master Mixture for Competitive Binding Assay

Substance	Target Volume/Tube (µl)		# of Tubes		Total Volume Needed (ml)		Master Mix Volumes (ml)
	Assay Tubes	Trace Tubes	Assay Tubes	Trace Tubes	Assay Tubes	Trace Tubes	
TEDG Buffer + PI	370	48.72	155	6	57.35	0.292	59.192
Diluted [3H]-17β-estradiol (50 nM)	10	1.28	155	6	1.55	0.008	1.558
Total	380	50			58.9	0.3	60.75

3.5.4 Individual Tubes

For the assay tubes, 380 µl of the master mixture above was added and kept on ice. For the total radioligand added (TRA) tubes, 50 µl (1 nM [^{3}H]-17β-estradiol) final was added directly to 10 ml of scintillation fluid in scintillation vials and counted immediately. The standards, weak positive, negative and test chemicals were prepared as described and added to the assay tubes. Twenty microliters of chemical was added per tube. After all chemicals were added to the tubes, 100 µl of cytosol was added to each tube for a final volume of 500 µl. The temperature of the tubes and contents were kept at approximately 4°C prior to the

addition of the cytosol. The assay tubes were vortexed after additions and incubated at approximately 4°C for 16 to 20 hours on a rotator.

Competitive Binding Assay Additions

Volume (µl)	Component
20	Unlabeled 17β-estradiol, weak positive control, negative control, or test substance
380	Master mixture (TEDG + PI assay buffer + [^{3}H]-17β-estradiol
100	Uterine cytosol (diluted to appropriate protein concentration)
500	**Total volume in each assay tube**

3.5.5 Separation of bound [^{3}H]-17β-estradiol from free [^{3}H]-17β-estradiol

The ER assay tubes were removed from the rotator and placed in an ice-water bath. A repeating pipette was used to add approximately 250 µl of ice cold HAP slurry (60% in TEDG + PI) to each assay tube. The tubes were vortexed for approximately 10 seconds at approximately 5 minute intervals for a total of approximately 15 minutes with tubes remaining in the ice-water bath between vortexing. Following the vortexing step, approximately 2 ml of the cold (approximately 4°C) TEDG + PI buffer was added, quickly vortexed, and centrifuged at approximately 4°C for approximately 10 minutes at 1000 x g. After centrifugation, the supernatant containing the free [^{3}H]-17β-estradiol was immediately decanted and discarded. The HAP pellet contained the estrogen receptor bound [^{3}H]-17β-estradiol. Approximately 2 ml of ice-cold TEDG + PI buffer was added to each tube and vortexed to resuspend the pellet. The tubes were centrifuged again at approximately 4°C for approximately 10 minutes at approximately 1000 x g. The supernatant was quickly decanted and discarded. The wash and centrifugation steps were repeated once more. After the final wash, the supernatant was decanted. The assay tubes were allowed to drain briefly for approximately 30 seconds.

3.5.6 Extraction and Quantification of [^{3}H]-17β-estradiol bound to ER.

Approximately 1.5 ml of absolute ethanol was added to each assay tube. The tubes were allowed to sit at room temperature for approximately 15 to 20 minutes, vortexing for approximately 10 seconds at approximately 5-minute intervals. The assay tubes were centrifuged for approximately 10 minutes at approximately 1000 x g. An approximately 1 ml aliquot was pipetted, taking care to avoid the centrifuged pellet, into a 20 ml scintillation vial containing approximately 10 ml scintillation cocktail (Perkin Elmer Opti-Fluor, cat# 6013199, lot# 47-11421 for the first valid run and lot# 47-12261 for the second and third valid runs). The vial was capped and shaken. The vials were placed in a scintillation counter (Perkin Elmer Tri-Carb 2910TR Liquid Scintillation Analyzer Model B2910) and each vial was counted for at least one minute with quench correction for determination of DPMs per vial.

Standards (^{3}H, ^{14}C and background) were used to verify accurate counting, and the liquid scintillation analyzer has an enhanced Instrument Performance Assessment (IPA) for monitoring efficiencies, backgrounds, E2/B and Chi-square values for ^{3}H and ^{14}C over the life of the instrument. The most recent IPA time and date stamped data are available on

demand for reporting purposes. Each IPA printout includes instrument model, serial number, software version number and calibration standard information.

3.6 Competitive Binding Data Analysis and Interpretation

3.6.1 Analysis and Considerations

For each of the three valid independent runs of the competitive binding assays, the DPM values were added to a locked data spreadsheet (Microsoft EXCEL 2010 Version 14.0.6123.5001; Redmond, WA). The following statistics were assessed; mean specific binding (%), standard deviation (SD), standard error of the mean (SEM), percent coefficient of variation (% CV), residuals, squared residuals, and the Loge(Syx) (ie. Loge(residual standard deviation)) using XLfit (Version 5.2.0.0; Guildford, Surrey, UK). XLfit was also used for graphing the results and determining the bottom, top, and hill slope and IC_{50} (if applicable) for each curve generated.

The competitive binding assay was functioning correctly if all of the following criteria had been met, according to OPPTS 890.1250:

Increasing concentrations of unlabeled 17β-estradiol displaced [^{3}H]-17β-estradiol from the receptor in a manner consistent with one-site competitive binding. Specifically, the curve fitted to the radioinert estradiol data points using non-linear regression descended from 90% to 10% over approximately an 81-fold increase in the concentration of the test chemicals.

Ligand depletion was minimal. Specifically, the ratio of total binding in the absence of competitor to the total amount of [^{3}H]-17β-estradiol added per assay tube was no greater than 15%.

The parameter values (top, bottom, and slope) for 17β-estradiol and the concurrent positive control (19-norethindrone) were within the tolerance bounds outlined in the OPPTS guideline and are provided below.

The solvent control substance did not alter the sensitivity or reliability of the assay. Specifically, the acceptable limit of ethanol concentration in the assay tube was 3%; the acceptable limit of DMSO concentration was ≤ 10%. All tubes must have contained equal amounts of solvent.

The negative control substance (octyltriethoxysilane) did not displace more than 25% of the radioligand from the ER on average across all concentrations.

The test chemical was tested over a concentration range that fully defined the top of the curve (i.e. a range that showed that a top plateau was achieved), and the top was within 25 percentage points of either the solvent control or the value for the lowest concentration of the estradiol standard for that run.

Upper and Lower Limits for Parameters in Competitive Binding Assay Curves for the Standards (Radioinert Estradiol and 19-Norethindrone)

Parameter	Unit	Estradiol		19-Norethindrone	
		Lower Limit	Upper Limit	Lower Limit	Upper Limit
Loge(Syx)	--	NA	2.35	NA	2.60
Bottom plateau level	% binding	-4	1	-5	1
Top plateau level	% binding	94	111	90	110
(Hill) Slope	Log10(M)-1	-1.1	-0.7	-1.1	-0.7

3.6.2 Classification

The classification of a chemical as a binder or non-binder was made on the basis of the average results of three non-concurrent runs, each of which met the performance criteria and taken together, were consistent with each other, as per OPPTS guideline 890.1250. Each run was classified as "interacting," "not interacting," "equivocal," or "equivocal up to the limit of the concentrations tested."

A run was classified as "interactive" with the ERs if the lowest point on the fitted response curve within the range of the data was less than 50%. "Percent" refers to binding of the radiolabeled estradiol. Thus, "less than 50%" means that less than 50% of the radiolabeled estradiol was bound, or equivalently, that more than 50% of the radiolabeled estradiol had been displaced from the receptor. In other words, a run was classified as "interactive" if a Log(IC_{50}) was obtained.

A run was classified as "equivocal up to the limit of concentrations tested" if there were no data points at or above a test chemical concentration of 10^{-6} M and one of the two following conditions held:

A binding curve could be fit but 50% or less of the radiolabeled estradiol was displaced by concentration of 10^{-6} M.
OR
A binding curve could not be fit and lowest average percent binding among the concentration groups in the data was above 50%.

A run was classified as "not interactive" if there were usable data points at or above 10^{-6} M and either:

The lowest point on the fitted response curve within the range of the data was above 75%.
OR
A binding curve could not be fitted and the lowest average percent binding among the concentration groups in the data was above 75%.

A run was classified as "equivocal" if it fell in none of the categories above.

After each run was classified, the chemical was classified by assigning the following values to each run and averaging across runs:

Interactive: 2
Equivocal: 1
Not Interactive: 0

Chemical classification, based on the average of all the runs performed for a chemical:
Interactive: average ≥ 1.5
Equivocal: 0.5 ≤ average < 1.5
Not Interactive: average < 0.5

For example, if a chemical was tested in three runs in one lab and is determined to be interactive in 2 runs and equivocal in 1 run, to classify this chemical one would average 2, 2, and 1 = ~1.67 and the chemical would be considered interactive because the average was greater than 1.5.

4.0 RESULTS AND DISCUSSION

4.1 Concentration Range for the Test Substance

In order to identify a suitable top concentration for use in the binding assays, preliminary assessments of precipitation were conducted as described in Sections 4.2. The final concentrations of ensulizole, avobenzone, homosalate and padimate-O to assess precipitation were 10^{-5}, 10^{-4} and 10^{-3} M.

The suitable top concentration of ensulizole was 10^{-3} M for use in all three valid independent runs (26-January-2013, 30-January-2013 and 18-February-2013), while the suitable top concentration of avobenzone was 10^{-5} M as precipitation was seen at 10^{-4} and 10^{-3} M. The suitable top concentration of homosalate and padimate-O for use in all three valid independent runs was 10^{-4} M as precipitation was seen at 10^{-3} M.

The final concentrations of ensulizole assessed in the binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M for all three valid independent runs (26-January-2013, 30-January-2013 and 18-February-2013), while the final concentrations of homosalate and padimate-O assessed in all three valid binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} because of precipitation of the test substances at 10^{-3} M. Finally, the final concentrations of avobenzone assessed in the three valid binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} because of precipitation of the test substances at 10^{-4} and 10^{-3} M.

4.2 Binding Assay Acceptance Criteria

In all three independent runs of the assay, increasing concentrations of unlabeled 17β-estradiol displaced [^{3}H]-17β-estradiol from the receptor in a manner consistent with one-site competitive binding, and the ligand depletion was held below 15%. Also, the solvent did not alter the assay sensitivity or reliability. The negative control, octyltriethoxysilane, did not displace more than 25% of the radioligand from the ERs

(maximum effective displacement of 15.1%). Finally, the data were within the acceptable ranges specified in Section 4.6.1

4.3 Results

The suitable top concentration of ensulizole was 10^{-3} M for use in all three valid independent runs (26-January-2013, 30-January-2013 and 18-February-2013), while the suitable top concentration of avobenzone was 10^{-5} M as precipitation was seen at 10^{-4} and 10^{-3} M. The suitable top concentration of homosalate and padimate-O for use in all three valid independent runs was 10^{-4} M as precipitation was seen at 10^{-3} M.
In all three valid independent runs, the mean specific binding was > 80% for all concentrations of the negative control octyltriethoxysilane. In the first valid independent run (26-January-2013), the mean specific binding was > 75% at every soluble concentration tested for ensulizole, avobenzone, homosalate and padimate-O, classifying them as "non-interacting" for this run. The weak positive control 19-norethindrone had a $LogIC_{50}$ of -5.5 M while the $LogIC_{50}$ of 17β-estradiol was -10.0 M.

In the second valid independent run (30-January-2013), the mean specific binding was > 75% at every soluble concentration tested for ensulizole, avobenzone, homosalate and padimate-O, classifying them as "non-interacting" for this run. The weak positive control 19-norethindrone had a $LogIC_{50}$ of -5.6 M while the $LogIC_{50}$ of 17β-estradiol was -9.0 M.

Finally, in the third valid independent run (18-February-2013), the mean specific binding was > 75% every soluble concentration tested for ensulizole, avobenzone, homosalate and padimate-O, classifying them as "non-interacting" for this run. The weak positive control 19-norethindrone had a $LogIC_{50}$ of -4.9 M while the $LogIC_{50}$ of 17β-estradiol was -9.0 M.

The mean relative binding affinity, or RBA (calculated by dividing the $LogIC_{50}$ of the control/test material by the $LogIC_{50}$ of the positive control 17β-estradiol) was 0.6 for 19-norethindrone.

5.0 CONCLUSIONS

Ensulizole, avobenzone, homosalate and padimate-O were classified as "non-interacting" with the ERs in all three independent runs and thus have a final classification of "non-interacting."

6.0 REFERENCES

Endocrine Disruptor Screening Program Test Guidelines. *OPPTS 890.1250: Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC).* EPA 740-C-09-005. October, 2009.

TABLES SECTION

TABLE 1 Results of 1st Valid Binding Assay – Controls – January 26, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Estradiol (NSB)	-7	0.0	0.3	0.2	-1.7E+18
	-8	1.3	0.4	0.2	28.8
	-8.5	3.1	0.3	0.2	8.8
	-9	8.8	0.3	0.1	2.9
	-9.5	22.4	0.2	0.1	0.9
	-10	47.4	0.4	0.2	0.8
	-11	86.9	1.8	1.0	2.0
19-Norethindrone	-4	2.8	0.2	0.1	6.5
	-4.5	7.6	0.1	0.1	1.7
	-5.5	44.8	1.9	1.1	4.2
	-6	70.2	1.7	1.0	2.4
	-6.5	88.0	1.3	0.7	1.4
	-7	86.4	10.6	6.1	12.2
	-7.5	92.7	0.5	0.3	0.5
	-8.5	96.3	3.7	2.2	3.9
Octyltriethoxysilane	-3	82.6	7.5	4.3	9.1
	-4	96.6	2.4	1.4	2.5
	-5	97.1	4.0	2.3	4.1
	-6	94.0	1.5	0.9	1.6
	-7	94.4	7.0	4.0	7.4
	-8	98.2	1.2	0.7	1.3
	-9	97.8	1.4	0.8	1.4
	-10	98.1	3.5	2.0	3.6

TABLE 2 Results of 1st Valid Binding Assay – Test Articles – January 26, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Ensulizole	-3	89.8	2.1	1.2	2.3
	-4	100.3	3.4	1.9	3.4
	-5	98.4	1.1	0.6	1.1
	-6	102.8	2.8	1.6	2.7
	-7	95.5	3.6	2.1	3.8
	-8	96.6	1.8	1.0	1.8
	-9	94.6	2.6	1.5	2.8
	-10	97.6	1.5	0.9	1.6
Avobenzone	-3	75.4	4.6	2.6	6.1
	-4	85.9	10.4	6.0	12.1
	-5	100.7	0.7	0.4	0.7
	-6	95.6	1.7	1.0	1.8
	-7	97.2	1.9	1.1	2.0
	-8	97.9	2.9	1.7	2.9
	-9	97.5	1.5	0.9	1.6
	-10	90.1	10.8	6.2	12.0
Homosalate	-3	78.0	2.9	1.7	3.7
	-4	89.3	1.6	0.9	1.8
	-5	94.1	2.0	1.2	2.2
	-6	94.6	1.8	1.1	1.9
	-7	91.9	4.2	2.4	4.6
	-8	91.9	0.3	0.2	0.3
	-9	94.2	1.4	0.8	1.5
	-10	95.6	1.7	1.0	1.8
Padimate-O	-3	102.8	10.5	6.0	10.2
	-4	91.3	1.5	0.9	1.7
	-5	95.3	4.5	2.6	4.7
	-6	95.5	0.4	0.3	0.5
	-7	96.0	0.5	0.3	0.6
	-8	96.9	2.4	1.4	2.5
	-9	96.6	3.9	2.3	4.0
	-10	98.5	4.5	2.6	4.5

Red lettering indicates where significant precipitation of test material was observed.

TABLE 3 Results of 1st Valid Binding Assay - Upper and Lower Parameters in Competitive Assay Binding Curves for the Standards – January 26, 2013

Parameter	Unit	17β-Estradiol	19-Norethindrone
$Log_e(S_{yx})$	--	-0.39	1.47
Bottom Plateau Level	% binding	0	0
Top Plateau Level	% binding	96	94
Hill Slope	$Log_{10}(M)^{-1}$	-1.0	-1.0

TABLE 4 Results of 2nd Valid Binding Assay – Controls – January 30, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Estradiol (NSB)	-7	0.0	1.1	0.7	-4.4E+17
	-8	7.5	0.4	0.2	5.7
	-8.5	22.3	0.8	0.5	3.7
	-9	47.6	2.5	1.5	5.3
	-9.5	74.6	2.1	1.2	2.8
	-10	88.7	2.6	1.5	2.9
	-11	97.9	1.2	0.7	1.2
19-Norethindrone	-4	1.3	0.4	0.3	34.0
	-4.5	7.3	0.3	0.2	3.7
	-5.5	45.6	0.2	0.1	0.5
	-6	72.2	1.2	0.7	1.7
	-6.5	90.5	2.1	1.2	2.3
	-7	96.7	2.7	1.5	2.8
	-7.5	96.7	2.2	1.3	2.2
	-8.5	98.5	3.7	2.1	3.7
Octyltriethoxysilane	-3	83.6	0.9	0.5	1.1
	-4	95.9	3.7	2.1	3.8
	-5	98.4	2.0	1.2	2.1
	-6	98.4	2.1	1.2	2.1
	-7	97.0	1.7	1.0	1.7
	-8	97.6	1.7	1.0	1.7
	-9	95.8	1.8	1.0	1.9
	-10	98.4	1.6	0.9	1.7

TABLE 5 Results of 2nd Valid Binding Assay – Test Articles – January 30, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Ensulizole	-3	89.8	2.9	1.7	3.2
	-4	98.1	1.2	0.7	1.2
	-5	98.0	1.7	1.0	1.8
	-6	94.7	2.4	1.4	2.6
	-7	93.9	6.2	3.6	6.6
	-8	98.0	1.2	0.7	1.2
	-9	98.4	1.2	0.7	1.2
	-10	102.7	1.7	1.0	1.6
Avobenzone	-3	73.3	1.6	0.9	2.2
	-4	83.3	2.7	1.6	3.3
	-5	122.6	21.3	12.3	17.3
	-6	100.3	2.2	1.2	2.2
	-7	100.2	1.9	1.1	1.9
	-8	96.6	2.5	1.4	2.6
	-9	98.4	2.8	1.6	2.9
	-10	106.3	13.3	7.7	12.5
Homosalate	-3	77.4	1.2	0.7	1.6
	-4	91.8	2.9	1.6	3.1
	-5	94.1	1.5	0.8	1.5
	-6	96.9	3.0	1.7	3.1
	-7	97.0	3.7	2.1	3.8
	-8	99.6	1.0	0.6	1.0
	-9	98.7	1.1	0.6	1.1
	-10	97.3	1.4	0.8	1.4
Padimate-O	-3	82.9	1.2	0.7	1.4
	-4	89.1	4.2	2.4	4.7
	-5	104.6	10.3	6.0	9.9
	-6	91.4	8.6	5.0	9.4
	-7	106.0	16.7	9.7	15.8
	-8	97.7	2.8	1.6	2.9
	-9	95.6	2.1	1.2	2.2
	-10	106.6	16.3	9.4	15.3

Red lettering indicates where significant precipitation of test material was observed.

TABLE 6 Results of 2nd Valid Binding Assay - Upper and Lower Parameters in Competitive Assay Binding Curves for the Standards – January 30, 2013

Parameter	Unit	17β-Estradiol	19-Norethindrone
$Log_e(S_{yx})$	--	0.48	0.68
Bottom Plateau Level	% binding	-1	0
Top Plateau Level	% binding	99	99
Hill Slope	$Log_{10}(M)^{-1}$	-1.0	-1.0

TABLE 7 Results of 3rd Valid Binding Assay - Controls – February 18, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Estradiol (NSB)	-7	0.0	0.5	0.3	-1.3E+18
	-8	6.6	0.0	0.0	0.4
	-8.5	20.2	0.5	0.3	2.3
	-9	44.0	1.2	0.7	2.8
	-9.5	70.8	1.7	1.0	2.3
	-10	86.7	2.3	1.3	2.6
	-11	93.3	1.7	1.0	1.8
19-Norethindrone	-4	10.0	0.0	0.0	0.4
	-4.5	25.0	0.2	0.1	0.9
	-5.5	77.7	2.7	1.6	3.5
	-6	88.6	0.6	0.4	0.7
	-6.5	93.6	1.7	1.0	1.8
	-7	94.7	1.6	0.9	1.7
	-7.5	93.5	1.6	0.9	1.7
	-8.5	98.2	4.9	2.9	5.0
Octyltriethoxysilane	-3	80.6	2.1	1.2	2.6
	-4	88.1	6.2	3.6	7.0
	-5	97.1	1.2	0.7	1.2
	-6	94.2	1.4	0.8	1.5
	-7	100.1	1.9	1.1	1.9
	-8	98.0	1.2	0.7	1.2
	-9	95.3	0.6	0.4	0.7
	-10	95.3	2.9	1.7	3.1

TABLE 8 Results of 3rd Valid Binding Assay – Test Articles – February 18, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Ensulizole	-3	86.1	3.1	1.8	3.6
	-4	98.7	3.4	2.0	3.5
	-5	96.6	1.4	0.8	1.5
	-6	92.6	2.9	1.6	3.1
	-7	97.0	1.0	0.6	1.1
	-8	92.5	1.0	0.6	1.1
	-9	94.7	0.8	0.5	0.8
	-10	95.5	2.3	1.3	2.4
Avobenzone	-3	65.4	1.2	0.7	1.9
	-4	74.6	1.8	1.0	2.4
	-5	97.5	0.2	0.1	0.2
	-6	96.4	3.2	1.8	3.3
	-7	98.3	1.6	0.9	1.6
	-8	96.4	2.5	1.5	2.6
	-9	94.3	2.8	1.6	3.0
	-10	93.7	1.6	0.9	1.7
Homosalate	-3	69.9	2.9	1.7	4.2
	-4	89.9	3.4	1.9	3.7
	-5	93.7	1.6	0.9	1.7
	-6	97.0	2.9	1.7	3.0
	-7	93.5	1.8	1.0	1.9
	-8	96.8	4.0	2.3	4.1
	-9	96.5	0.3	0.2	0.3
	-10	97.5	1.9	1.1	2.0
Padimate-O	-3	86.9	3.9	2.3	4.5
	-4	88.1	7.2	4.2	8.2
	-5	97.1	3.9	2.2	4.0
	-6	98.0	1.7	1.0	1.7
	-7	98.3	4.1	2.4	4.2
	-8	97.0	1.8	1.0	1.9
	-9	98.0	0.9	0.5	0.9
	-10	95.7	2.9	1.7	3.0

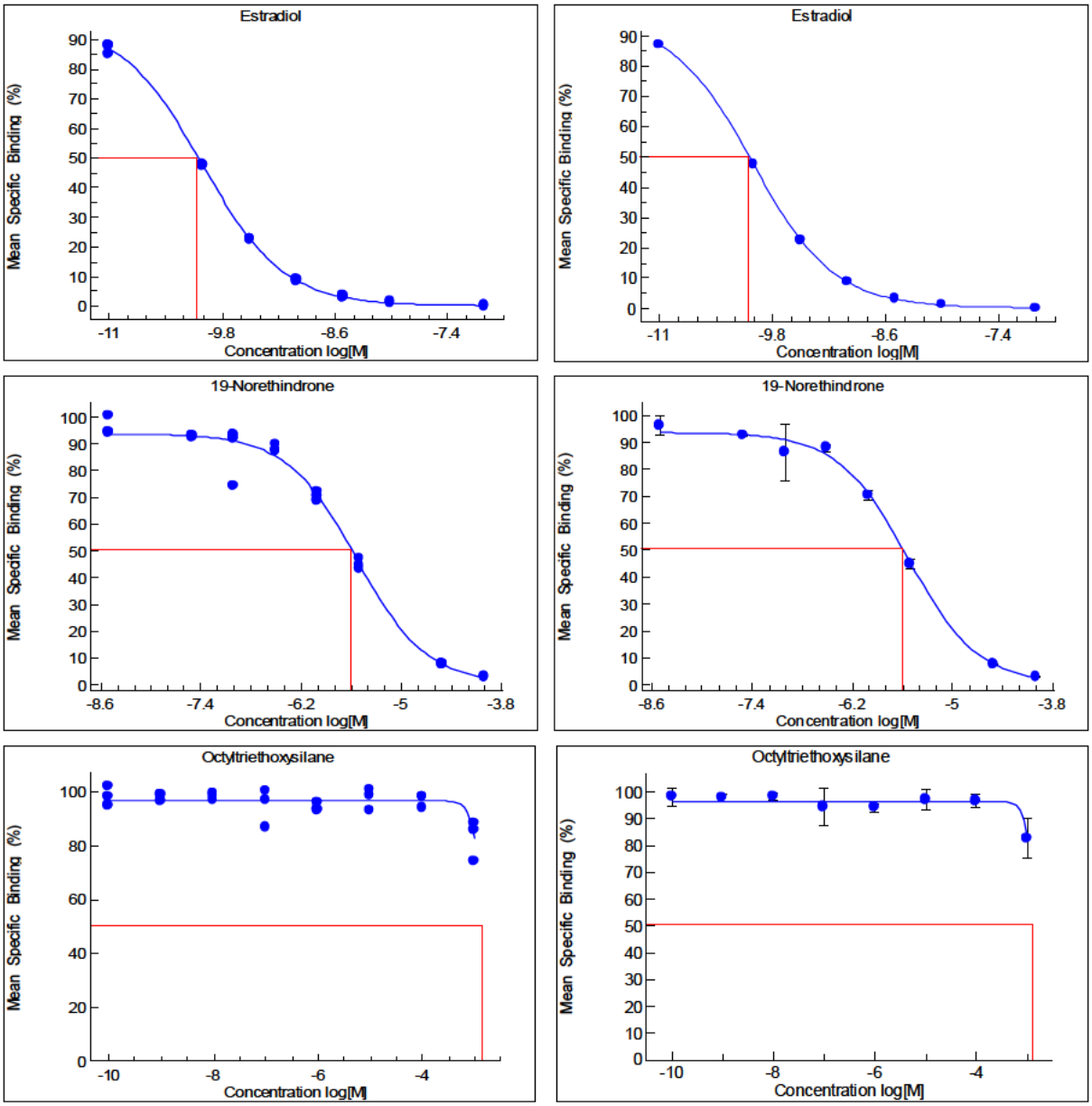
Red lettering indicates where significant precipitation of test material was observed.

TABLE 9 Results of 3rd Valid Binding Assay - Upper and Lower Parameters in Competitive Assay Binding Curves for the Standards – February 18, 2013

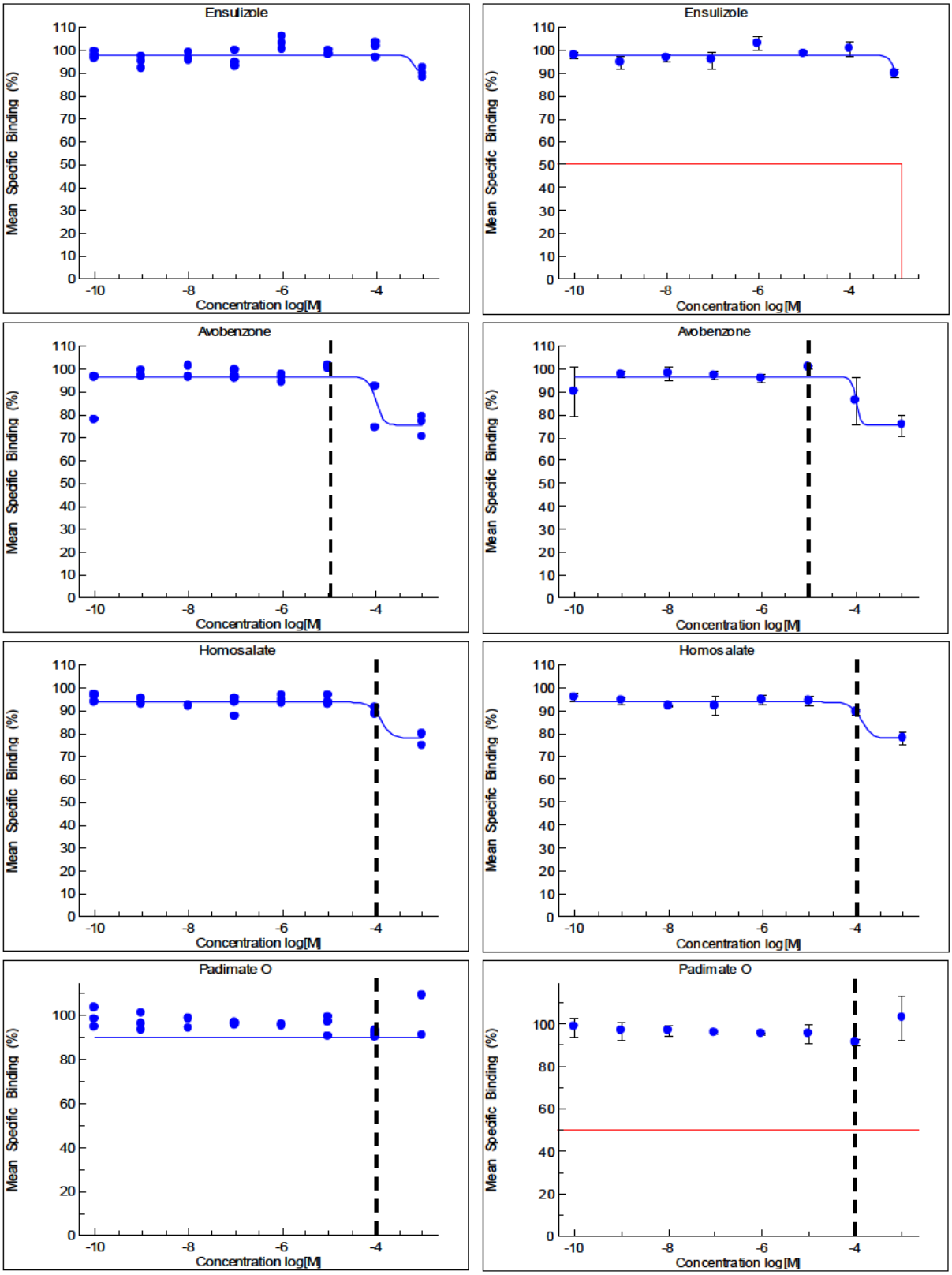
Parameter	Unit	17β-Estradiol	19-Norethindrone
$Log_e(S_{yx})$	--	0.28	0.88
Bottom Plateau Level	% binding	0	1
Top Plateau Level	% binding	95	96
Hill Slope	$Log_{10}(M)^{-1}$	-1.0	-1.1

FIGURES SECTION

FIGURE 1 Specific Binding for 1st Valid Run - Controls and Test Articles – January 26, 2013

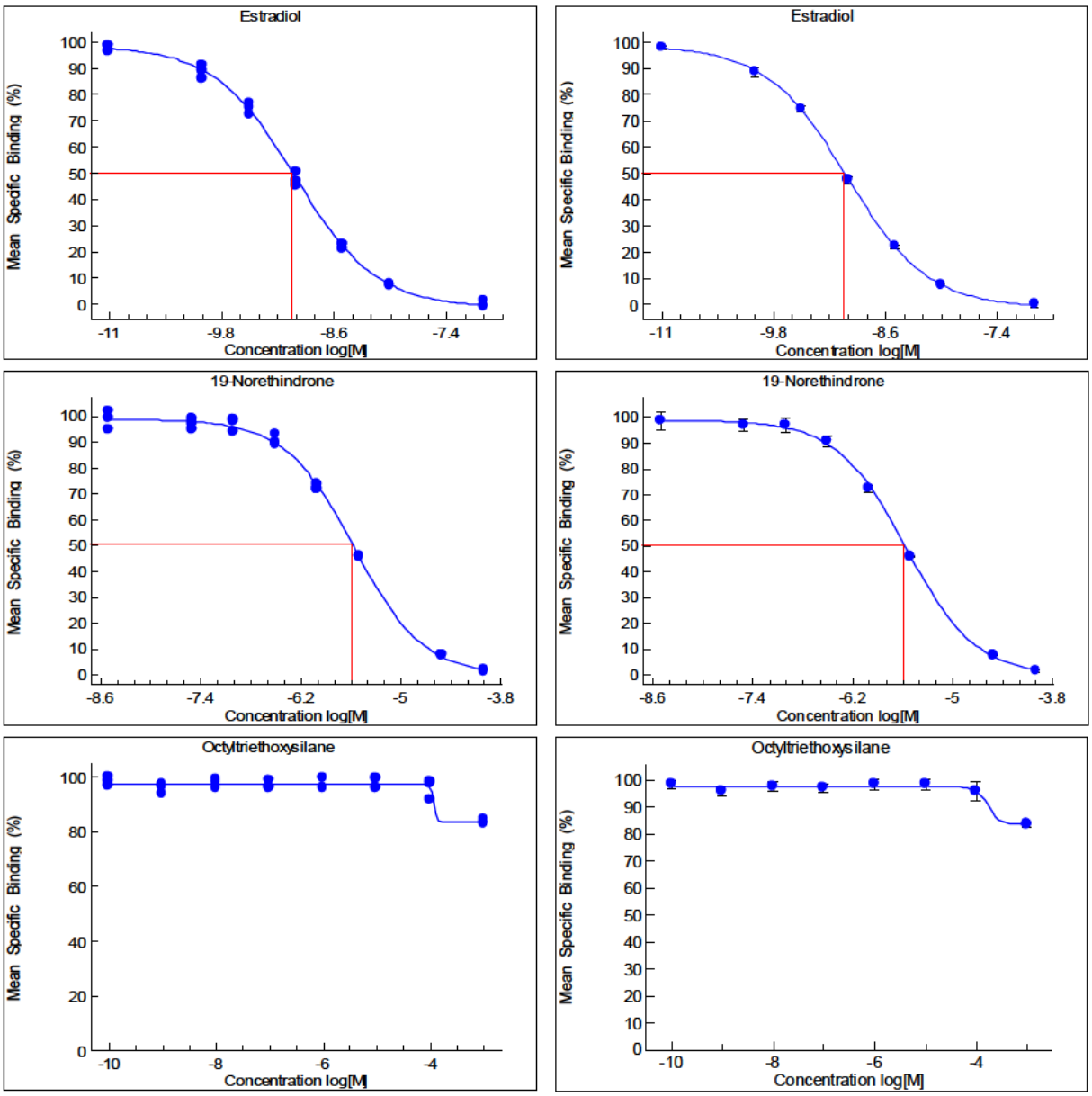


The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the first valid independent run of the assay (n=3).

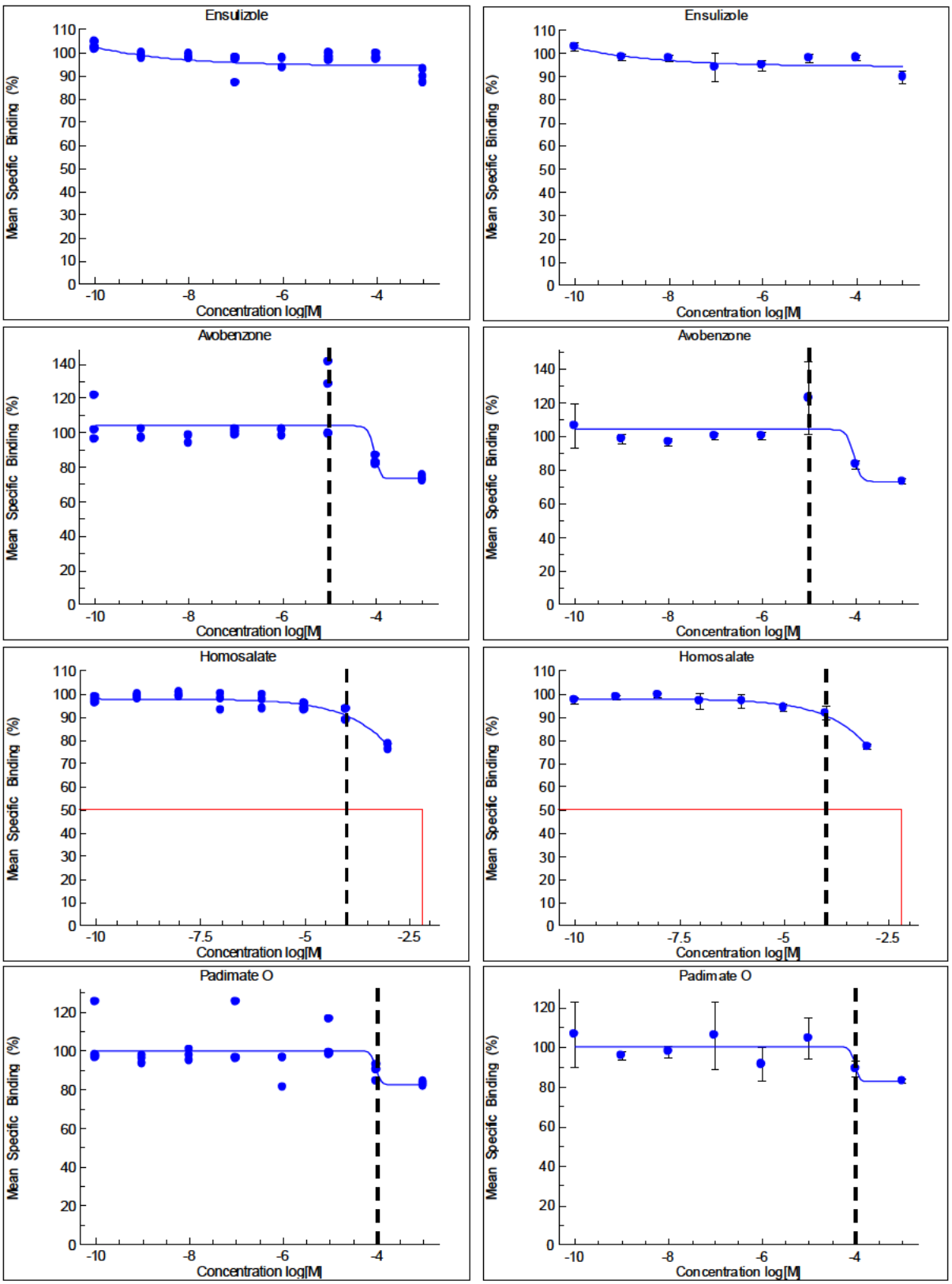


The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the first valid independent run of the assay (n=3). The vertical black dotted lined represents the limit of solubility as tested in this system. Any concentration at or below this line was considered soluble.

FIGURE 2 Specific Binding for 2nd Valid Run - Controls and Test Articles – January 30, 2013

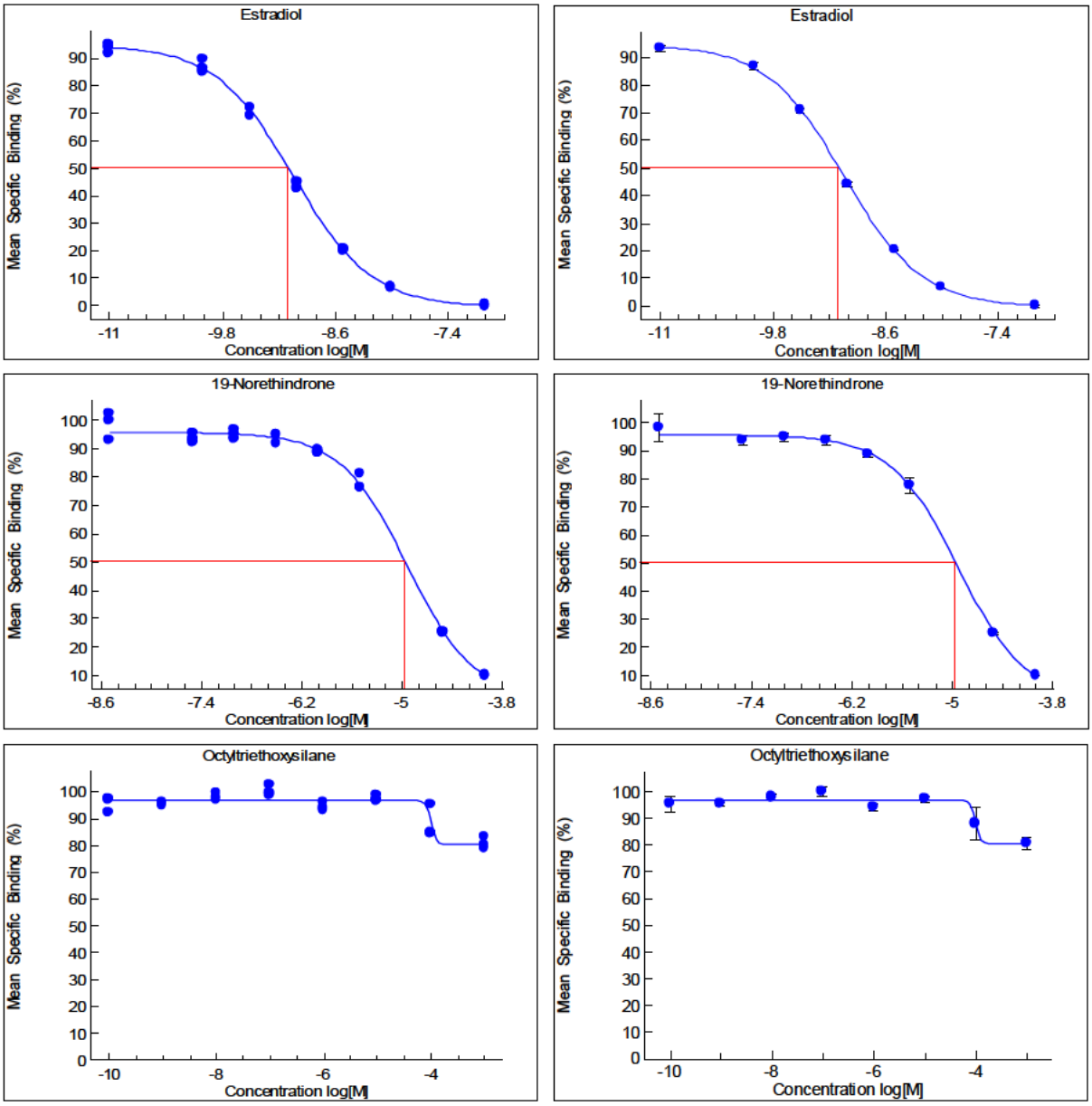


The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the second valid independent run of the assay (n=3).

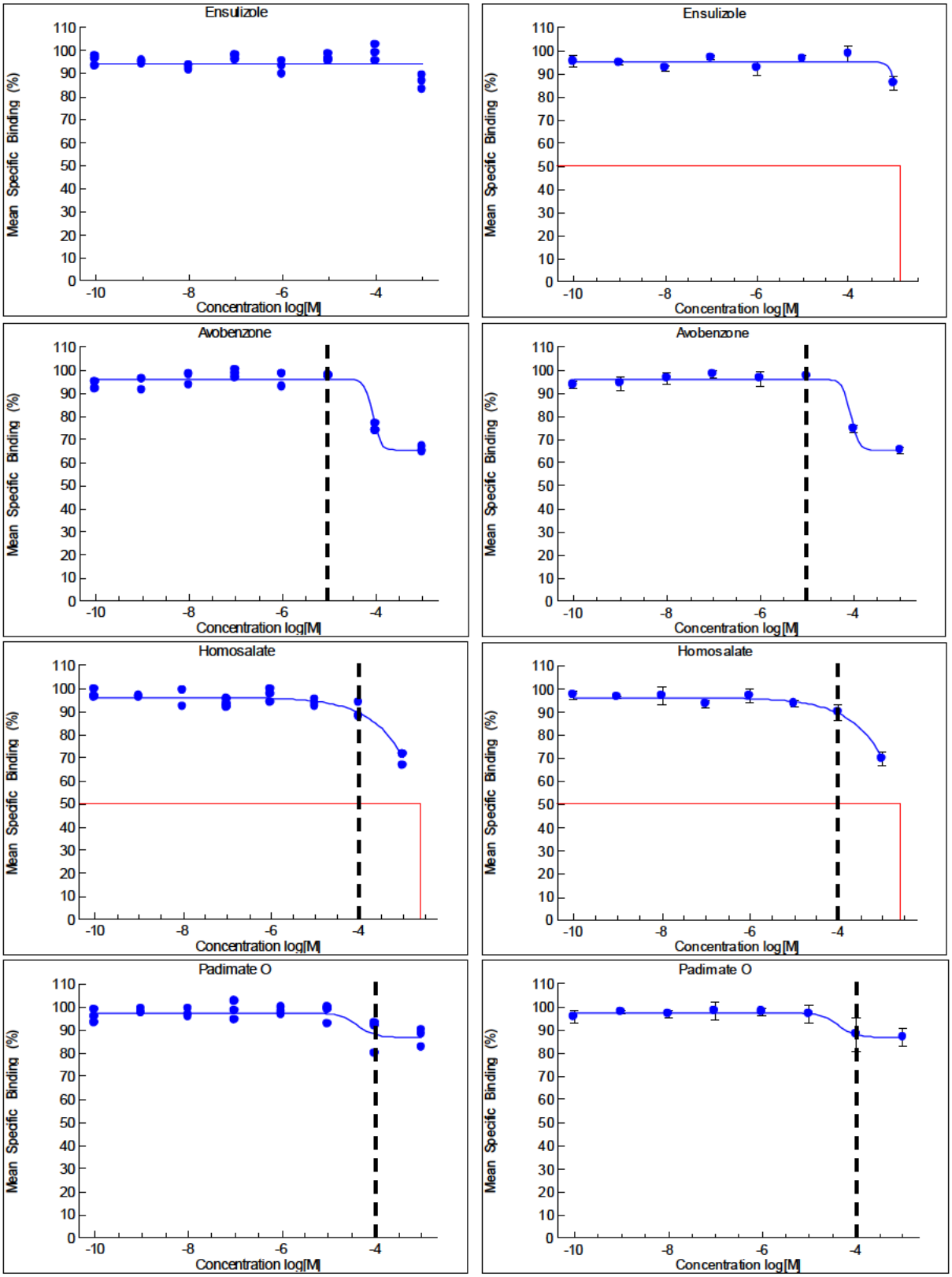


The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the second valid independent run of the assay (n=3). The vertical black dotted lined represents the limit of solubility as tested in this system. Any concentration at or below this line was considered soluble.

FIGURE 3 Specific Binding for 3rd Valid Run - Controls and Test Articles – February 18, 2013



The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the third valid independent run of the assay (n=3).



The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the third valid independent run of the assay (n=3). The vertical black dotted lined represents the limit of solubility as tested in this system. Any concentration at or below this line was considered soluble.

APPENDICES SECTION

APPENDIX 1 Raw and Normalized Data 1st Valid Run – January 26, 2013

Experiment Date:	29-Jan-13	Study Number:	9070-100794ERB	Assays Conducted by:	[redacted]
Test substance:	Ensulizole				
3/7/2013 18:44					
	10 uL of 50 nM E2=	N/A	DPM and 0.5 x10-12 moles		
	Therefore there are	N/A	DPM/mole		
	ug protein/assay tube =	1.000E+02			

Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.5mL)	Mean
1	Total Activity (Master Mix)	20160	--	157248.0	160537.1
2		20577	--	160500.6	
3		20776	--	162052.8	
4		20555	--	160329.0	
5		20925	--	162435.0	
6		20574	--	161257.2	
7	Total Binding (Solvent Control)	14377	13966.0	20992.0	20392.0
8		14004	13706.0	20557.5	
9		13480	13091.0	19635.5	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.5mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
393.0	10	Estradiol (NSB)	-7	4.0	6.0	0.0	-0.2	0.1	0.0	0.3	0.2	-1.7E+18	0.4
430.0	11		-7	41.0	61.5	0.3	0.0	0.0					0.4
344.0	12		-7	-45.0	-67.5	-0.3	-0.6	0.4					0.3
612.0	13	Estradiol	-8	223.0	334.5	1.6	0.5	0.3	1.3	0.4	0.2	28.8	0.6
559.0	14		-8	169.0	253.5	1.2	0.1	0.0					0.5
513.0	15		-8	124.0	186.0	0.9	-0.2	0.0					0.5
656.0	16	Estradiol	-8.5	467.0	700.5	3.4	0.4	0.2	3.1	0.3	0.2	8.8	0.9
795.0	17		-8.5	406.0	609.0	3.0	0.0	0.0					0.7
739.0	18		-8.5	399.0	598.5	2.9	-0.1	0.0					0.7
1542.0	19	Estradiol	-9	1153.0	1729.5	8.5	-0.1	0.0	8.8	0.3	0.1	2.9	1.4
1588.0	20		-9	1199.0	1798.5	8.8	0.2	0.0					1.5
1610.0	21		-9	1221.0	1831.5	9.0	0.4	0.1					1.5
3449.0	22	Estradiol	-9.5	3060.0	4590.0	22.5	-0.1	0.0	22.4	0.2	0.1	0.9	3.2
3450.0	23		-9.5	3061.0	4591.5	22.5	-0.1	0.0					3.2
3403.0	24		-9.5	3014.0	4521.0	22.2	-0.4	0.2					3.2
6780.0	25	Estradiol	-10	6391.0	9586.5	47.0	-0.3	0.1	47.4	0.4	0.2	0.8	6.3
6879.0	26		-10	6490.0	9735.0	47.7	0.4	0.2					6.4
6846.0	27		-10	6457.0	9685.5	47.5	0.2	0.0					6.4
11935.0	28	Estradiol	-11	11546.0	17319.0	84.9	-2.0	4.1	86.9	1.8	1.0	2.0	11.1
12320.0	29		-11	11931.0	17896.5	87.8	0.8	0.6					11.5
12370.0	30		-11	11981.0	17971.5	88.1	1.2	1.4					11.6
793.0	31	19-Norethindrone	-4	404.0	606.0	3.0	0.3	0.1	2.8	0.2	0.1	6.5	0.7
787.0	32		-4	378.0	567.0	2.8	0.1	0.0					0.7
744.0	33		-4	355.0	532.5	2.6	-0.1	0.0					0.7
1434.0	34	19-Norethindrone	-4.5	1045.0	1567.5	7.7	0.1	0.0	7.6	0.1	0.1	1.7	1.3
1414.0	35		-4.5	1025.0	1537.5	7.5	-0.1	0.0					1.3
1399.0	36		-4.5	1010.0	1515.0	7.4	-0.2	0.0					1.3
6769.0	37	19-Norethindrone	-5.5	6379.0	9568.5	46.9	2.0	3.9	44.8	1.9	1.1	4.2	6.3
6418.0	38		-5.5	6029.0	9043.5	44.3	-0.6	0.4					6.0
6264.0	39		-5.5	5876.0	8812.5	43.2	-1.7	3.0					5.8
9712.0	40	19-Norethindrone	-6	9323.0	13984.5	68.6	-2.0	4.0	70.2	1.7	1.0	2.4	9.1
9932.0	41		-6	9543.0	14314.5	70.2	-0.4	0.1					9.3
10188.0	42		-6	9779.0	14668.5	71.9	1.4	1.9					9.5
12556.0	43	19-Norethindrone	-6.5	12167.0	18250.5	89.6	4.2	17.7	88.0	1.3	0.7	1.4	11.7
12270.0	44		-6.5	11881.0	17821.5	87.4	2.1	4.4					11.5
12250.0	45		-6.5	11861.0	17791.5	87.2	2.0	3.8					11.4
10477.0	46	19-Norethindrone	-7	10088.0	15132.0	74.2	-18.8	293.2	86.4	10.6	6.1	12.2	9.8
12947.0	47		-7	12468.0	18697.0	91.6	0.6	0.4					12.0
13069.0	48		-7	12590.0	19020.0	93.3	2.2	5.0					12.2
12970.0	49	19-Norethindrone	-7.5	12581.0	18871.5	92.5	-0.4	0.1	92.7	0.5	0.3	0.5	12.1
13060.0	50		-7.5	12671.0	19006.5	93.2	0.3	0.1					12.2
12939.0	51		-7.5	12560.0	18825.0	92.3	-0.6	0.4					12.1
14060.0	52	19-Norethindrone	-8.5	13671.0	20506.5	100.6	6.9	47.3	96.3	3.7	2.2	3.9	13.1
13139.0	53		-8.5	12760.0	19125.0	93.8	0.1	0.0					12.3
13224.0	54		-8.5	12836.0	19252.5	94.4	0.7	0.5					12.3
12377.0	55	Octyltriethoxysilane	-3	11988.0	17982.0	88.2	5.5	30.7	82.6	7.5	4.3	9.1	11.6
10466.0	56		-3	10077.0	15115.5	74.1	-8.6	72.5					9.8
12030.0	57		-3	11641.0	17461.5	85.6	3.0	8.9					11.2
13712.0	58	Octyltriethoxysilane	-4	13323.0	19984.5	98.0	1.4	2.0	96.6	2.4	1.4	2.6	12.8
13144.0	59		-4	12766.0	19132.5	93.8	-2.8	7.7					12.3
13719.0	60		-4	13330.0	19995.0	98.1	1.5	2.1					12.8
14035.0	61	Octyltriethoxysilane	-5	13646.0	20469.0	100.4	3.8	14.2	97.1	4.0	2.3	4.1	13.1
13746.0	62		-5	13357.0	20035.5	98.3	1.6	2.7					12.8
12993.0	63		-5	12604.0	18906.0	92.7	-3.9	15.1					12.1
13402.0	64	Octyltriethoxysilane	-6	13013.0	19519.5	95.7	-0.9	0.8	94.0	1.5	0.9	1.6	12.5
13014.0	65		-6	12626.0	18937.5	92.9	-3.7	14.0					12.2
13084.0	66		-6	12696.0	19042.5	93.4	-3.2	10.4					12.2
13985.0	67	Octyltriethoxysilane	-7	13596.0	20394.0	100.0	3.4	11.6	94.4	7.0	4.0	7.4	13.1
13527.0	68		-7	13138.0	19707.0	96.6	0.0	0.0					12.6
12164.0	69		-7	11775.0	17662.5	86.6	-10.0	99.8					11.4
13548.0	70	Octyltriethoxysilane	-8	13159.0	19738.5	96.8	0.2	0.0	98.2	1.2	0.7	1.3	12.7
13791.0	71		-8	13402.0	20103.0	98.6	2.0	3.9					12.9
13873.0	72		-8	13484.0	20226.0	99.2	2.6	6.7					13.0
13800.0	73	Octyltriethoxysilane	-9	13411.0	20116.5	98.6	2.0	4.2	97.8	1.4	0.8	1.4	12.9
13486.0	74		-9	13077.0	19615.5	96.2	-0.4	0.2					12.6
13780.0	75		-9	13391.0	20086.5	98.5	1.9	3.6					12.9
14210.0	76	Octyltriethoxysilane	-10	13821.0	20731.5	101.7	5.1	25.6	98.1	3.6	2.0	3.6	13.3
13263.0	77		-10	12874.0	19311.0	94.7	-1.9	3.6					12.4
13703.0	78		-10	13314.0	19971.0	97.9	1.3	1.8					12.8
12330	79	Ensulizole	-3	11941.0	17911.5	87.8	-1.9	3.6	89.8	2.1	1.2	2.3	11.5
12662	80		-3	12173.0	18259.5	89.5	-0.2	0.1					11.7
12850	81		-3	12501.0	18751.5	92.0	2.2	4.7					12.0
14402	82	Ensulizole	-4	14013.0	21019.5	103.1	5.1	26.0	100.3	3.4	1.9	3.4	13.4
14172	83		-4	13783.0	20674.5	101.4	3.4	11.6					13.2
13519	84		-4	13130.0	19695.0	96.6	-1.4	2.0					12.6
13693	85	Ensulizole	-5	13304.0	19956.0	97.9	-0.1	0.0	98.4	1.1	0.6	1.1	12.8
13946	86		-5	13556.0	20334.0	99.7	1.7	3.0					13.0
13676	87		-5	13287.0	19930.5	97.7	-0.2	0.1					12.8
14738	88	Ensulizole	-6	14349.0	21523.5	105.6	7.6	57.3	102.8	2.8	1.6	2.7	13.8
14367	89		-6	13968.0	20952.0	102.7	4.8	22.7					13.4
13978	90		-6	13589.0	20383.5	100.0	2.0	3.9					13.1
13930	91	Ensulizole	-7	13541.0	20311.5	99.6	1.6	2.6	95.5	3.8	2.1	3.8	13.0
13195	92		-7	12806.0	19209.0	94.2	-3.8	14.3					12.3
12992	93		-7	12603.0	18904.5	92.7	-5.3	27.8					12.1
13436	94	Ensulizole	-8	13049.0	19573.5	96.0	-2.0	4.0	96.6	1.8	1.0	1.6	12.5
13795	95		-8	13406.0	20109.0	98.6	0.6	0.4					12.9
13337	96		-8	12948.0	19422.0	95.2	-2.7	7.5					12.5
12863	97	Ensulizole	-9	12474.0	18711.0	91.8	-6.2	38.7	94.6	2.6	1.5	2.8	12.0
13571	98		-9	13182.0	19773.0	97.0	-1.0	1.0					12.7
13303	99		-9	12914.0	19371.0	95.0	-3.0	8.9					12.4
13638	100	Ensulizole	-10	13249.0	19873.5	97.5	-0.6	0.3	97.6	1.5	0.9	1.6	12.7
13880	101		-10	13491.0	20236.5	99.2	1.3	1.6					13.0
13466	102		-10	13077.0	19615.5	96.2	-1.6	3.2					12.6

APPENDIX 1 Raw and Normalized Data 1st Valid Run (continued) – January 26, 2013

11137	79	Avobenzone	-3	10748.0	16122.0	79.1	3.7	13.4	75.4	4.6	2.6	6.1	10.4
10830	80		-3	10441.0	15661.5	76.8	1.4	2.0					10.1
9939	81		-3	9550.0	14325.0	70.2	-5.1	26.5					9.3
12886	82	Avobenzone	-4	12497.0	18745.5	91.9	6.0	36.0	85.9	10.4	6.0	12.1	12.0
10442	83		-4	10053.0	15079.5	73.9	-12.0	143.5					9.8
12890	84		-4	12501.0	18751.5	92.0	6.0	36.3					12.0
13971	85	Avobenzone	-5	13582.0	20373.0	99.9	3.4	11.7	100.7	0.7	0.4	0.7	13.0
14121	86		-5	13732.0	20598.0	101.0	4.5	20.5					13.2
14160	87		-5	13771.0	20656.5	101.3	4.8	23.2					13.2
13134	88	Avobenzone	-6	12745.0	19117.5	93.8	-2.7	7.5	95.6	1.7	1.0	1.8	12.3
13605	89		-6	13216.0	19824.0	97.2	0.7	0.5					12.7
13427	90		-6	13038.0	19557.0	95.9	-0.6	0.3					12.5
13530	91	Avobenzone	-7	13141.0	19711.5	96.7	0.2	0.0	97.2	1.9	1.1	2.0	12.6
13888	92		-7	13499.0	20248.5	99.3	2.8	7.9					13.0
13377	93		-7	12988.0	19482.0	95.5	-0.9	0.9					12.5
14142	94	Avobenzone	-8	13753.0	20629.5	101.2	4.7	21.9	97.9	2.9	1.7	2.9	13.2
13488	95		-8	13099.0	19648.5	96.4	-0.1	0.0					12.6
13447	96		-8	13058.0	19587.0	96.1	-0.4	0.2					12.6
13876	97	Avobenzone	-9	13487.0	20230.5	99.2	2.7	7.4	97.5	1.5	0.9	1.6	13.0
13542	98		-9	13153.0	19729.5	96.8	0.3	0.1					12.6
13496	99		-9	13107.0	19660.5	96.4	-0.1	0.0					12.6
13499	100	Avobenzone	-10	13110.0	19665.0	96.4	0.0	0.0	90.1	10.8	6.2	12.0	12.6
13462	101		-10	13073.0	19609.5	96.2	-0.3	0.1					12.6
10937	102		-10	10548.0	15822.0	77.6	-18.9	357.0					10.2
10532	79	Homosalate	-3	10143.0	15214.5	74.6	-3.3	11.2	78.0	2.9	1.7	3.7	9.8
11181	80		-3	10792.0	16188.0	79.4	1.4	2.0					10.4
11249	81		-3	10860.0	16290.0	79.9	1.9	3.7					10.5
12772	82	Homosalate	-4	12383.0	18574.5	91.1	1.8	3.4	89.3	1.6	0.9	1.8	11.9
12386	83		-4	11997.0	17995.5	88.2	-1.0	1.0					11.6
12410	84		-4	12021.0	18031.5	88.4	-0.8	0.7					11.6
12957	85	Homosalate	-5	12568.0	18852.0	92.4	-1.3	1.6	94.1	2.0	1.2	2.2	12.1
13496	86		-5	13107.0	19660.5	96.4	2.7	7.2					12.6
13108	87		-5	12719.0	19078.5	93.6	-0.2	0.0					12.2
13506	88	Homosalate	-6	13117.0	19675.5	96.5	2.8	7.6	94.6	1.8	1.1	1.9	12.6
13010	89		-6	12621.0	18931.5	92.8	-0.9	0.8					12.1
13234	90		-6	12845.0	19267.5	94.5	0.8	0.6					12.4
13085	91	Homosalate	-7	12696.0	19044.0	93.4	-0.3	0.1	91.9	4.2	2.4	4.6	12.2
13326	92		-7	12937.0	19405.5	95.2	1.4	2.1					12.4
12238	93		-7	11849.0	17773.5	87.2	-6.6	43.1					11.4
12841	94	Homosalate	-8	12452.0	18678.0	91.6	-2.1	4.5	91.9	0.3	0.2	0.3	12.0
12874	95		-8	12485.0	18727.5	91.8	-1.9	3.6					12.0
12919	96		-8	12530.0	18795.0	92.2	-1.6	2.4					12.1
13282	97	Homosalate	-9	12893.0	19339.5	94.8	1.1	1.2	94.2	1.4	0.8	1.5	12.4
12977	98		-9	12588.0	18882.0	92.6	-1.1	1.3					12.1
13322	99		-9	12933.0	19399.5	95.1	1.4	2.0					12.4
13126	100	Homosalate	-10	12737.0	19105.5	93.7	0.0	0.0	95.6	1.7	1.0	1.8	12.3
13455	101		-10	13066.0	19599.0	96.1	2.4	5.7					12.6
13588	102		-10	13199.0	19798.5	97.1	3.4	11.3					12.7
15202	79	Padimate O	-3	14813.0	22219.5	109.0	19.2	369.7	102.8	10.5	6.0	10.2	14.2
12718	80		-3	12329.0	18493.5	90.7	1.0	0.9					11.9
15168	81		-3	14779.0	22168.5	108.7	19.0	360.2					14.2
12808	82	Padimate O	-4	12419.0	18628.5	91.4	1.6	2.6	91.3	1.5	0.9	1.7	12.0
12588	83		-4	12199.0	18298.5	89.7	0.0	0.0					11.8
13007	84		-4	12618.0	18927.0	92.8	3.1	9.5					12.1
12658	85	Padimate O	-5	12269.0	18403.5	90.2	0.5	0.3	95.3	4.5	2.6	4.7	11.8
13834	86		-5	13445.0	20167.5	98.9	9.2	84.0					12.9
13537	87		-5	13148.0	19722.0	96.7	7.0	48.7					12.6
13304	88	Padimate O	-6	12915.0	19372.5	95.0	5.3	27.7	95.5	0.4	0.3	0.5	12.4
13424	89		-6	13035.0	19552.5	95.9	6.1	37.8					12.5
13380	90		-6	12991.0	19486.5	95.6	5.8	33.9					12.5
13363	91	Padimate O	-7	12974.0	19461.0	95.4	5.7	32.5	96.0	0.5	0.3	0.6	12.5
13505	92		-7	13116.0	19674.0	96.5	6.7	45.5					12.6
13459	93		-7	13070.0	19605.0	96.1	6.4	41.0					12.6
13188	94	Padimate O	-8	12799.0	19198.5	94.1	4.4	19.5	96.9	2.4	1.4	2.5	12.3
13726	95		-8	13337.0	20005.5	98.1	8.4	70.1					12.8
13782	96		-8	13393.0	20089.5	98.5	8.8	77.1					12.9
13434	97	Padimate O	-9	13045.0	19567.5	96.0	6.2	38.7	96.6	3.9	2.3	4.0	12.5
13044	98		-9	12655.0	18982.5	93.1	3.4	11.3					12.2
14096	99		-9	13707.0	20560.5	100.8	11.1	123.0					13.2
13223	100	Padimate O	-10	12834.0	19251.0	94.4	4.7	21.8	98.5	4.5	2.6	4.5	12.3
14432	101		-10	14043.0	21064.5	103.3	13.6	184.0					13.5
13698	102		-10	13309.0	19963.5	97.9	8.2	66.7					12.8

APPENDIX 1 Raw and Normalized Data 2nd Valid Run – January 30, 2013

Experiment Date:	30-Jan-13	Study Number:	9070-100794ERB	Assays Conducted by:	[redacted]
Test substance:	Ensulizole				
3/7/2013 18:44					
	10 uL of 50 nM E2=	N/A	DPM and 0.5 x10-12 moles		
	Therefore there are	N/A	DPM/mole		
	ug protein/assay tube =	1.000E+02			

Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.5mL)	Mean
1	Total Activity (Master Mix)	19858	--	154892.4	156295.4
2		19840	--	154752.0	
3		20081	--	156631.8	
4		20124	--	156967.2	
5		20093	--	156725.4	
6		20232	--	157809.6	
7	Total Binding (Solvent Control)	13925	13231.7	19847.5	20469.6
8		14674	13979.7	20969.5	
9		14422	13727.7	20591.5	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.5mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
823.0	10	Estradiol (NSB)	-7	-71.3	-107.0	-0.5	-0.4	0.2	0.0	1.1	0.7	-4.4E+17	0.5
874.0	11		-7	179.7	269.5	1.3	1.4	2.0					0.8
586.0	12		-7	-108.3	-162.5	-0.8	-0.7	0.5					0.6
1737.0	13	Estradiol	-8	1042.7	1564.0	7.6	0.0	0.0	7.6	0.4	0.2	5.7	1.7
1649.0	14		-8	954.7	1432.0	7.0	-0.6	0.4					1.6
1759.0	15		-8	1064.7	1597.0	7.8	0.2	0.0					1.7
3793.0	16	Estradiol	-8.5	3098.7	4648.0	22.7	0.6	0.3	22.3	0.8	0.5	3.7	3.6
3810.0	17		-8.5	3115.7	4673.5	22.8	0.7	0.5					3.7
3806.0	18		-8.5	2911.7	4367.5	21.3	-0.8	0.6					3.5
7096.0	19	Estradiol	-9	6401.7	9602.5	46.9	-0.9	0.8	47.6	2.5	1.5	5.3	6.8
7553.0	20		-9	6858.7	10303.0	50.3	2.5	6.3					7.3
6894.0	21		-9	6199.7	9299.5	45.4	-2.4	5.8					6.6
10816.0	22	Estradiol	-9.5	10221.7	15332.5	74.9	0.8	0.7	74.6	2.1	1.2	2.8	10.5
11141.0	23		-9.5	10446.7	15670.0	76.6	2.5	6.1					10.7
10576.0	24		-9.5	9881.7	14822.5	72.4	-1.7	2.8					10.1
12842.0	25	Estradiol	-10	12147.7	18221.5	89.0	-0.3	0.1	88.7	2.6	1.5	2.9	12.3
13132.0	26		-10	12437.7	18656.5	91.1	1.8	3.2					12.6
12439.0	27		-10	11744.7	17617.0	86.1	-3.3	10.9					11.9
14132.0	28	Estradiol	-11	13437.7	20156.5	98.5	0.8	0.7	97.9	1.2	0.7	1.2	13.6
13874.0	29		-11	13179.7	19769.5	96.6	-1.1	1.2					13.3
14186.0	30		-11	13471.7	20207.5	98.7	1.1	1.1					13.6
935.0	31	19-Norethindrone	-4	240.7	361.0	1.8	-0.1	0.0	1.3	0.4	0.3	34.0	0.9
869.0	32		-4	174.7	262.0	1.3	-0.6	0.3					0.8
814.0	33		-4	119.7	179.5	0.9	-1.0	1.0					0.8
1715.0	34	19-Norethindrone	-4.5	1020.7	1531.0	7.5	0.7	0.5	7.3	0.3	0.2	3.7	1.5
1653.0	35		-4.5	958.7	1438.0	7.0	0.3	0.1					1.5
1720.0	36		-4.5	1025.7	1538.5	7.5	0.8	0.6					1.7
6357.0	37	19-Norethindrone	-5.5	6262.7	9394.0	45.9	0.4	0.2	46.6	0.2	0.1	0.5	6.7
6308.0	38		-5.5	6213.7	9320.5	45.6	0.1	0.0					6.6
6300.0	39		-5.5	6205.7	9308.5	45.5	0.0	0.0					6.6
10732.0	40	19-Norethindrone	-6	10037.7	15056.5	73.6	0.4	0.2	72.2	1.2	0.7	1.7	10.3
10435.0	41		-6	9740.7	14611.0	71.4	-1.7	3.1					10.0
10461.0	42		-6	9766.7	14650.0	71.6	-1.6	2.4					10.0
12813.0	43	19-Norethindrone	-6.5	12118.7	18178.0	88.8	-0.6	0.3	90.6	2.1	1.2	2.3	12.3
12955.0	44		-6.5	12260.7	18391.0	89.8	0.5	0.2					12.4
13364.0	46		-6.5	12669.7	19004.5	92.8	3.5	11.9					12.8
14018.0	46	19-Norethindrone	-7	13323.7	19985.5	97.6	1.9	3.5	96.7	2.7	1.5	2.8	13.5
13479.0	47		-7	12784.7	19177.0	93.7	-2.1	4.4					12.9
14172.0	48		-7	13477.7	20216.5	98.6	3.0	8.9					13.6
13901.0	49	19-Norethindrone	-7.5	13206.7	19810.0	96.6	-1.1	1.2	96.7	2.2	1.3	2.2	13.3
14169.0	50		-7.5	13493.7	20240.5	98.9	1.0	1.0					13.6
13597.0	51		-7.5	12902.7	19354.0	94.6	-3.3	11.0					13.0
14226.0	52	19-Norethindrone	-8.5	13531.7	20297.5	99.2	0.4	0.2	98.5	3.7	2.1	3.7	13.7
14598.0	53		-8.5	13903.7	20855.5	101.9	3.2	10.0					14.0
13804.0	54		-8.5	12909.7	19364.5	94.6	-4.1	16.9					13.1
12240.0	55	Octyltriethoxysilane	-3	11545.7	17318.5	84.6	1.0	1.0	83.6	0.9	0.5	1.1	11.7
12068.0	56		-3	11374.7	17062.0	83.4	-0.3	0.1					11.6
12005.0	57		-3	11310.7	16966.0	82.9	-0.7	0.5					11.5
14118.0	58	Octyltriethoxysilane	-4	13423.7	20135.5	98.4	2.5	6.1	95.9	3.7	2.1	3.8	13.5
13204.0	59		-4	12509.7	18764.5	91.7	-4.2	17.9					12.7
14021.0	60		-4	13326.7	19990.0	97.7	1.8	3.1					13.5
14250.0	61	Octyltriethoxysilane	-5	13555.7	20333.5	99.3	1.7	3.0	98.4	2.0	1.2	2.1	13.7
13801.0	62		-5	13106.7	19660.0	96.0	-1.6	2.4					13.2
14303.0	63		-5	13608.7	20413.0	99.7	2.1	4.5					13.7
13798.0	64	Octyltriethoxysilane	-6	13103.7	19655.5	96.0	-1.6	2.5	98.4	2.1	1.2	2.1	13.2
14279.0	65		-6	13584.7	20377.0	99.5	1.9	3.8					13.7
14301.0	66		-6	13606.7	20410.0	99.7	2.1	4.4					13.7
14193.0	67	Octyltriethoxysilane	-7	13498.7	20248.0	98.9	1.3	1.7	97.0	1.7	1.0	1.7	13.6
13767.0	68		-7	13072.7	19609.0	95.8	-1.8	3.3					13.2
13848.0	69		-7	13153.7	19730.5	96.4	-1.2	1.5					13.3
13777.0	70	Octyltriethoxysilane	-8	13082.7	19624.0	95.9	-1.7	3.0	97.6	1.7	1.0	1.7	13.2
14241.0	71		-8	13546.7	20320.0	99.3	1.7	2.8					13.7
14041.0	72		-8	13346.7	20020.0	97.8	0.2	0.0					13.5
13975.0	73	Octyltriethoxysilane	-9	13280.7	19921.0	97.3	-0.3	0.1	96.8	1.8	1.0	1.9	13.4
13493.0	74		-9	12798.7	19198.0	93.8	-3.8	14.6					12.9
13816.0	75		-9	13121.7	19682.5	96.2	-1.4	2.1					13.3
14395.0	76	Octyltriethoxysilane	-10	13640.7	20461.0	100.0	2.4	5.5	98.4	1.6	0.9	1.7	13.8
13890.0	77		-10	13196.7	19793.5	96.7	-0.9	0.8					13.3
14141.0	78		-10	13446.7	20170.0	98.5	0.9	0.9					13.6
12666	79	Ensulizole	-3	11871.7	17807.5	87.0	-7.6	56.7	89.8	2.9	1.7	3.2	12.1
13368	80		-3	12563.7	18995.5	92.8	-1.7	3.0					12.8
12902	81		-3	12207.7	18311.5	89.5	-5.1	25.7					12.4
14267	82	Ensulizole	-4	13572.7	20359.0	99.5	4.8	23.3	98.1	1.2	0.7	1.2	13.7
14006	83		-4	13310.7	19966.0	97.5	2.9	8.4					13.4
13996	84		-4	13261.7	19892.5	97.2	2.5	6.5					13.4
14046	85	Ensulizole	-5	13351.7	20027.5	97.8	3.0	9.1	98.0	1.7	1.0	1.8	13.5
14313	86		-5	13618.7	20439.0	99.8	5.0	24.7					13.7
13948	87		-5	13148.7	19723.0	96.4	1.5	2.3					13.3
14005	88	Ensulizole	-6	13310.7	19966.0	97.5	2.9	8.4	94.7	2.4	1.4	2.6	13.4
13426	89		-6	12731.7	19097.5	93.3	-1.9	3.6					12.9
13426	90		-6	12731.7	19097.5	93.3	-1.9	3.6					12.9
12532	91	Ensulizole	-7	11837.7	17755.5	86.7	-9.1	82.3	93.9	6.2	3.6	6.6	12.0
14031	92		-7	13336.7	20005.0	97.7	1.9	3.7					13.5
13946	93		-7	13261.7	19877.5	97.1	1.3	1.7					13.4
13973	94	Ensulizole	-8	13278.7	19918.0	97.3	0.4	0.1	98.0	1.2	0.7	1.2	13.4
13961	95		-8	13266.7	19900.0	97.2	0.3	0.1					13.4
14263	96		-8	13568.7	20338.0	99.4	2.4	5.8					13.7
14092	97	Ensulizole	-9	13397.7	20096.5	98.2	-0.8	0.6	98.4	1.2	0.7	1.2	13.5
13978	98		-9	13283.7	19925.5	97.3	-1.6	2.6					13.4
14307	99		-9	13612.7	20419.0	99.8	0.8	0.7					13.7
14628	100	Ensulizole	-10	13833.7	20750.5	101.4	-1.0	1.1	102.7	1.7	1.0	1.6	13.9
14969	101		-10	14274.7	21412.0	104.6	2.2	4.8					14.4
14629	102		-10	13934.7	20902.0	102.1	-0.3	0.1					14.0

APPENDIX 1 Raw and Normalized Data 2nd Valid Run (continued) – January 30, 2013

10651	79	Avobenzone	-3	9956.7	14935.0	73.0	-0.3	0.1	73.3	1.6	0.9	2.2	10.2
10937	80		-3	10242.7	15364.0	75.1	1.8	3.1					10.5
10500	81		-3	9805.7	14708.5	71.9	-1.4	2.1					10.1
12478	82	Avobenzone	-4	11783.7	17675.5	86.4	3.1	9.4	83.3	2.7	1.6	3.3	12.0
11771	83		-4	11076.7	16615.0	81.2	-2.1	4.5					11.3
11930	84		-4	11235.7	16853.5	82.3	-1.0	0.9					11.4
19911	85	Avobenzone	-5	19216.7	28825.0	140.8	36.7	1349.9	122.6	21.3	12.3	17.3	19.1
18139	86		-5	17444.7	26167.0	127.8	23.8	564.3					17.4
14242	87		-5	13547.7	20321.5	99.3	-4.8	23.1					13.7
14555	88	Avobenzone	-6	13860.7	20791.0	101.6	-2.5	6.3	100.3	2.2	1.2	2.2	14.0
14559	89		-6	13864.7	20797.0	101.6	-2.5	6.1					14.0
14047	90		-6	13352.7	20029.0	97.8	-6.2	38.8					13.5
14365	91	Avobenzone	-7	13670.7	20506.0	100.2	-3.9	15.2	100.2	1.9	1.1	1.9	13.8
14620	92		-7	13925.7	20888.5	102.0	-2.0	4.1					14.0
14102	93		-7	13407.7	20111.5	98.3	-5.8	34.0					13.5
13490	94	Avobenzone	-8	12795.7	19193.5	93.8	-10.3	106.3	96.6	2.5	1.4	2.6	12.9
14117	95		-8	13422.7	20134.0	98.4	-5.7	32.7					13.5
14034	96		-8	13339.7	20009.5	97.8	-6.3	40.0					13.5
14574	97	Avobenzone	-9	13879.7	20819.5	101.7	-2.4	5.6	98.4	2.8	1.6	2.9	14.0
13919	98		-9	13224.7	19837.0	96.9	-7.2	51.4					13.4
13884	99		-9	13189.7	19784.5	96.7	-7.4	55.1					13.3
14524	100	Avobenzone	-10	13829.7	20744.5	101.3	-2.7	7.5	106.3	13.3	7.7	12.5	13.9
13817	101		-10	13122.7	19684.0	96.2	-7.9	62.7					13.3
17252	102		-10	16557.7	24836.5	121.3	17.3	297.7					16.6
11365	79	Homosalate	-3	10670.7	16006.0	78.2	0.6	0.3	77.4	1.2	0.7	1.6	10.9
11071	80		-3	10376.7	15565.0	76.0	-1.6	2.5					10.6
11344	81		-3	10649.7	15974.5	78.0	0.4	0.2					10.9
12770	82	Homosalate	-4	12075.7	18113.5	88.5	-2.3	5.1	91.8	2.9	1.6	3.1	12.3
13431	83		-4	12736.7	19105.0	93.3	2.6	6.7					12.9
13456	84		-4	12761.7	19142.5	93.5	2.8	7.7					12.9
13450	85	Homosalate	-5	12755.7	19133.5	93.5	-2.0	3.9	94.1	1.5	0.8	1.5	12.9
13404	86		-5	12709.7	19064.5	93.1	-2.3	5.4					12.9
13768	87		-5	13073.7	19610.5	95.8	0.3	0.1					13.2
14285	88	Homosalate	-6	13590.7	20386.0	99.6	2.5	6.0	96.9	3.0	1.7	3.1	13.7
13472	89		-6	12777.7	19166.5	93.6	-3.5	12.3					12.9
13980	90		-6	13285.7	19928.5	97.4	0.2	0.0					13.4
14358	91	Homosalate	-7	13663.7	20495.5	100.1	2.4	5.7	97.0	3.7	2.1	3.8	13.8
14040	92		-7	13345.7	20018.5	97.8	0.1	0.0					13.5
13381	93		-7	12686.7	19030.0	93.0	-4.8	22.7					12.8
14250	94	Homosalate	-8	13555.7	20333.5	99.3	1.4	1.9	99.6	1.0	0.6	1.0	13.7
14163	95		-8	13468.7	20203.0	98.7	0.8	0.6					13.6
14427	96		-8	13732.7	20599.0	100.6	2.7	7.2					13.8
14024	97	Homosalate	-9	13329.7	19994.5	97.7	-0.3	0.1	98.7	1.1	0.6	1.1	13.5
14314	98		-9	13619.7	20429.5	99.8	1.8	3.2					13.7
14151	99		-9	13456.7	20185.0	98.6	0.6	0.3					13.6
13971	100	Homosalate	-10	13276.7	19915.0	97.3	-0.8	0.6	97.3	1.4	0.8	1.4	13.4
13777	101		-10	13082.7	19624.0	95.9	-2.2	4.8					13.2
14159	102		-10	13464.7	20197.0	98.7	0.6	0.4					13.6
12178	79	Padimate O	-3	11483.7	17225.5	84.2	1.3	1.6	82.9	1.2	0.7	1.4	11.7
11983	80		-3	11288.7	16933.0	82.7	-0.2	0.0					11.5
11855	81		-3	11160.7	16741.0	81.8	-1.1	1.2					11.4
12228	82	Padimate O	-4	11533.7	17300.5	84.5	-4.6	21.0	89.1	4.2	2.4	4.7	11.7
13338	83		-4	12643.7	18965.5	92.7	3.5	12.6					12.8
12995	84		-4	12300.7	18451.0	90.1	1.0	1.1					12.5
16591	85	Padimate O	-5	15896.7	23845.0	116.5	16.2	261.8	104.6	10.3	6.0	9.9	15.9
14093	86		-5	13398.7	20098.0	98.2	-2.1	4.5					13.5
14206	87		-5	13511.7	20267.5	99.0	-1.3	1.7					13.6
13854	88	Padimate O	-6	13159.7	19739.5	96.4	-3.9	15.0	91.4	8.6	5.0	9.4	13.3
13850	89		-6	13155.7	19733.5	96.4	-3.9	15.3					13.3
11810	90		-6	11115.7	16673.5	81.5	-18.9	355.6					11.3
13890	91	Padimate O	-7	13195.7	19793.5	96.7	-3.6	13.1	106.0	16.7	9.7	15.8	13.3
13800	92		-7	13105.7	19658.5	96.0	-4.3	18.3					13.2
17795	93		-7	17100.7	25651.0	125.3	25.0	625.1					17.1
14430	94	Padimate O	-8	13735.7	20603.5	100.7	0.3	0.1	97.7	2.8	1.6	2.9	13.8
14000	95		-8	13305.7	19958.5	97.5	-2.8	7.9					13.4
13669	96		-8	12974.7	19462.0	95.1	-5.2	27.4					13.1
13789	97	Padimate O	-9	13094.7	19642.0	96.0	-4.4	19.0	95.6	2.1	1.2	2.2	13.2
13983	98		-9	13288.7	19933.0	97.4	-2.9	8.6					13.4
13430	99		-9	12735.7	19103.5	93.3	-7.0	48.8					12.9
14039	100	Padimate O	-10	13344.7	20017.0	97.8	-2.5	6.4	106.6	16.3	9.4	15.3	13.5
17799	101		-10	17104.7	25657.0	125.3	25.0	626.6					17.1
13869	102		-10	13174.7	19762.0	96.5	-3.8	14.2					13.3

APPENDIX 1 Raw and Normalized Data 3rd Valid Run – February 18, 2013

Experiment Date:	18-Feb-13	Study Number:	9070-100794ERB	Assays Conducted by:	[redacted]
Test substance:	Ensulizole				
3/7/2013 18:44					
	10 uL of 50 nM E2=	N/A	DPM and 0.5 x10-12 moles		
	Therefore there are	N/A	DPM/mole		
	ug protein/assay tube =	1.000E+02			

Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.5mL)	Mean
1	Total Activity (Master Mix)	19782	--	154299.6	152821.5
2		19637	--	153168.6	
3		19340	--	150852.0	
4		19528	--	152318.4	
5		19662	--	153363.6	
6		19606	--	152926.8	
7	Total Binding (Solvent Control)	12924	12495.0	18742.5	17873.5
8		12287	11858.0	17787.0	
9		11823	11394.0	17091.0	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.5mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
369.0	10	Estradiol (NSB)	-7	-40.0	-60.0	-0.3	-0.6	0.3	0.0	0.6	0.3	-1.3E+19	0.4
495.0	11		-7	86.0	99.0	0.6	0.3	0.1					0.5
403.0	12		-7	-26.0	-39.0	-0.2	-0.4	0.2					0.4
1218.0	13	Estradiol	-8	789.0	1183.5	6.6	0.0	0.0	6.6	0.0	0.0	0.4	1.2
1218.0	14		-8	789.0	1183.5	6.6	0.0	0.0					1.2
1224.0	15		-8	795.0	1192.5	6.7	0.1	0.0					1.2
2782.0	16	Estradiol	-8.5	2353.0	3529.5	19.7	0.1	0.0	20.2	0.6	0.3	2.3	2.7
2822.0	17		-8.5	2393.0	3589.5	20.1	0.4	0.2					2.8
2892.0	18		-8.5	2463.0	3694.5	20.7	1.0	1.0					2.8
5739.0	19	Estradiol	-9	5310.0	7965.0	44.6	0.0	0.0	44.0	1.2	0.7	2.8	5.6
5781.0	20		-9	5352.0	8028.0	44.9	0.4	0.1					5.7
5507.0	21		-9	5078.0	7617.0	42.6	-1.9	3.7					5.4
8969.0	22	Estradiol	-9.5	8540.0	12810.0	71.7	0.8	0.6	70.8	1.7	1.0	2.3	8.8
8992.0	23		-9.5	8563.0	12844.5	71.9	1.0	0.9					8.8
8639.0	24		-9.5	8210.0	12315.0	68.9	-2.0	4.0					8.5
10539.0	25	Estradiol	-10	10110.0	15165.0	84.8	-1.2	1.4	86.7	2.3	1.3	2.6	10.3
10686.0	26		-10	10257.0	15385.5	86.1	0.1	0.0					10.5
11065.0	27		-10	10636.0	15954.0	89.3	3.2	10.5					10.9
11395.0	28	Estradiol	-11	10966.0	16359.0	91.5	-2.3	5.1	93.3	1.7	1.0	1.8	11.1
11593.0	29		-11	11139.0	16708.5	93.5	-0.3	0.1					11.4
11745.0	30		-11	11316.0	16974.0	95.0	1.2	1.4					11.5
1617.0	31	19-Norethindrone	-4	1188.0	1782.0	10.0	0.2	0.0	10.0	0.0	0.0	0.4	1.6
1624.0	32		-4	1195.0	1792.5	10.0	0.2	0.0					1.6
1614.0	33		-4	1185.0	1777.5	9.9	0.1	0.0					1.6
3380.0	34	19-Norethindrone	-4.5	2951.0	4426.5	24.8	-0.6	0.3	25.0	0.2	0.1	0.9	3.3
3432.0	35		-4.5	3003.0	4504.5	25.2	-0.1	0.0					3.4
3421.0	36		-4.5	2992.0	4488.0	25.1	-0.2	0.0					3.4
10050.0	37	19-Norethindrone	-5.5	9631.0	14446.5	80.8	3.9	14.4	77.7	2.7	1.6	3.6	9.9
9497.0	38		-5.5	9078.0	13597.0	76.0	-1.0	1.0					9.3
9469.0	39		-5.5	9050.0	13605.0	76.1	-0.9	0.8					9.3
10927.0	40	19-Norethindrone	-6	10508.0	15747.0	88.1	-1.2	1.5	88.6	0.6	0.4	0.7	10.7
10952.0	41		-6	10523.0	15784.5	88.3	-1.0	1.1					10.7
11085.0	42		-6	10636.0	15954.0	89.3	-0.1	0.0					10.9
11733.0	43	19-Norethindrone	-6.5	11304.0	16956.0	94.9	1.2	1.4	93.6	1.7	1.0	1.8	11.6
11348.0	44		-6.5	10919.0	16378.5	91.6	-2.1	4.2					11.1
11655.0	45		-6.5	11226.0	16839.0	94.2	0.5	0.3					11.4
11706.0	46	19-Norethindrone	-7	11277.0	16915.5	94.6	-0.4	0.1	94.7	1.6	0.9	1.7	11.5
11518.0	47		-7	11089.0	16633.5	93.1	-2.0	3.8					11.3
11910.0	48		-7	11481.0	17221.5	96.4	1.3	1.8					11.7
11407.0	49	19-Norethindrone	-7.5	10978.0	16467.0	92.1	-3.3	10.8	93.5	1.6	0.9	1.7	11.2
11532.0	50		-7.5	11103.0	16654.5	93.2	-2.2	5.0					11.3
11774.0	51		-7.5	11346.0	17017.5	95.2	-0.2	0.0					11.6
12307.0	52	19-Norethindrone	-8.5	12178.0	18267.0	102.2	6.6	44.1	98.2	4.9	2.9	5.0	12.4
12316.0	53		-8.5	11887.0	17830.5	99.8	4.2	17.6					12.1
11473.0	54		-8.5	11044.0	16566.0	92.7	-2.9	8.3					11.3
10307.0	55	Octyltriethoxysilane	-3	9878.0	14817.0	82.9	2.3	5.4	80.6	2.1	1.2	2.6	10.1
9925.0	56		-3	9396.0	14094.0	78.9	-1.7	3.0					9.6
9961.0	57		-3	9532.0	14298.0	80.0	-0.6	0.3					9.8
10491.0	58	Octyltriethoxysilane	-4	10062.0	15093.0	84.4	-3.6	13.1	88.1	6.2	3.6	7.0	10.3
10503.0	59		-4	10074.0	15111.0	84.6	-3.6	12.3					10.3
11771.0	60		-4	11342.0	17013.0	95.2	7.1	50.8					11.6
11950.0	61	Octyltriethoxysilane	-5	11521.0	17281.5	96.7	0.0	0.0	97.1	1.2	0.7	1.2	11.7
11895.0	62		-5	11466.0	17199.0	96.2	-0.4	0.2					11.7
12161.0	63		-5	11732.0	17598.0	98.6	1.8	3.2					11.9
11522.0	64	Octyltriethoxysilane	-6	11093.0	16639.5	93.1	-3.6	12.7	94.2	1.4	0.8	1.5	11.3
11848.0	65		-6	11419.0	17128.5	95.8	-0.8	0.7					11.6
11596.0	66		-6	11167.0	16750.5	93.7	-2.9	8.7					11.4
12612.0	67	Octyltriethoxysilane	-7	12163.0	18274.5	102.2	5.6	31.1	100.1	1.9	1.1	1.9	12.4
12183.0	68		-7	11754.0	17631.0	98.6	2.0	3.9					12.0
12279.0	69		-7	11849.0	17773.5	99.4	2.8	7.7					12.1
11983.0	70	Octyltriethoxysilane	-8	11554.0	17331.0	97.0	0.3	0.1	98.0	1.2	0.7	1.2	11.8
12093.0	71		-8	11659.0	17488.5	97.7	1.0	1.0					11.8
12253.0	72		-8	11824.0	17736.0	99.2	2.6	6.6					12.0
11706.0	73	Octyltriethoxysilane	-9	11277.0	16915.5	94.6	-2.0	4.1	95.3	0.6	0.4	0.7	11.5
11793.0	74		-9	11364.0	17046.0	95.4	-1.3	1.7					11.6
11959.0	75		-9	11430.0	17145.0	95.9	-0.7	0.5					11.6
11384.0	76	Octyltriethoxysilane	-10	10965.0	16432.5	91.9	-4.7	22.4	95.3	2.9	1.7	3.1	11.2
11944.0	77		-10	11515.0	17272.5	96.6	0.0	0.0					11.7
12017.0	78		-10	11588.0	17382.0	97.3	0.6	0.3					11.8
10297	79	Ensulizole	-3	9868.0	14802.0	82.8	-11.4	130.0	86.1	3.1	1.8	3.6	10.1
10737	80		-3	10308.0	15462.0	86.5	-7.7	59.5					10.5
11032	81		-3	10603.0	15904.5	89.0	-5.2	27.4					10.8
12605	82	Ensulizole	-4	12176.0	18264.0	102.2	8.0	63.6	98.7	3.4	2.0	3.5	12.4
12174	83		-4	11746.0	17617.5	98.6	4.3	18.9					11.9
11789	84		-4	11360.0	17040.0	95.3	1.1	1.2					11.6
11890	85	Ensulizole	-5	11461.0	17191.5	96.2	2.0	3.9	96.6	1.4	0.8	1.5	11.7
11797	86		-5	11368.0	17052.0	95.4	1.2	1.4					11.6
12131	87		-5	11702.0	17553.0	98.2	4.0	15.9					11.9
11497	88	Ensulizole	-6	11068.0	16602.0	92.9	-1.3	1.8	92.6	2.9	1.6	3.1	11.3
11103	89		-6	10674.0	16011.0	89.6	-4.6	21.5					10.9
11760	90		-6	11351.0	17026.5	95.3	1.0	1.1					11.6
12084	91	Ensulizole	-7	11655.0	17482.5	97.8	3.6	12.9	97.0	1.0	0.6	1.1	11.9
11843	92		-7	11414.0	17121.0	95.8	1.6	2.5					11.6
12021	93		-7	11592.0	17388.0	97.3	3.1	9.4					11.8
11480	94	Ensulizole	-8	11051.0	16576.5	92.7	-1.5	2.2	92.5	1.0	0.6	1.1	11.3
11564	95		-8	11135.0	16702.5	93.4	-0.8	0.6					11.4
11323	96		-8	10894.0	16341.0	91.4	-2.8	7.8					11.1
11609	97	Ensulizole	-9	11180.0	16770.0	93.8	-0.4	0.2	94.7	0.8	0.5	0.8	11.4
11793	98		-9	11364.0	17046.0	95.4	1.2	1.3					11.6
11737	99		-9	11308.0	16962.0	94.9	0.7	0.5					11.5
11898	100	Ensulizole	-10	11469.0	17203.5	96.3	2.0	4.1	95.5	2.3	1.3	2.4	11.7
12020	101		-10	11691.0	17386.5	97.3	3.1	9.3					11.8
11493	102		-10	11064.0	16596.0	92.9	-1.4	1.9					11.3

APPENDIX 1 Raw and Normalized Data 3rd Valid Run (continued) – February 18, 2013

8388	79	Avobenzone	-3	7959.0	11938.5	66.8	1.4	1.9	65.4	1.2	0.7	1.9	8.2
8168	80		-3	7739.0	11608.5	64.9	-0.5	0.2					8.0
8109	81		-3	7680.0	11520.0	64.5	-0.9	0.9					8.0
9562	82	Avobenzone	-4	9133.0	13699.5	76.6	2.1	4.4	74.6	1.8	1.0	2.4	9.4
9183	83		-4	8754.0	13131.0	73.5	-1.1	1.2					9.0
9192	84		-4	8763.0	13144.5	73.5	-1.0	1.0					9.0
12052	85	Avobenzone	-5	11623.0	17434.5	97.5	1.4	2.0	97.5	0.2	0.1	0.2	11.8
12059	86		-5	11630.0	17445.0	97.6	1.5	2.2					11.8
12020	87		-5	11591.0	17386.5	97.3	1.2	1.3					11.8
11478	88	Avobenzone	-6	11049.0	16573.5	92.7	-3.4	11.5	96.4	3.2	1.8	3.3	11.3
12132	89		-6	11703.0	17554.5	98.2	2.1	4.4					11.9
12146	90		-6	11717.0	17575.5	98.3	2.2	4.9					11.9
11953	91	Avobenzone	-7	11524.0	17286.0	96.7	0.6	0.4	98.3	1.6	0.9	1.6	11.7
12151	92		-7	11722.0	17583.0	98.4	2.3	5.1					11.9
12326	93		-7	11897.0	17845.5	99.8	3.7	13.9					12.1
12066	94	Avobenzone	-8	11637.0	17455.5	97.7	1.5	2.4	96.4	2.5	1.5	2.6	11.8
11575	95		-8	11146.0	16719.0	93.5	-2.6	6.6					11.4
12119	96		-8	11690.0	17535.0	98.1	2.0	4.0					11.9
11280	97	Avobenzone	-9	10851.0	16276.5	91.1	-5.1	25.5	94.3	2.8	1.6	3.0	11.1
11866	98		-9	11437.0	17155.5	96.0	-0.1	0.0					11.6
11867	99		-9	11438.0	17157.0	96.0	-0.1	0.0					11.6
11376	100	Avobenzone	-10	10947.0	16420.5	91.9	-4.2	18.0	93.7	1.6	0.9	1.7	11.2
11708	101		-10	11279.0	16918.5	94.7	-1.5	2.1					11.5
11700	102		-10	11271.0	16906.5	94.6	-1.5	2.3					11.5
8952	79	Homosalate	-3	8523.0	12784.5	71.5	1.6	2.6	69.9	2.9	1.7	4.2	8.8
8960	80		-3	8531.0	12796.5	71.6	1.7	2.8					8.8
8354	81		-3	7925.0	11887.5	66.5	-3.4	11.7					8.2
10891	82	Homosalate	-4	10462.0	15693.0	87.8	-1.7	3.0	89.9	3.4	1.9	3.7	10.7
11602	83		-4	11173.0	16759.5	93.8	4.2	17.9					11.4
10924	84		-4	10495.0	15742.5	88.1	-1.5	2.1					10.7
11790	85	Homosalate	-5	11361.0	17041.5	95.3	0.8	0.6	93.7	1.6	0.9	1.7	11.6
11411	86		-5	10982.0	16473.0	92.2	-2.4	5.8					11.2
11590	87		-5	11161.0	16741.5	93.7	-0.9	0.8					11.4
12300	88	Homosalate	-6	11871.0	17806.5	99.6	3.8	14.4	97.0	2.9	1.7	3.0	12.1
11613	89		-6	11184.0	16776.0	93.9	-2.0	3.9					11.4
12048	90		-6	11619.0	17428.5	97.5	1.7	2.8					11.8
11536	91	Homosalate	-7	11107.0	16660.5	93.2	-2.9	8.6	93.5	1.8	1.0	1.9	11.3
11801	92		-7	11372.0	17058.0	95.4	-0.7	0.5					11.6
11372	93		-7	10943.0	16414.5	91.8	-4.3	18.6					11.2
11419	94	Homosalate	-8	10990.0	16485.0	92.2	-4.0	16.0	96.8	4.0	2.3	4.1	11.2
12226	95		-8	11797.0	17695.5	99.0	2.8	7.7					12.0
12247	96		-8	11818.0	17727.0	99.2	3.0	8.7					12.0
11952	97	Homosalate	-9	11523.0	17284.5	96.7	0.5	0.2	96.5	0.3	0.2	0.3	11.7
11879	98		-9	11450.0	17175.0	96.1	-0.2	0.0					11.7
11943	99		-9	11514.0	17271.0	96.6	0.4	0.1					11.7
12308	100	Homosalate	-10	11879.0	17818.5	99.7	3.4	11.8	97.5	1.9	1.1	2.0	12.1
11918	101		-10	11489.0	17233.5	96.4	0.2	0.0					11.7
11900	102		-10	11471.0	17206.5	96.3	0.0	0.0					11.7
11144	79	Padimate O	-3	10715.0	16072.5	89.9	3.0	9.1	86.9	3.9	2.3	4.5	10.9
10253	80		-3	9824.0	14736.0	82.4	-4.5	19.9					10.1
10957	81		-3	10528.0	15792.0	88.4	1.4	2.1					10.8
9935	82	Padimate O	-4	9506.0	14259.0	79.8	-8.3	69.2	88.1	7.2	4.2	8.2	9.8
11494	83		-4	11065.0	16597.5	92.9	4.8	22.7					11.3
11349	84		-4	10920.0	16380.0	91.6	3.6	12.6					11.1
11468	85	Padimate O	-5	11039.0	16558.5	92.6	-4.4	19.5	97.1	3.9	2.2	4.0	11.3
12194	86		-5	11765.0	17647.5	98.7	1.7	2.8					12.0
12319	87		-5	11890.0	17835.0	99.8	2.7	7.4					12.1
12090	88	Padimate O	-6	11661.0	17491.5	97.9	0.4	0.2	98.0	1.7	1.0	1.7	11.9
11920	89		-6	11491.0	17236.5	96.4	-1.0	1.0					11.7
12322	90		-6	11893.0	17839.5	99.8	2.4	5.7					12.1
12633	91	Padimate O	-7	12204.0	18306.0	102.4	5.0	25.0	98.3	4.1	2.4	4.2	12.4
11647	92		-7	11218.0	16827.0	94.1	-3.3	10.7					11.4
12155	93		-7	11726.0	17589.0	98.4	1.0	1.0					11.9
11825	94	Padimate O	-8	11396.0	17094.0	95.6	-1.8	3.2	97.0	1.8	1.0	1.9	11.6
12234	95		-8	11805.0	17707.5	99.1	1.6	2.7					12.0
11916	96		-8	11487.0	17230.5	96.4	-1.0	1.0					11.7
12057	97	Padimate O	-9	11628.0	17442.0	97.6	0.2	0.0	98.0	0.9	0.5	0.9	11.8
12226	98		-9	11797.0	17695.5	99.0	1.6	2.5					12.0
12024	99		-9	11595.0	17392.5	97.3	-0.1	0.0					11.8
11513	100	Padimate O	-10	11084.0	16626.0	93.0	-4.4	19.4	95.7	2.9	1.7	3.0	11.3
11808	101		-10	11379.0	17068.5	95.5	-1.9	3.7					11.6
12193	102		-10	11764.0	17646.0	98.7	1.3	1.7					12.0

APPENDIX 2 Rat Uterine Cytosol Preparation and Information

Supplier	Harlan Laboratories
Strain	Sprague-Dawley
Age	85 - 100
Days after ovariectomy	7
Protein Concentration	1.10 mg/mL
Method of Determination	Bradford Method
Supplier and Product	Thermo Scientific Pierce® BCA Protein Assay Kit
Catalog Number	23227
Batch/Lot Number	ND172051
Method of Transport	FedEx – priority overnight
Conditions of Transport	Dry Ice

Rat uteri were purchased from Harlan Laboratories. Collection of rat uteri was not performed according to GLP. The cytosol preparation was performed on May 11, 2012. The homogenizer probe was pre-chilled by placing it in an ice cold beaker of TEDG buffer on ice. The uterine tissue was checked for signs of residual ovarian tissue after ovariectomy (*e.g.*, uterine imbibition) and any tissues that appeared compromised were discarded. The uterine tissues were added to a beaker of TEDG+PI buffer in ice bath, at 10 ml of buffer/g tissue. Uteri were minced with fine scissors until all pieces were small 1-2 mm cubes. Then the minced tissue was homogenized at ~4°C using a pre-chilled Polytron homogenizer. For the Polytron PT 10-35GT, setting 3 with 3 to 5 short 5 sec bursts of power spaced at 20 sec intervals was used. The homogenates were transferred to pre-cooled centrifuge tubes, balanced, and centrifuged at 2,500 x g for 10 minutes in a centrifuge cooled to 4°C. The supernatant from all samples was pooled and centrifuged at 105,000 x g for 60 minutes at ~4°C, and the pellet discarded. The resulting supernatant containing the cytosolic receptors was aliquoted into labeled tubes and stored at approximately -80ºC. The cytosol preparations used in the acetone study were thawed immediately prior to use in the assay and any leftover cytosol was discarded. The protein content for each batch of cytosol was determined using the Bradford method immediately after isolation was completed.

Raw Data Plate Map

The green color refers to the protein optimization and the blue color is the bovine serum albumin (BSA) standard curve used to determine protein concentration of the isolated prostate cytosol.

	1	2	3	4	5	6	7	8	9	10	11	12
A	buffer bkg	buffer blank	water blank	0.5	0.5	0.5	3x dil buffer	3x dil buffer	3x dil buffer	empty	empty	empty
B	buffer bkg	buffer blank	water blank	0.25	0.25	0.25	5x dil buffer	5x dil buffer	5x dil buffer	empty	empty	empty
C	buffer bkg	buffer blank	water blank	0.125	0.125	0.125	10x dil buffer	10x dil buffer	10x dil buffer	empty	empty	empty
D	buffer bkg	buffer blank	water blank	0.06	0.06	0.06	20x dil buffer	20x dil buffer	20x dil buffer	empty	empty	empty
E	buffer bkg	buffer blank	water blank	0.5	0.5	0.5	40x dil buffer	40x dil buffer	40x dil buffer	empty	empty	empty
F	buffer bkg	buffer blank	water blank	0.25	0.25	0.25	80x dil buffer	80x dil buffer	80x dil buffer	empty	empty	empty
G	buffer bkg	buffer blank	water blank	0.125	0.125	0.125	5x dil water	5x dil water	5x dil water	empty	empty	empty
H	buffer bkg	buffer blank	water blank	0.06	0.06	0.06	40x dil water	40x dil water	40x dil water	empty	empty	empty

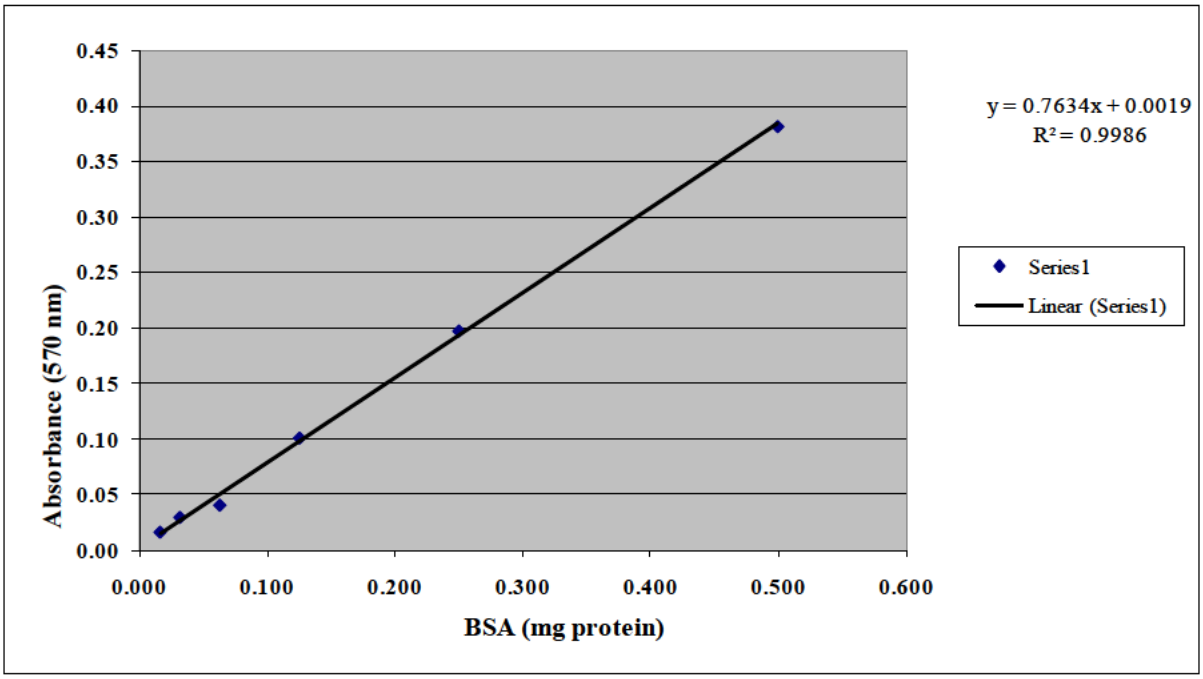
Raw Data

Absorbance values at a wavelength of 570 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.056	0.412	0.414	0.839	0.922	0.943	1.544	1.711	1.369	0.044	0.042	0.045
B	0.05	0.416	0.414	0.629	0.695	0.707	1.154	1.226	1.194	0.048	0.042	0.047
C	0.053	0.414	0.428	0.517	0.574	0.573	0.841	0.881	0.876	0.044	0.043	0.04
D	0.057	0.416	0.416	0.447	0.465	0.476	0.63	0.688	0.662	0.046	0.052	0.042
E	0.054	0.416	0.419	0.839	0.909	0.902	0.516	0.553	0.547	0.044	0.043	0.044
F	0.044	0.412	0.426	0.67	0.701	0.707	0.459	0.493	0.467	0.045	0.042	0.045
G	0.055	0.412	0.411	0.53	0.558	0.569	1.462	1.288	1.986	0.043	0.04	0.042
H	0.06	0.421	0.42	0.451	0.478	0.48	0.528	0.543	0.544	0.042	0.041	0.04

Calibration Curve

Bovine serum albumin (BSA) standard curve used to determine protein concentration of the isolated prostate cytosol.



Protein Optimization

The optimal protein concentration to use in the assays was determined by incubating increasing concentrations of cytosol with 0.03 nM [^{3}H]-17β-estradiol. This allowed for the determination of the protein concentration that binds no more than 25-35% of the total radiolabel added.

Code (N=3 of each)	Receptor Dilution	Receptor concentration	Inert R1881	^{3}H-R1881	Cytosol
TB 0.25nM	undiluted	Conc 1	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:1.25	Conc 2	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:1.5	Conc 3	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:2	Conc 4	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:3	Conc 5	none	7.5 µL of 10 nM	300 µL
NSB 0.25nM	undiluted	Conc 1	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:1.25	Conc 2	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:1.5	Conc 3	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:2	Conc 4	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:3	Conc 5	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
TB 1.0 nM	undiluted	Conc 1	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:1.25	Conc 2	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:1.5	Conc 3	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:2	Conc 4	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:3	Conc 5	none	30 µL of 10 nM	300 µL
NSB 1.0 nM	undiluted	Conc 1	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:1.25	Conc 2	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:1.5	Conc 3	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:2	Conc 4	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:3	Conc 5	30 µL of 1 µM	30 µL of 10 nM	300 µL

Saturation Binding Methods

A Saturation Binding Experiment measuring total and non-specific binding of [^{3}H]-17β-estradiol was performed to demonstrate that the estrogen receptor (ER) was present in reasonable concentrations and had the appropriate affinity for the native ligand. The conditions for the saturation binding experiment are summarized in Table 1.

TABLE 1. Summary of Conditions for Saturation Binding Experiment		
Source of receptor		Rat uterine cytosol
Concentration of radioligand (as serial dilutions)		0.03-3 nM
Concentration of non-labeled ligand (100X [radioligand])		3-300 nM
Concentration of receptor		Sufficient to bind ~25-35% of radioligand at 0.03 nM
Temperature		~4ºC
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM

The [^{3}H]-17β-estradiol was manufactured on March 23, 2012 and the specific activity was 140.0 Ci/mmol. On the day of the assay the specific activity of the stock solution [^{3}H]-17β-estradiol was adjusted for decay over time, and serial dilutions in TEDG + PMSF buffer were prepared to achieve the final concentrations of 0.03, 0.06, 0.08, 0.1, 0.3, 0.6, 1, and 3 nM. Solutions of non-labeled 17β-estradiol were prepared in a similar manner to achieve concentrations that were 100-fold greater than each respective radiolabeled concentration to result in final concentrations of 3, 6, 8, 10, 30, 60, 100 and 300 nM. For each batch of cytosol, the optimal protein concentration was determined by calculating specific binding to differing amounts of protein per tube, using 0.03 nM radiolabeled estradiol, until a concentration was reached that bound ~25-35% of the total radioactivity added. The protein concentration was 15 µg per assay tube for the three saturation binding experiments. Each assay consisted of three non-concurrent binding assay runs (May 19, 2012, May 26, 2012 and May 27, 2012), and each run contained three concurrent replicates at each concentration, resulting in the 72 sample tubes depicted in Table 2.

TABLE 2. Saturation Binding Experiment Run For all Three Runs			
Total binding [a]	Non-specific binding [b]	Radioligand alone [c]	Assay Components
Tubes 1-24	Tubes 25-48	Tubes 49-72	
350 µL	300 µL	---	TEDG + PMSF buffer
50 µL	50 µL	50 µL	[^{3}H]-17β-estradiol (8 serial dilutions) [d]
---	50 µL	---	Non-labeled 17β-estradiol (8 serial dilutions, 100x each respective labeled concentration) [e]
100 µL	100 µL	---	Uterine cytosol (diluted to appropriate conc.)
500 µL	500 µL	50 µL	Total volume in each assay tube

a Total binding = [^{3}H]-17β-estradiol bound to ER
b Non-specific binding = [^{3}H]-17β-estradiol and 100-fold greater non-labeled bound to ER
c Total [^{3}H]-17β-estradiol alone for dpm determination at each concentration
d Final concentrations of [^{3}H]-17β-estradiol = [0.03, 0.06, 0.08, 0.1, 0.3, 0.6, 1, and 3] nM.
e Final concentrations of non-labeled 17β-estradiol = [3, 6, 8, 10, 30, 60, 100, and 300] nM.

Tubes were incubated at approximately 4°C, with gentle vortexing, for 20 hr 0 min, 19 hr 05 min and 19 hr 35 min for the first, second and third saturation binding experiments, respectively. To separate bound from free estradiol, 250 µL of hydroxyapatite (HAP) slurry was added to each tube and vortexed (3 times with 5-minute intervals). Subsequently, the contents of each tube were washed three times as follows: 2 mL of ice cold TEDG +PMSF buffer was added, vortexed and centrifuged for 10 min at 1000 x *g*. The supernatant decanted and discarded. The HAP pellet remaining in each tube was resuspended in 1.5 mL absolute ethanol to extract the [^{3}H]-17β-estradiol, followed by vortexing, and centrifugation for 10 min at 1000 x *g*. 20 mL scintillation vials were filled with 10 mL scintillation cocktail and a 1 mL aliquot of supernatant was radioassayed by scintillation counting. The temperature was maintained at approximately 4ºC throughout the assay prior to extraction with ethanol. This was repeated two more times for a total of three saturation binding runs.

Data Analysis

For the Saturation Binding Experiment, total binding and non-specific binding data were modeled via non-linear regression using Graph Pad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA), incorporating automatic outlier elimination according to the method of Motulsky and Brown (2006) implemented by using the ROUT procedure in Prism v. 5 with a Q value of 1.0. Scatchard plots were also generated using Graph Pad Prism v. 5. Receptor binding data plots were corrected for ligand depletion using the method of Swillens (1995). Parameters reported from the Saturation Binding Experiment (K_d and B_{max}), means and standard deviations, were calculated for each run and the means and standard errors were calculated for the composite three runs using Microsoft Excel 2007 (Redmond, WA; version 12.0.6557.5000).

Saturation Binding Results

Non-specific binding was 9.9% of total binding (mean value for all three saturation binding runs). This is within the suggested range of 8.1% – 10.0%. The mean dissociation constant (K_d) for [^{3}H]-17β-estradiol was 0.046 ± 0.012 nM. The mean estimated B_{max} was 0.008 ± 0.002 nM (75.98 ± 17.06 fmol/100 µg protein) for the single batch of prostate cytosol that was prepared. The K_d and B_{max} were within the range reported in the OPPTS estrogen receptor binding guidelines. Confidence in these numbers is high according to the goodness of fit (r^2 = 0.863 – 0.915) and the relatively small variation among runs.

TABLE 5. Saturation Binding Experiment of 17β-estradiol with Estrogen Receptor from Rat Uterine Cytosol

Parameter	Run 1	Run 2	Run 3	Runs 1-3 [a]
R^2 (unweighted)	0.863	0.904	0.915	0.863 – 0.915
B_{max} (nM)	0.006	0.009	0.008	0.008 ± 0.002
B_{max} (fmol/100 µg protein)	57.39	90.90	79.66	75.98 ± 17.05
K_d (nM)	0.034	0.058	0.045	0.046 ± 0.012

a The range of R^2 is reported and the mean ± SEM is reported for the other parameters.
R^2 = Goodness of fit for curve calculated for specific binding

Figure 1. Binding of [^{3}H]-17β-estradiol to the Estrogen Receptor during the Saturation Binding Experiment, Run 1 (19-May-2012).

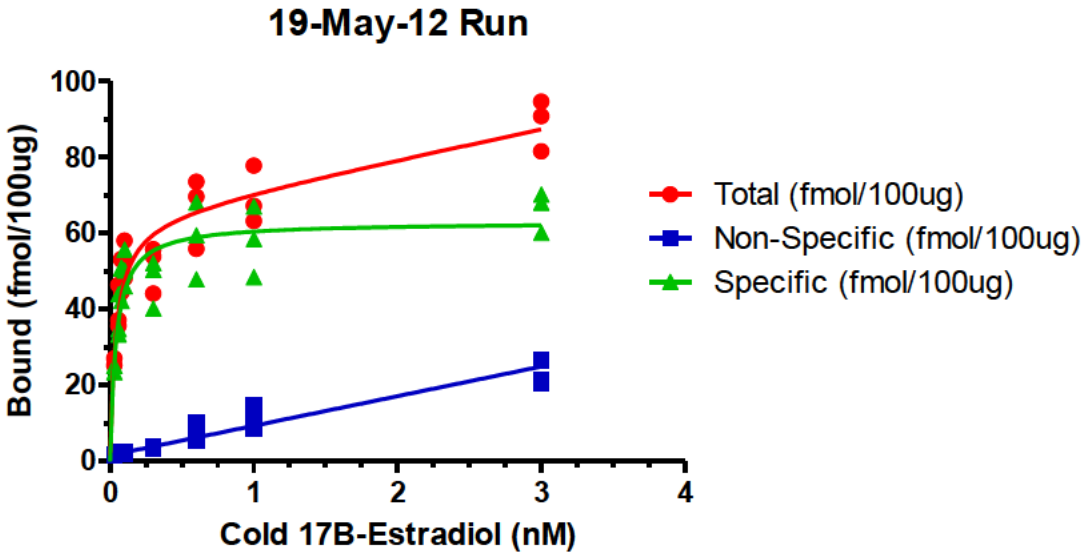


Figure 2. Scatchard Plot of the Binding of [^{3}H]-17β-estradiol to the Estrogen Receptor, Run 1 (19-May-2012).

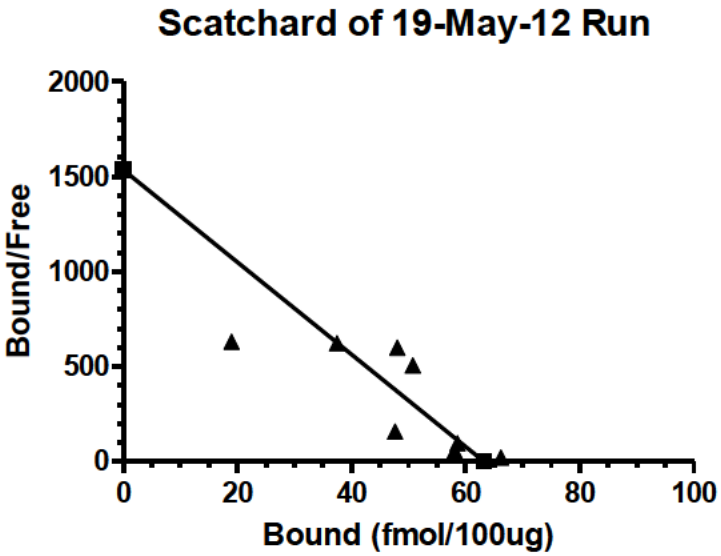


Figure 3. Binding of [^{3}H]-17β-estradiol to the Estrogen Receptor during the Saturation Binding Experiment, Run 2 (26-May-2012).

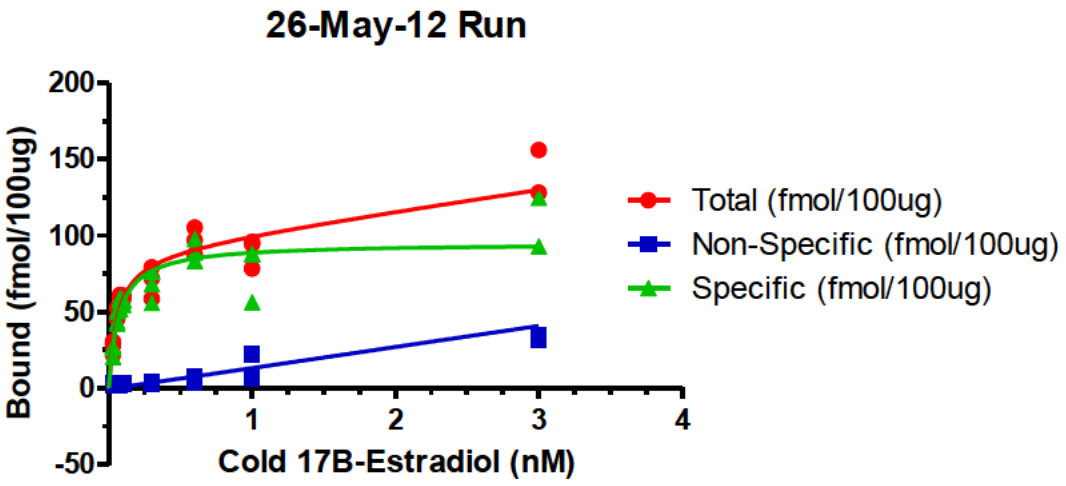


Figure 4. Scatchard Plot of the Binding of [^{3}H]-17β-estradiol to the Estrogen Receptor, Run 2 (26-May-2012).

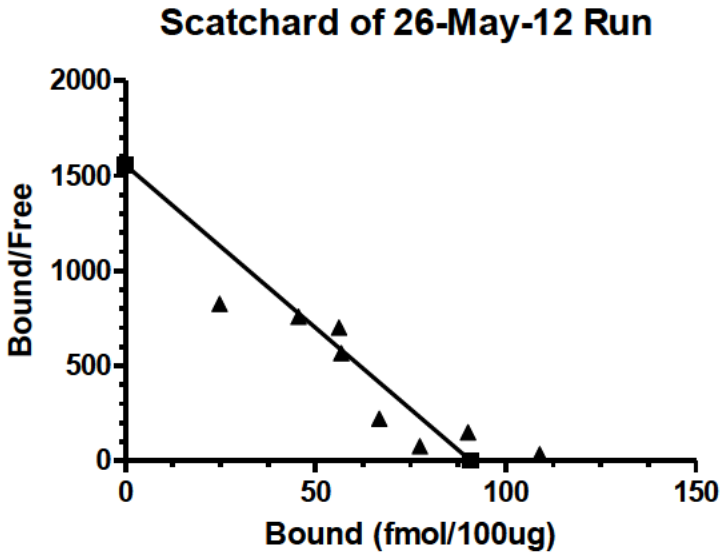


Figure 5. Binding of [^{3}H]-17β-estradiol to the Estrogen Receptor during the Saturation Binding Experiment, Run 3 (27-May-2012).

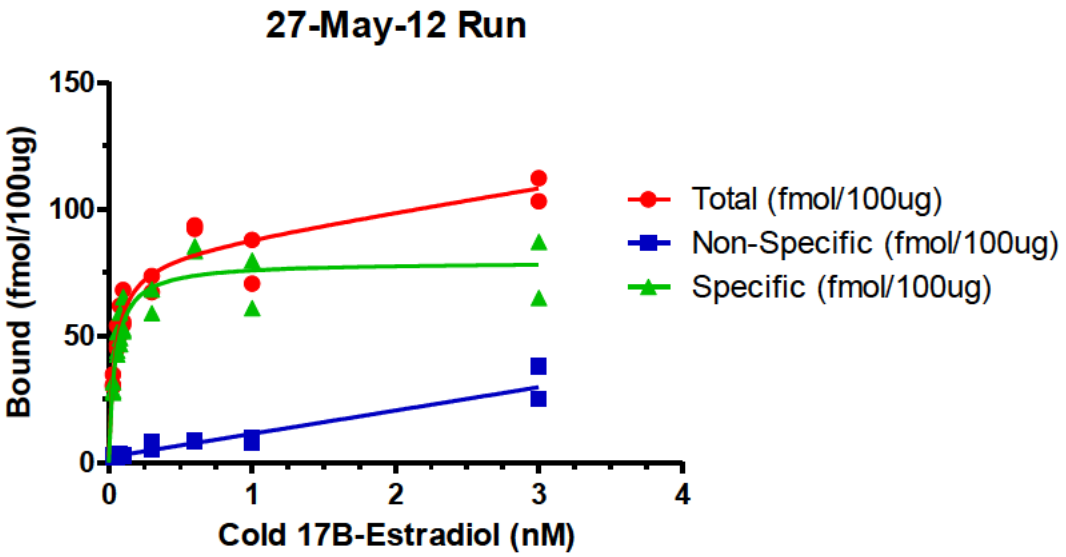
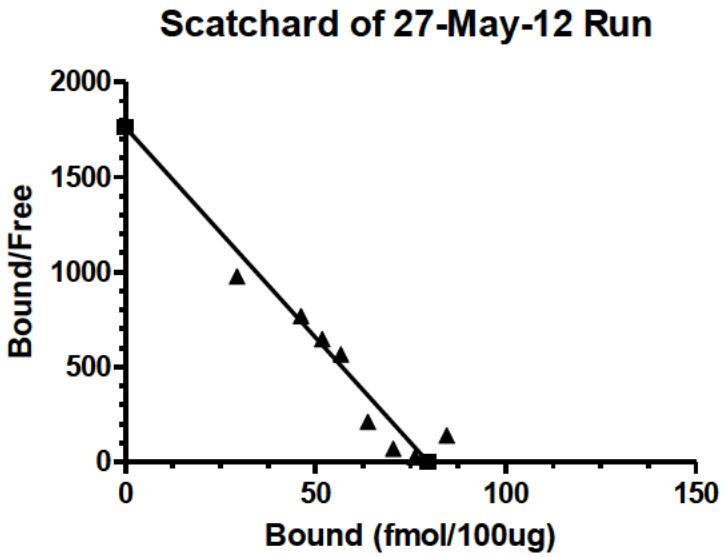


Figure 6. Scatchard Plot of the Binding of [^{3}H]-17β-estradiol to the Estrogen Receptor, Run 3 (27-May-2012).



APPENDIX 3 Deviation Form

Form #: SOP-1003-F-1.2

CeeTox
In vitro models to predict toxicity

Deviation and Investigation

Study Number (if applicable): 9070-100794ERB

SOP Number (if applicable): N/A

Equipment Serial Number (if applicable): N/A

Date of Reporting: 08-March-13 Reporting Associate: [redacted]

Date of Occurrence: 26-Jan-13, 30-Jan-13 and 18-Feb-13 Associate Involved: [redacted]

Description of Deviation:

Solubility was determined visually as per the OPPTS guideline, not by nephelometry per the protocol.

Signature [redacted] Date: 08-March-13

Reporting Associate

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

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

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

Solubility was determined visually as per the OPPTS guideline, not by nephelometry per the protocol.

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

None, no impact on data or integrity of study.

Signature [redacted] Date: 08-March-13

SD/PI/Management Acknowledgement

Signature [redacted] Date: 08-March-13

Quality Assurance Review

If study specific, sponsor notified on: 08 March 2013 By: [redacted]

See Attached Documentation (email documentation is sufficient) ☒

Standard Operating Procedure Page 1 of 1

APPENDIX 4 Certificate of Analysis

TECHNICAL DATA SHEET ^3H

9070-100794 [redacted]

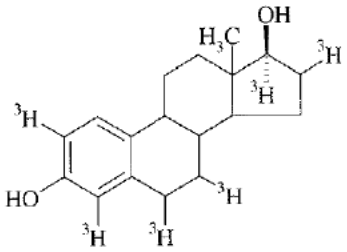
Caution: For Laboratory Use. A product for research purposes only.

ESTRADIOL, [2,4,6,7,16,17-^{3}H(N)]-

Product Number: NET517

LOT SPECIFIC INFORMATION

Lot Number:	1664345
Specific Activity:	140 Ci/mmol
	5180 GBq/mmol
Production Date:	13-Sep-2012



M.W. 272
$C_{18}H_{24}O_2$

PACKAGING: 1.0 mCi/ml (37 MBq/ml) in ethanol, shipped on dry ice.

STABILITY AND STORAGE RECOMMENDATIONS: When estradiol, [2,4,6,7,16,17-^{3}H(N)]- is stored at -20°C in its original solvent and at its original concentration, the rate of decomposition is initially 2-3% over the first three months from date of purification. Stability is nonlinear and not correlated to isotope half-life. Lot to lot variation may occur.

SPECIFIC ACTIVITY RANGE: 110-170 Ci/mmol (4070-6290 GBq/mmol)

RADIOCHEMICAL PURITY: This product was initially found to be greater than 97% when determined by the following methods. The rate of decomposition can accelerate. It is advisable to check purity prior to use:

High pressure liquid chromatography on a Zorbax ODS column using the following mobile phase:
acetonitrile : water, (1:1).

Thin layer chromatography on silica gel G using the following solvent system:
toluene : ethanol, (9:1).

QUALITY CONTROL: The radiochemical purity of estradiol, [2,4,6,7,16,17-^{3}H(N)]- is checked at appropriate intervals using the first listed chromatography method.

HAZARD INFORMATION: WARNING: This product contains a chemical known to the state of California to cause cancer.

PerkinElmer, Inc.
549 Albany Street
Boston, MA 02118 USA
P: (800) 762-4000 or (+1) 203-925-4602
www.perkinelmer.com/tenradiochemicals
For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

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SIGMA-ALDRICH®

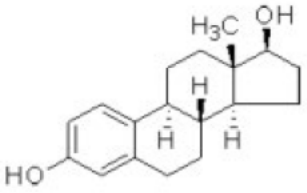
sigma-aldrich.com

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

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Certificate of Analysis

SIGMA-ALDRICH

Product Name	19-Norethindrone, ≥98%, powder
Product Number	N4128
Product Brand	SIGMA
CAS Number	68-22-4
Molecular Formula	$C_{20}H_{26}O_2$
Molecular Weight	298.42

TEST	SPECIFICATION	LOT 030m1359v RESULTS
Appearance (Color)	White to Off-White	Off-White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless to Faint Yellow	Faint Yellow
Solubility (Turbidity)	Clear	Clear
	at 50 mg/mL in Chloroform	
EmM	16.5 - 17.1	16.9
	in EtOH	
Wavelength	240 - 241 nm	240 nm
Purity (HPLC)	≥98 %	99 %
Recommended Retest Period	----------	----------
	4 years	
Specification Date:		MAR 2010
Date of QC Release:		APR 2010
Recommended Retest Date:		MAR 2014
Print Date:		APR 06 2010

[redacted]

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

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Triethoxy(octyl)silane, ≥97.5%
Product Number	440213
Product Brand	ALDRICH
CAS Number	2943-75-1
Molecular Formula	$CH_3(CH_2)_7Si(OC_2H_5)_3$
Molecular Weight	276.49

TEST	SPECIFICATION	LOT 24996kk RESULTS
Appearance (Color)	Colorless to Very Faint Yellow	Colorless
Appearance (Form)	Liquid	Liquid
Infrared spectrum	Conforms to Structure	Conforms
Purity (GC)	≥97.50 % *Dow Corning Data	99.34 %
Color Test	≤50 APHA *Dow Corning Data	1 APHA
Specification Date:		JUL 2008
Date of QC Release:		SEP 2009
Print Date:		SEP 16 2009

[redacted]

[redacted] Senior Chemist
Quality Control
Sheboygan Falls, Wisconsin, USA

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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:

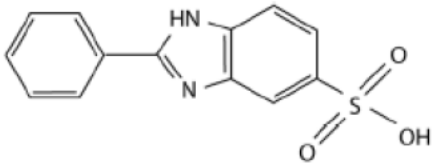
[redacted] 09/05/12
Reshan Fernando, Ph.D. Date
Principal Investigator

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

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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.

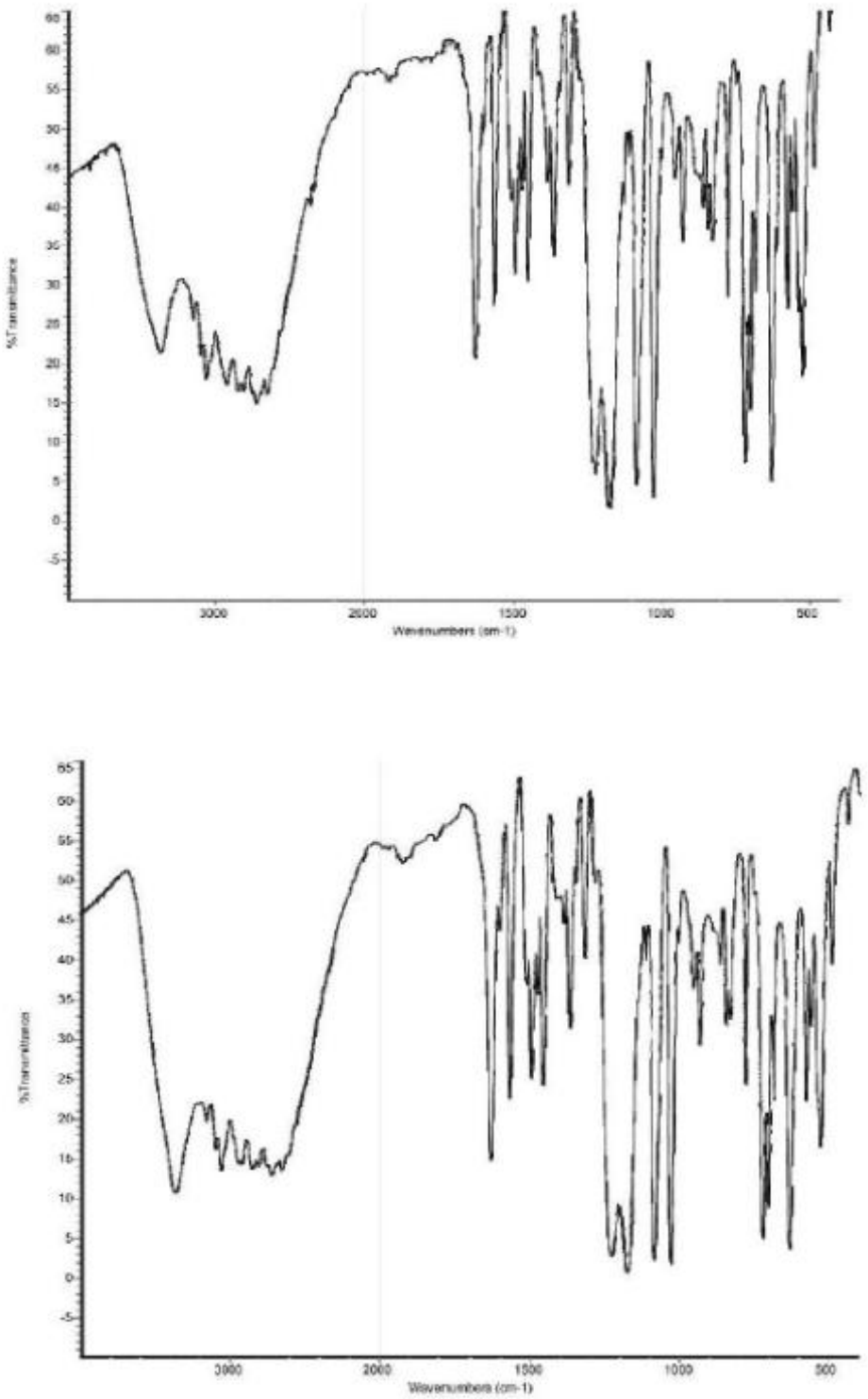


Figure 1: Infrared Spectrum of Ensulizole Frozen Reference (top spectrum) and Bulk Sample (bottom spectrum)

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.

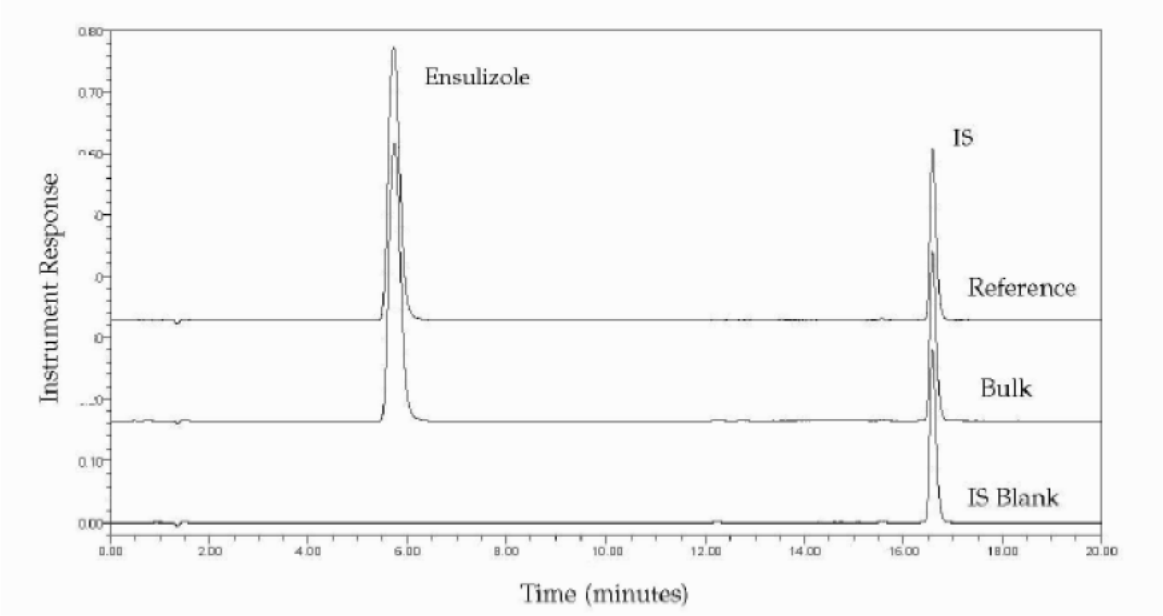


Figure 2: Example Liquid Chromatograms of Ensulizole Reference and Bulk Sample, and a Blank

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:

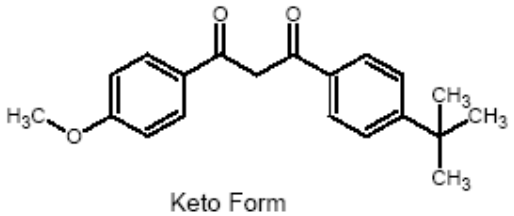
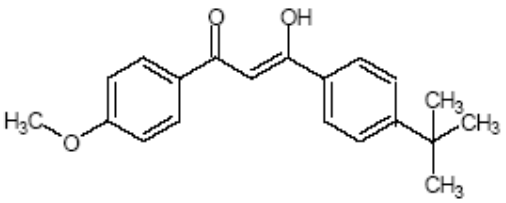
[redacted]
Group Leader

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

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
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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]

09-05-12
Date

Task Leader

Approved by:

[redacted]

09/05/12
Date

Reshan Fernando, Ph.D.
Principal Investigator

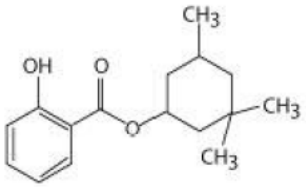
Submitted to:

[redacted]

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

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]

Quality Assurance Specialist

9/5/12

Date

Reviewed by:

[redacted]

Quality Assurance Specialist

9/5/12

Date

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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.

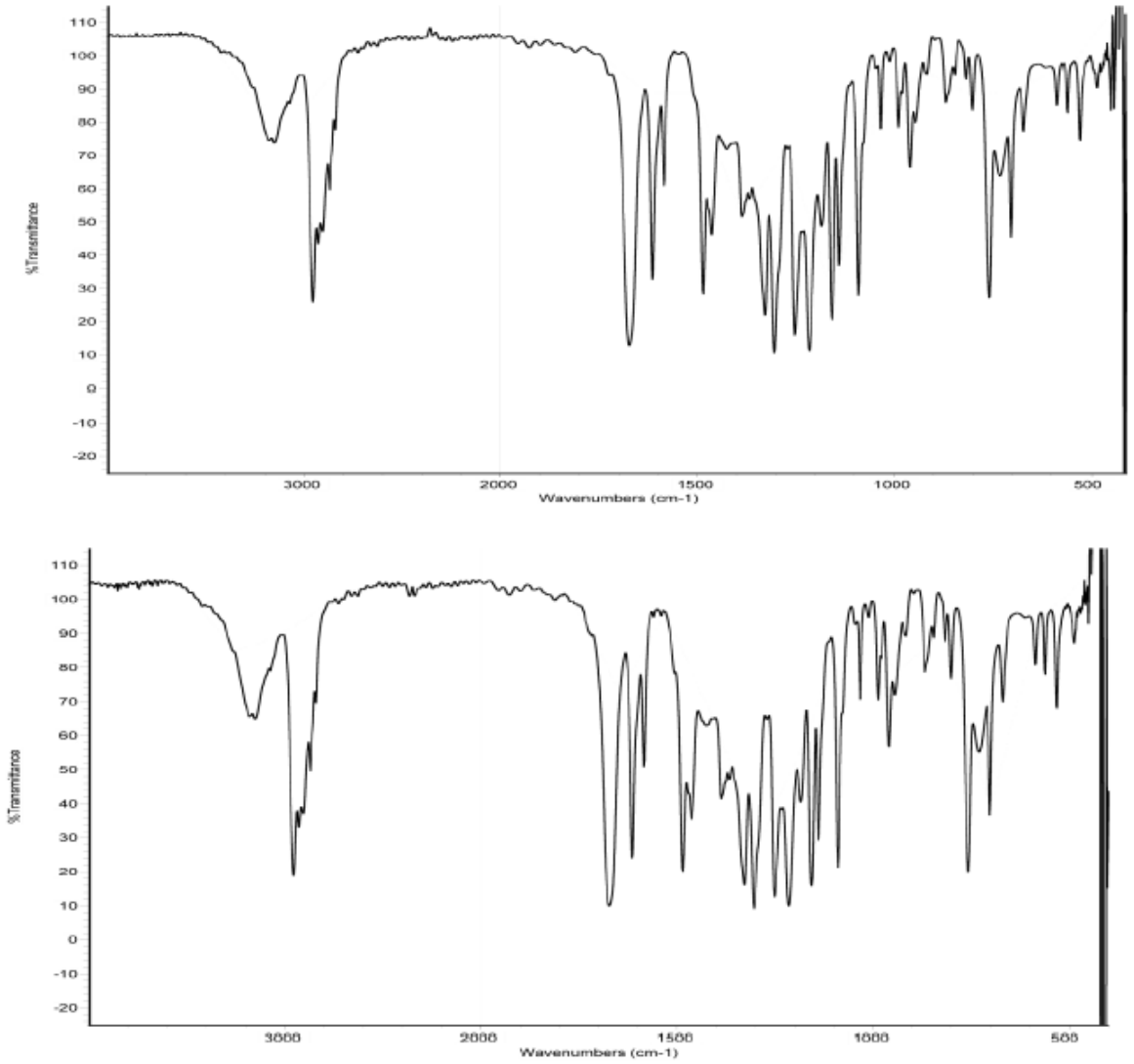

Figure 1: Infrared Spectrum of Homosalate Bulk (top spectrum)
and Frozen Reference (bottom spectrum)

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.

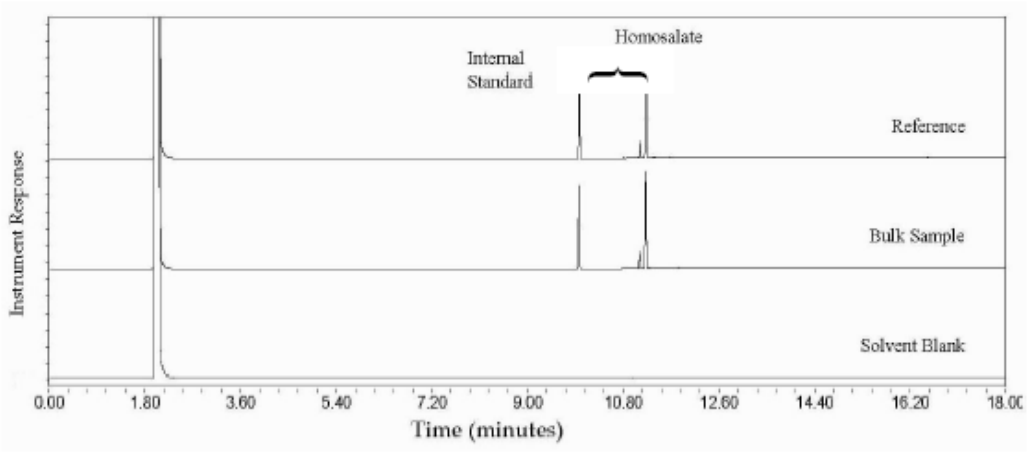

Figure 2: Example Gas Chromatograms of Homosalate Reference and Bulk Sample, and a Blank

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
NTP Analytical Chemistry Services
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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
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

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)

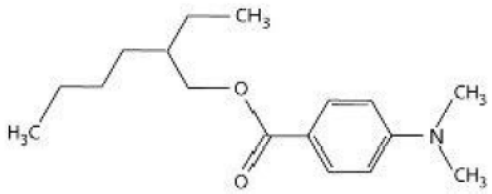
Receipt Date: Aug 20, 2010 (Bulk)

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

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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.

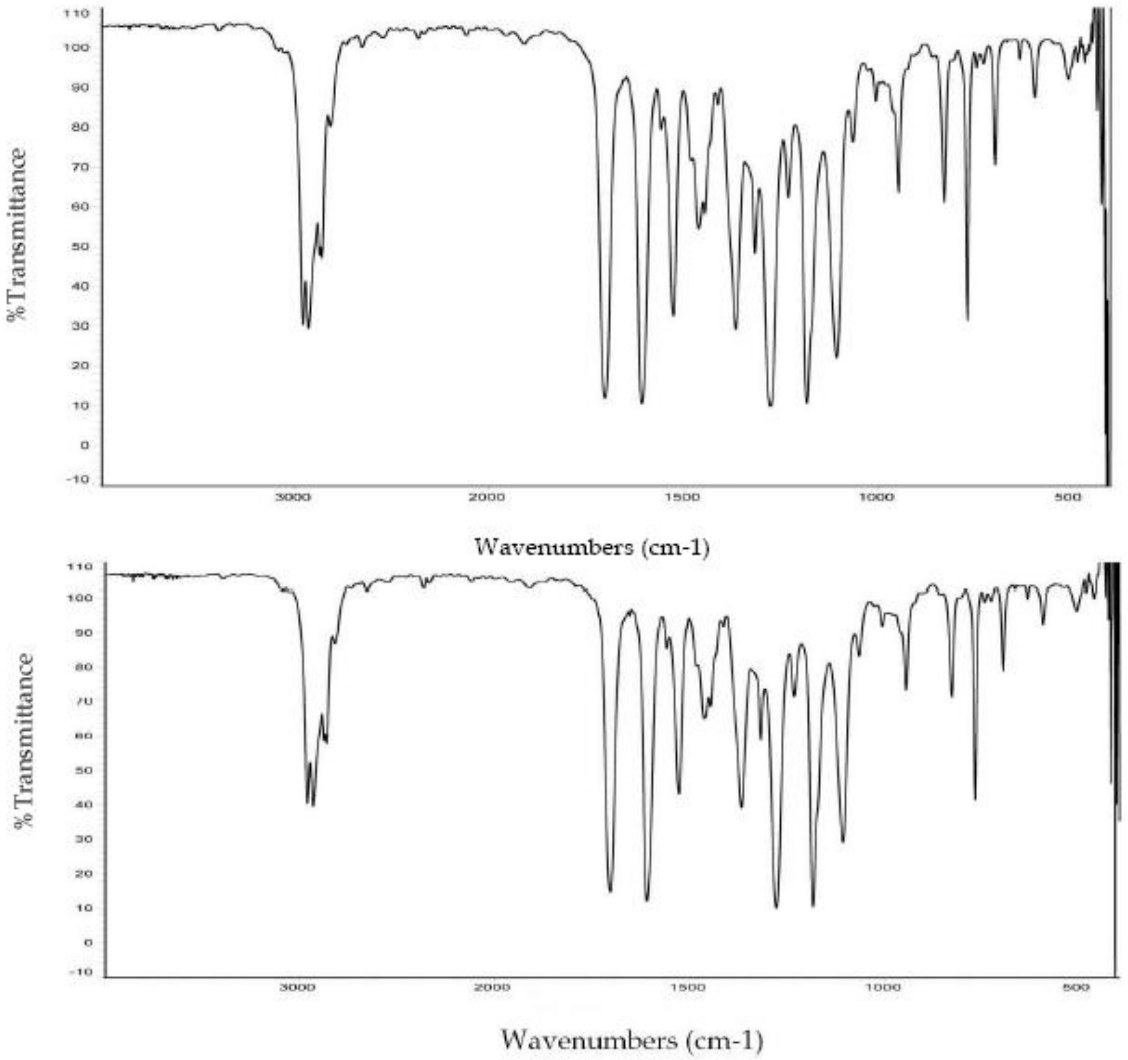

Figure 1: Infrared Spectrum of Padimate O Bulk (top spectrum)
and Frozen Reference (bottom spectrum)

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.

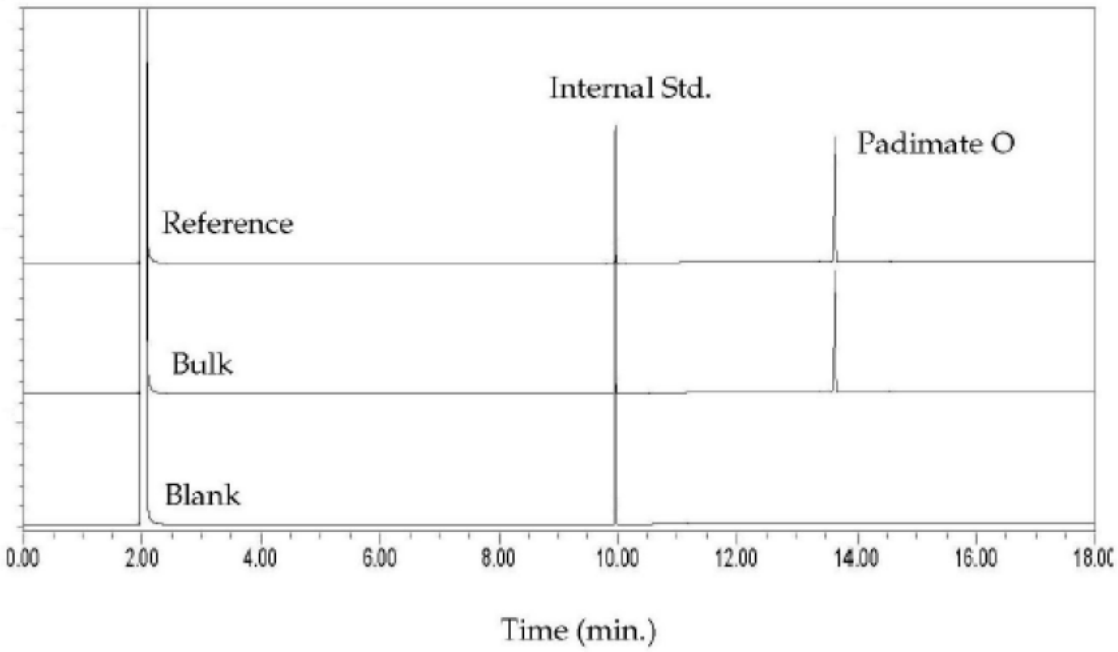

Figure 2: Example Gas Chromatograms of Padimate O Reference and Bulk Sample, and an IS Blank

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 5 Protocol and Protocol Amendment

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

PROTOCOL

Estrogen Receptor Binding (Rat Uterine Cytosol)

Data Requirements: *OPPTS 890.1250*

Study Number:
9070-100794ERB

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 Binding (Rat Uterine Cytosol)

2. Purpose of Study

The objective of this study is to evaluate four test substances for the ability to compete with [^{3}H] ligand for binding estrogen receptors (ERs) in rat uterine tissue homogenate. The endpoint is the decays per minute (DPM) of the radioligand.

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

Endocrine Disruptor Screening Program Test Guidelines. *OPPTS 890.1250: Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC).* EPA 740-C-09-005. October, 2009.

6. Test Facility

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

7. Experimental Design

The estrogen receptor binding 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 systems. This assay is intended to identify the

ability of test compounds to interact with the estrogen receptors (ERs) isolated from Sprague-Dawley rat uteri. In this assay the test materials and the controls are incubated with rat uterine cytosol (containing the ERs) and radiolabeled 17β-estradiol (competitor) for approximately 16-20 hours at approximately 4°C. The amount of bound radiolabeled 17β-estradiol is assessed using a scintillation counter to determine the decays per minute (DPM), and specific binding is then determined. A complete concentration response curves for the positive control 17β-estradiol, the weak positive control (wPC) 19-norethindrone, and the negative control, octyltriethoxysilane, and if applicable, the test materials, will be generated each time the binding assay is performed.

8. Justification of the Test System

As per the guideline (OPPTS 890.1250) the uteri from ovariectomized female Sprague-Dawley rats (85 to 100 days of age at time of kill) will be used to prepare the cytosol. The cytosol will be prepared, and deemed acceptable, per EPA guideline and CeeTox SOP for use in this study. Bias is not a factor in this test system. The test system (cytosol) will be identified by the isolation date. Cytosol preparation and saturation binding data will be included in the appendices of the final report.

9. Identification of the Test System

Uteri from ovariectomized Sprague-Dawley female rats will be used to prepare the cytosol. The uteri will be purchased from an outside vendor. The cytosol will be prepared, and deemed acceptable, per EPA guideline and CeeTox SOP for use in this study. The test system (cytosol) will be identified by the isolation date. Cytosol preparation information, saturation binding data and any other information deemed necessary will be included in the appendices of the final report.

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 stock test substances will be formulated in dimethyl sulfoxide (DMSO) or appropriate solvent. Fresh 50X dilutions of the stock solution will be prepared on the day of use such that the target concentration of test substance can be achieved by the addition of approximately 10 µL of the dilution to approximately 500 µL total assay volume. Dose concentrations of test and control substances will not be verified using analytical methods.

Serial Dilutions of Test Substances

Serial dilutions of test substances will be prepared in the TEDG (Tris, EDTA, DTT, Glycerol) assay buffer to yield the final concentrations indicated in Table 2, unless solubility limits the top concentration tested.

Table 1. Test Substance Dilution Procedure

Tube #	Volume of stock to add for diluted concentration	Volume of TEDG assay buffer to add	Total volume of diluted test substance	Diluted test substance concentration	*Final test substance concentration in ER assay tube
TS1	Use 500 µl of stock test substance (100 mM)	500 µl	1 ml	5×10^{-2} M	1×10^{-3} M
TS2	Use 100 µl of dilution TS1 (50 mM)	900 µl	1 ml	5×10^{-3} M	1×10^{-4} M
TS3	Use 100 µl of dilution TS2 (5 mM)	900 µl	1 ml	5×10^{-4} M	1×10^{-5} M
TS4	Use 100 µl of dilution TS3 (500 µM)	900 µl	1 ml	5×10^{-5} M	1×10^{-6} M
TS5	Use 100 µl of dilution TS4 (50 µM)	900 µl	1 ml	5×10^{-6} M	1×10^{-7} M
TS6	Use 100 µl of dilution TS5 (5 µM)	900 µl	1 ml	5×10^{-7} M	1×10^{-8} M
TS7	Use 100 µl of dilution TS6 (500 nM)	900 µl	1 ml	5×10^{-8} M	1×10^{-9} M
TS8	Use 100 µl of dilution TS7 (50 nM)	900 µl	1 ml	5×10^{-9} M	1×10^{-10} M

*Final concentration of test substance in assay tube when 10 µl of diluted concentration is used in a total volume of 500 µl.

10.3 Reference Substances

When testing substances for their ability to bind to the estrogen receptor (ER), a solvent control, positive control (standard), negative control and a weak positive control will be included in each experiment. One set of standards is needed in each run on a given day.

A standard curve using 17β-estradiol (CAS 50-28-2; positive control) will be included to allow for an assessment of variability in the conduct of the experiment across time. The concentration range for 17β-estradiol will be 1 x 10^{-11} to 1 x 10^{-7} M. The supplier, catalog number, lot number and purity will be included in the final report.

Table 2. Example of Dilution Procedure for Reference Standard 17β-estradiol

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of 17β-estradiol	Diluted 17β-estradiol concentration	Final 17β-estradiol concentration in ER assay tube
NSB1	Use 100 µl of stock 17β-estradiol (50 µM)	900 µl	1 ml	5 x 10^{-6} M	1 x 10^{-7} M
S2	Use 100 µl of dilution NSB1 (5 µM)	900 µl	1 ml	5 x 10^{-7} M	1 x 10^{-8} M
S3	Use 277 µl of dilution S2 (500 nM)	600 µl	877 µl	1.58 x 10^{-7} M	3.16 x 10^{-9} M
S4	Use 100 µl of dilution S2 (500 nM)	900 µl	1 ml	5 x 10^{-8} M	1 x 10^{-9} M
S5	Use 100 µl of dilution S3 (158 nM)	900 µl	1 ml	1.58 x 10^{-8} M	3.16 x 10^{-10} M
S6	Use 100 µl of dilution S4 (50 nM)	900 µl	1 ml	5 x 10^{-9} M	1 x 10^{-10} M
S7	Use 100 µl of dilution S6 (5 nM)	900 µl	1 ml	5 x 10^{-10} M	1 x 10^{-11} M

The weak positive reference (19-norethindrone; CAS 68-22-4) will be included to demonstrate the sensitivity of each experiment and to allow an assessment of variability of the conduct of the experiment across time. The final concentration range tested for 19-norethindrone will be from 3.16 x 10^{-9} to 1 x 10^{-4} M. The supplier, catalog number, lot number and purity will be included in the final report.

Table 3. Example Dilution Procedure for 19-Norethindrone

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted weak positive control	Weak Positive Control Concentration	
				Diluted	Final in ER assay tube
P1	Use 400 µl of stock positive control (10 mM)	400 µl	800 µl	5×10^{-3} M	1×10^{-4} M
P2	Use 150 µl of stock positive control (10 mM)	800 µl	950 µl	1.58×10^{-3} M	3.16×10^{-5} M
P3	Use 100 µl of P2 (1.58 mM)	900 µl	1 ml	1.58×10^{-4} M	3.16×10^{-6} M
Intermed	Use 100 µl of P1 (5 mM)	900 µl	1 ml	5×10^{-4} M	Not used
P4	Use 100 µl of Intermed (500 µM)	900 µl	1 ml	5×10^{-5} M	1×10^{-6} M
P5	Use 100 µl of P3 (158 µM)	900 µl	1 ml	1.58×10^{-5} M	3.16×10^{-7} M
P6	Use 100 µl of P4 (50 µM)	900 µl	1 ml	5×10^{-6} M	1×10^{-7} M
P7	Use 100 µl of P5 (15.8 µM)	900 µl	1 ml	1.58×10^{-6} M	3.16×10^{-8} M
P8	Use 100 µl of P7 (1.58 µM)	900 µl	1 ml	1.58×10^{-7} M	3.16×10^{-9} M

A negative reference control (octyltriethoxysilane; CAS 2943-75-1) will be included to allow for an assessment of variability in the conduct of the experiment across time. The concentration range for the negative reference is 1×10^{-10} to 1×10^{-3} M. The supplier, catalog number, lot number and purity will be included in the final report.

Table 4. Example Dilution Procedure for Octyltriethoxysilane

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted negative control	Diluted negative control concentration	
				Diluted	Final in ER assay tube
NC1	Use 500 µl of stock NC (100 mM)	500 µl	1 ml	5×10^{-2} M	1×10^{-3} M
NC2	Use 100 µl of dilution NC1 (50 mM)	900 µl	1 ml	5×10^{-3} M	1×10^{-4} M
NC3	Use 100 µl of dilution NC2 (5 mM)	900 µl	1 ml	5×10^{-4} M	1×10^{-5} M
NC4	Use 100 µl of dilution NC3 (500 µM)	900 µl	1 ml	5×10^{-5} M	1×10^{-6} M
NC5	Use 100 µl of dilution NC4 (50 µM)	900 µl	1 ml	5×10^{-6} M	1×10^{-7} M
NC6	Use 100 µl of dilution NC5 (5 µM)	900 µl	1 ml	5×10^{-7} M	1×10^{-8} M
NC7	Use 100 µl of dilution NC6 (500 nM)	900 µl	1 ml	5×10^{-8} M	1×10^{-9} M
NC8	Use 100 µl of dilution NC7 (50 nM)	900 µl	1 ml	5×10^{-9} M	1×10^{-10} M

The Radioactive Ligand ([^{3}H]–17β-estradiol) supplier, catalog number and batch number will be included in the final report. The specific activity (SA) and date for which that SA

was certified by the supplier will be included along with the concentration as received from the supplier (Ci/mmol) and the concentrations tested (nM).

11. Stock Solution Preparation

200 mM EDTA Stock Solution:

- For example, 7.444 g disodium EDTA will be added to 100 ml purified H_2O. The solution will be stored at approximately 4°C.

1M Tris Buffer:

- For example, 147.24 g Tris-HCl and 8.0 g Tris base will be added to 800 ml purified H_2O. The final volume will be brought to 1 Liter. The pH will be adjusted to approximately 7.4 and stored at approximately 4°C for up to 12 months.

Preparation of 2X TEG Buffer (20 mM Tris, 3 mM EDTA, 20% glycerol, pH ~7.4):

For example, to make 100 ml of 2X TEG Buffer, the following will be added in this order:

- 70 ml purified H_2O
- 2.0 ml 1M Tris Buffer
- 20 ml glycerol
- 1.5 ml 200 mM EDTA

The buffer will be cooled to approximately 4°C before the pH is adjusted to ~7.4, and the volume brought to 100 ml with purified H^2O and stored at approximately 4°C for up to 3 months.

Preparation of Working Assay Buffer (10 mM Tris, 1.5 mM EDTA (Ethylenediaminetetraacetic acid), 1 mM DTT(Dithiothreitol), 0.5% Protease Inhibitor (with PMSF)(v/v), 10% glycerol, pH 7.4) [TEDG + PI]:

This will be prepared daily as needed. For example to make 100 ml, the following will be added in this order:

- 50 ml 2X TEG buffer (prepared as above and cooled to approximately 4°C)
- 15.43 mg DTT (added immediately before use)
- 1.0 ml Protease Inhibitor added immediately before use
- Brought to 100 ml with ice cold purified H_2O.

Any unused 1X buffer will be discarded

Preparation of 60% hydroxyapatite (HAP) slurry:

- HAP will be mixed gently to resuspend and approximately 25 ml of slurry will be added to a 100 ml graduated cylinder for washing.

- TEDG + PI buffer will be added to a final volume of approximately 100 ml, the container capped, mixed by inversion and refrigerated for at least 2 hours.
- The supernatant will be aspirated or decanted and the HAP resuspended in fresh TEDG + PI buffer (~4°C) to 100 ml. This will be mixed gently. The HAP will be allowed to settle for ~2 hours at ~4°C and the wash step repeated.
- After the last wash, the HAP slurry will be left to settle overnight (at least 8 to 10 hours at ~4°C).
- On the next day (the day of use), the volume of HAP will be noted on the graduated cylinder. The supernatant will be aspirated or decanted and the HAP resuspended to a final volume of 60% HAP and 40% cold TEDG + PI. The HAP slurry should be well-suspended and ice-cold when used in the separation procedure.

Preparation of [^{3}H]-17β-estradiol Stock Solutions

- For example the original stock of [^{3}H]–17β-estradiol will be diluted to 50 nM (i.e., 5 X 10-^{8} M) by pipetting 1 µl of the stock solution for every specific activity unit (Ci/mmol) and diluting this to 20.0 ml with TEDG + PI. Store the [^{3}H]–R1881 stock solution and dilutions at approximately -20°C.
- A copy of the Certificate of Analysis for [^{3}H]–17β-estradiol will be maintained with the study records.

Preparation of 100X Radioinert 17β-estradiol Solutions

- A 50 mM solution of 17β-estradiol will be prepared in DMSO. For example, 5.00 mg of radioinert 17β-estradiol will be weighed in a tared amber vial and 367.1 µl solvent added. The 50 mM stock will be diluted 1:10 in DMSO to get 5 mM stock.
- The 500 µM radioinert 17β-estradiol stock will be prepared by diluting the 5 mM stock 1:10 in an amber vial. This will be the 500 µM radioinert 17β-estradiol stock.
- The 50 µM radioinert 17β-estradiol stock will be prepared by pipetting the 500 µM stock 1:10 in an amber vial. This will be the 50 µM radioinert 17β-estradiol stock.

12. Competitive Radioligand Binding Assay

The competitive binding assay will be performed a minimum of three times. The optimal amount of cytosolic protein added contains enough receptor to bind 10 - 15% of the radiolabeled estradiol that has been added to the tube.

Preparation of test substance stock solutions

Test substances will be prepared at 50X the desired final concentration (listed in Table 2). Initial stocks will be prepared in TEDG assay buffer at a concentration of 50 mM.

Table 5. Summary of Assay Conditions

		Competitive Binding Assay Protocol
Source of receptor		Rat uterine cytosol
Concentration of radioligand		1 nM
Concentration of receptor		Sufficient to bind 10-15% of radioligand
Concentration of test substance (as serial dilutions)		100 pM to 1 mM
Temperature		~4°C
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH ~7.4)
	EDTA	1.5 mM
	Glycerol	10% (v/v)
	Protease Inhibitor	0.5% (v/v)
	DTT	1 mM

The specific activity (SA) of [^{3}H]–17β-estradiol will be adjusted for decay over time. The SA will be calculated on the day of the assay using the following equation:

$$SA_{adjusted} = SA * e^{-Kdecay*Time}$$

$SA_{adjusted}$/SA = Fraction Isotope Remaining (FIR)

Where:

SA is the specific activity on the packaging date (both SA and the packaging date are printed on the stock bottle from the manufacturer).

K_{decay} is the decay constant for tritium (equal to 1.54 x 10^{-4}/day)

Time = number of days since the printer date on the stock bottle from the manufacturer

The [^{3}H]-17β-estradiol will be diluted with TEDG + PI buffer

A stock dilution in TEDG + PI buffer will be prepared.

To calculate the amount of stock [^{3}H]-17β-estradiol to add to the dilution (for example having a final concentration of 1 nM in 500 µl assay tube volume) the following steps will be used:

The SA from Ci/mmole will be converted to nM. If SA = X Ci/mmole, and Y = concentration of radiolabel, then X Ci/mmole is converted to nM and the SA activity adjusted for decay over time by the following conversion:

(Y mCi/ml / X Ci/mmole) * 1 Ci/1000 mCi * 10^6 nmole/mmole * 1000 ml/L = (Y/X) * 10^6 nM

A 50 nM diluted stock of the [^{3}H]-17β-estradiol will be prepared for a final concentration of 1 nM.

The 50 nM [^{3}H]-17β-estradiol will be kept on ice until standards, test substances, and assay tubes are prepared.

Assay Preparations

12 x 75 mm (or appropriately sized) siliconized glass tubes will be used for the assay. A master mixture of radioligand and buffer will be prepared to be used for the assay. An example is shown below; 155 tubes are required for a run that includes the solvent control, three standards, and three unknowns. Trace tubes are also required. Trace tubes are 50 µl TEDG Buffer + PI with diluted [3H]-17β-estradiol. The following table describes the example preparation of a master mixture for 155 tubes.

Table 6. Master Mixture for Competitive Binding Assay

Substance	Target Volume/Tube (µl)		# of Tubes		Total Volume Needed (ml)		Master Mix Volumes (ml)
	Assay Tubes	Trace Tubes	Assay Tubes	Trace Tubes	Assay Tubes	Trace Tubes	
TEDG Buffer + PI	380	48.72	155	6	58.9	0.292	59.192
Diluted [^{3}H]-17β-estradiol (50 nM)	10	1.28	155	6	1.55	0.008	1.558
Total	390	50			60.45	0.3	60.75

Individual Tubes

For the assay individual tubes, approximately 390 µl of the master mixture above will be added to each assay tube and kept on ice. For the trace tubes, approximately 50 µl will be added directly to approximately 10 ml of scintillation fluid in scintillation vials and counted immediately. The standard, weak positive, negative and test substances will be prepared as described in section 10 and added to the assay tubes. Ten microliters of substance will be added by pipette per tube. After all substances have been added to the tubes, approximately 100 µl of cytosol will be added to each tube for a final volume of approximately 500 µl. Assay tubes will be vortexed after additions and incubated at approximately 4°C for approximately 16 to 20 hours on a rotary mixer.

Separation of bound [^{3}H]-17β-estradiol from free [^{3}H]-17β-estradiol

ER assay tubes will be removed from the rotator and placed in an ice-water bath. Using a repeating pipette, approximately 250 µl of ice cold HAP slurry (60% in TEDG + PI) will be added to each assay tube. The tubes will be vortexed for approximately 10 seconds at approximately 5 minute intervals for a total of approximately 15 minutes with tubes remaining in the ice-water bath between vortexing. Following the vortexing step, approximately 2 ml of the cold (approximately 4°C) TEDG + PI buffer will be added, vortexed quickly, and centrifuged at approximately 4°C for approximately 10 minutes at 1000 x g. After centrifugation, the tubes will be decanted immediately, and the supernatant containing the free [^{3}H]-17β-estradiol will be discarded. The HAP pellet will contain the estrogen receptor bound [^{3}H]-17β-estradiol. Two ml of ice-cold TEDG + PI buffer will be added to each tube and vortexed to resuspend the pellet. The tubes will be

centrifuged again at approximately 4°C for approximately 10 minutes at approximately 1000 x g. The tubes will be quickly decanted and the supernatant discarded. The tubes will be blotted. The wash and centrifugation will be repeated once more. After the final wash, the supernatant will be decanted. The assay tubes will be drained briefly.

Extraction and Quantification of [^{3}H]-17β-estradiol bound to ER

One and one half mL of absolute ethanol will be added to each assay tube. The tubes will be allowed to sit at room temperature for approximately 15 to 20 minutes; the tubes will be vortexed for approximately 10 seconds at approximately 5-minute intervals. The assay tubes will be centrifuged at room temperature for ~10 minutes at approximately 1000 x g. An approximately 1 ml aliquot will be pipetted, taking care to avoid the centrifuged pellet, into a 20 ml scintillation vial containing approximately 10 ml scintillation cocktail. The vial will be capped and shaken. The vials will be placed in the scintillation counter and counted for at least one minute with quench correction for determination of DPMs per vial.

13. 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 TEDG buffer alone (no cytosol) 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).

14. Competitive Binding Analysis

Estimating the IC_{50}

An ER competitive binding assay measures the binding of a single concentration of [^{3}H]-17β-estradiol in the presence of increasing concentrations of a test substance. The competitive binding curve is plotted as specific [^{3}H]-17β-estradiol binding (as a percent of total binding) versus the concentration (log_{10} units) of the competitor. The concentration of the test substance that inhibits 50% of the maximum specific [^{3}H]-17β-estradiol binding is the IC_{50} value. Estimates of IC_{50} values are determined using XLfit (Guildford, Surrey, UK).

Calculation of RBA

If necessary, the relative binding affinity (RBA) for each competitor will be calculated by dividing the IC_{50} for 17β-estradiol by the IC_{50} of the competitor and expressing as a percent.

$$\% \text{ RBA} = \frac{IC_{50}\ 17\beta\text{-estradiol}}{IC_{50}\ \text{Test Substance}} \times 100$$

Competitive Binding Performance Criteria

The competitive binding assay is functioning correctly if all of the following criteria have been met:

Increasing concentrations of unlabeled 17β-estradiol displace [^{3}H]-17β-estradiol from the receptor in a manner consistent with one-site competitive binding. Specifically, the curve fitted to the radioinert estradiol data points using non-linear regression descend from 90% to 10% over approximately an 81-fold increase in the concentration of the test substance.

Ligand depletion is minimal. Specifically, the ratio of total binding in the absence of competitor to the total amount of [^{3}H]-17β-estradiol added per assay tube is no greater than 15%.

The parameter values (top, bottom, and slope) for 17β-estradiol and the concurrent positive control (19-norethindrone) are within the tolerance bounds provided in Table 8.

The solvent control substance does not alter the sensitivity or reliability of the assay. Specifically, the acceptable limit of ethanol concentration in the assay tube is 3%; the acceptable limit of DMSO concentration is 10%. All control tubes must contain equal amounts of solvent.

The negative control substance (octyltriethoxysilane) does not displace more than 25% of the radioligand from the ER on average across all concentrations.

The test substance was tested over a concentration range that fully defines the top of the curve (i.e. a range that shows that a top plateau was achieved), and the top is within 25 percentage points of either the solvent control or the value for the lowest concentration of the estradiol standard for that run.

Table 7. Suggested Upper and Lower Limits for Parameters in Competitive Binding Assay Curves for the Standards (Radioinert Estradiol and 19-Norethindrone)

Parameter	Unit	Estradiol		Norethindrone	
		Lower Limit	Upper Limit	Lower Limit	Upper Limit
$Log_e(S_{yx})$	--	NA	2.35	NA	2.60
Bottom plateau level	% binding	-4	1	-5	1
Top plateau level	% binding	94	111	90	110
(Hill) Slope	$Log_{10}(M)^{-1}$	-1.1	-0.7	-1.1	-0.7

15. Proposed Statistical Methods

For each of the three valid independent runs of the competitive binding assays, the following statistics will be assessed; mean specific binding (%), standard deviation (SD), standard error of the mean (SEM), percent coefficient of variation (% CV), residuals, squared residuals, and the $Log_e(S_{yx})$ (ie. Log_e(residual standard deviation)) using XLfit (Guildford, Surrey, UK).

16. Classification Criteria

The classification of a substance as a binder or non-binder is made on the basis of the average results of three non-concurrent runs, each of which meet the performance criteria and taken together are consistent with each other. Each run is classified as

"interacting," "not interacting," "equivocal," or "equivocal up to the limit of the concentrations tested."

A run is classified as "interactive" with the ER if the lowest point on the fitted response curve within the range of the data is less than 50%. "Percent" refers to binding of the radiolabeled estradiol. Thus, "less than 50%" means that less than 50% of the radiolabeled estradiol is bound, or equivalently, that more than 50% of the radiolabeled estradiol has been displaced from the receptor. In other words, a run is classified as "interactive" if a log (IC_{50}) was obtained.

A run is classified as "equivocal up to the limit of concentrations tested" if there are no data points at or above a test substance concentration of 10^{-6} M and one of the two following conditions hold:

A binding curve can be fit but 50% or less of the radiolabeled estradiol is displaced by concentration 10^{-6} M.

OR

A binding curve cannot be fit and lowest average percent binding among the concentration groups in the data is above 50%.

A run is classified as "not interactive" if there are usable data points at or above 10^{-6} M and either:

The lowest point on the fitted response curve within the range of the data is above 75%.

OR

A binding curve cannot be fitted and the lowest average percent binding among the concentration groups in the data is above 75%.

A run is classified as "equivocal" if it falls in none of the categories above.

After each run is classified, the substance is classified by assigning the following values to each run and averaging across runs:

Interactive:	2
Equivocal:	1
Not Interactive:	0
Equivocal up to the limit of concentrations tested:	("missing")

Substance classification, based on the average of all the runs performed for a substance:

Interactive:	average ≥ 1.5
Equivocal:	0.5 ≤ average < 1.5
Not Interactive:	average < 0.5
Equivocal up to the limit of concentrations tested:	("missing")

For example, if a substance is tested in three runs in one lab and is determined to be interactive in 2 runs and equivocal in 1 run, to classify this substance one would average 2, 2, and 1 = ~1.67 and the substance would be considered interactive because the average is greater than 1.5.

17. Final Study Reports

The data to be reported 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, reference/test substance information (including but not limited to substance name, code, molecular weight, concentrations tested, notes regarding solubility), and data, data analysis and interpretation and classification of the test substances.

18. 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 the Sponsor's Representative. A copy of the protocol and all amendments will be issued to the Sponsor and originals will be placed in the study binder.

19. 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

20. Test Substance Disposition

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

Protocol Amendment 1

Study Number: 9070-100794ERB

Title of Study to be Amended: Estrogen Receptor Binding (Rat Uterine Cytosol)

Reason for Amendment to Protocol: This amendment is to correct numerous sections of the protocol that deal with the preparation and dilution scheme of the test substances and reference controls. The protocol states that the test substances and reference controls will be prepared at 50X stocks in vehicle (dimethyl sulfoxide [DMSO]) then diluted to 1X by adding 10 µL of the stock into a final volume of 500 µL. Due to solubility concerns, the stock test substances and reference controls will be prepared as 25X stocks in DMSO then diluted to 1X by adding 20 µL of the stock into a final volume of 500 µL. The final DMSO concentration, now 4%, is still well within the upper limit of 10% (OPPTS 890.1250 test guideline). Changes are indicated in bold red font.

Changes:

10.2 Preparation of Test Substance

The stock test substances will be formulated in dimethyl sulfoxide (DMSO) or appropriate solvent. Fresh **25X** dilutions of the stock solution will be prepared on the day of use such that the target concentration of test substance can be achieved by the addition of approximately **20 µL** of the dilution to approximately 500 µL total assay volume. Dose concentrations of test and control substances will not be verified using analytical methods.

Serial Dilutions of Test Substances

Serial dilutions of test substances will be prepared in the TEDG (Tris, EDTA, DTT, Glycerol) assay buffer to yield the final concentrations indicated in Table 2, unless solubility limits the top concentration tested.

Table 1. Test Substance Dilution Procedure

Tube #	Volume of stock to add for diluted concentration	Volume of TEDG assay buffer to add	Total volume of diluted test substance	Diluted test substance concentration	*Final test substance concentration in ER assay tube
TS1	Use 500 µl of stock test substance **(50 mM)**	500 µl	1 ml	**2.5×10^{-2} M**	1×10^{-3} M
TS2	Use 100 µl of dilution TS1 **(25 mM)**	900 µl	1 ml	**2.5×10^{-3} M**	1×10^{-4} M
TS3	Use 100 µl of dilution TS2 **(2.5 mM)**	900 µl	1 ml	**2.5×10^{-4} M**	1×10^{-5} M
TS4	Use 100 µl of dilution TS3 **(250 µM)**	900 µl	1 ml	**2.5×10^{-5} M**	1×10^{-6} M
TS5	Use 100 µl of dilution TS4 **(25 µM)**	900 µl	1 ml	**2.5×10^{-6} M**	1×10^{-7} M
TS6	Use 100 µl of dilution TS5 **(2.5 µM)**	900 µl	1 ml	**2.5×10^{-7} M**	1×10^{-8} M
TS7	Use 100 µl of dilution TS6 **(250 nM)**	900 µl	1 ml	**2.5×10^{-8} M**	1×10^{-9} M
TS8	Use 100 µl of dilution TS7 **(25 nM)**	900 µl	1 ml	**2.5×10^{-9} M**	1×10^{-10} M

*Final concentration of test substance in assay tube when **20 µl** of diluted concentration is used in a total volume of 500 µl.

10.3 ***Reference Substances***

When testing substances for their ability to bind to the estrogen receptor (ER), a solvent control, positive control (standard), negative control and a weak positive control will be included in each experiment. One set of standards is needed in each run on a given day.

A standard curve using 17β-estradiol (CAS 50-28-2; positive control) will be included to allow for an assessment of variability in the conduct of the experiment across time. The concentration range for 17β-estradiol will be 1×10^{-11} to 1×10^{-7} M. The supplier, catalog number, lot number and purity will be included in the final report.

Table 2. Example of Dilution Procedure for Reference Standard 17β-estradiol

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of 17β-estradiol	Diluted 17β-estradiol concentration	Final 17β-estradiol concentration in ER assay tube
NSB1	Use 100 µl of stock 17β-estradiol **(25 µM)**	900 µl	1 ml	**2.5×10^{-6} M**	1×10^{-7} M
S2	Use 100 µl of dilution NSB1 **(2.5 µM)**	900 µl	1 ml	**2.5×10^{-7} M**	1×10^{-8} M
S3	Use 277 µl of dilution S2 **(250 nM)**	600 µl	877 µl	**7.9×10^{-8} M**	3.16×10^{-9} M
S4	Use 100 µl of dilution S2 **(250 nM)**	900 µl	1 ml	**2.5×10^{-8} M**	1×10^{-9} M
S5	Use 100 µl of dilution S3 **(79 nM)**	900 µl	1 ml	**7.9×10^{-9} M**	3.16×10^{-10} M
S6	Use 100 µl of dilution S4 **(25 nM)**	900 µl	1 ml	**2.5×10^{-9} M**	1×10^{-10} M
S7	Use 100 µl of dilution S6 **(2.5 nM)**	900 µl	1 ml	**2.5×10^{-10} M**	1×10^{-11} M

The weak positive reference (19-norethindrone; CAS 68-22-4) will be included to demonstrate the sensitivity of each experiment and to allow an assessment of variability of the conduct of the experiment across time. The final concentration range tested for 19-norethindrone will be from 3.16×10^{-9} to 1×10^{-4} M. The supplier, catalog number, lot number and purity will be included in the final report.

Table 3. Example Dilution Procedure for 19-Norethindrone

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted weak positive control	Weak Positive Control Concentration	
				Diluted	Final in ER assay tube
P1	Use 400 µl of stock positive control **(5 mM)**	400 µl	800 µl	**2.5×10^{-3} M**	1×10^{-4} M
P2	Use 150 µl of stock positive control **(5 mM)**	800 µl	950 µl	**7.9×10^{-4} M**	3.16×10^{-5} M
P3	Use 100 µl of P2 **(790 µM)**	900 µl	1 ml	**7.9×10^{-5} M**	3.16×10^{-6} M
Intermed	Use 100 µl of P1 **(2.5 mM)**	900 µl	1 ml	**2.5×10^{-4} M**	Not used
P4	Use 100 µl of Intermed **(250 µM)**	900 µl	1 ml	**2.5×10^{-5} M**	1×10^{-6} M
P5	Use 100 µl of P3 **(79 µM)**	900 µl	1 ml	**7.9×10^{-6} M**	3.16×10^{-7} M
P6	Use 100 µl of P4 **(25 µM)**	900 µl	1 ml	**2.5×10^{-6} M**	1×10^{-7} M
P7	Use 100 µl of P5 **(7.9 µM)**	900 µl	1 ml	**7.9×10^{-7} M**	3.16×10^{-8} M
P8	Use 100 µl of P7 **(790 nM)**	900 µl	1 ml	**7.9×10^{-8} M**	3.16×10^{-9} M

A negative reference control (octyltriethoxysilane; CAS 2943-75-1) will be included to allow for an assessment of variability in the conduct of the experiment across time. The concentration range for the negative reference is 1×10^{-10} to 1×10^{-3} M. The supplier, catalog number, lot number and purity will be included in the final report.

Table 4. Example Dilution Procedure for Octyltriethoxysilane

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted negative control	Diluted negative control concentration	
				Diluted	Final in ER assay tube
NC1	Use 500 µl of stock NC **(50 mM)**	500 µl	1 ml	**2.5×10^{-2} M**	1×10^{-3} M
NC2	Use 100 µl of dilution NC1 **(25 mM)**	900 µl	1 ml	**2.5×10^{-3} M**	1×10^{-4} M
NC3	Use 100 µl of dilution NC2 **(2.5 mM)**	900 µl	1 ml	**2.5×10^{-4} M**	1×10^{-5} M
NC4	Use 100 µl of dilution NC3 **(250 µM)**	900 µl	1 ml	**2.5×10^{-5} M**	1×10^{-6} M
NC5	Use 100 µl of dilution NC4 **(25 µM)**	900 µl	1 ml	**2.5×10^{-6} M**	1×10^{-7} M
NC6	Use 100 µl of dilution NC5 **(2.5 µM)**	900 µl	1 ml	**2.5×10^{-7} M**	1×10^{-8} M
NC7	Use 100 µl of dilution NC6 **(250 nM)**	900 µl	1 ml	**2.5×10^{-8} M**	1×10^{-9} M
NC8	Use 100 µl of dilution NC7 **(25 nM)**	900 µl	1 ml	**2.5×10^{-9} M**	1×10^{-10} M

The Radioactive Ligand ([^{3}H]–17β-estradiol) supplier, catalog number and batch number will be included in the final report. The specific activity (SA) and date for which that SA was certified by the supplier will be included along with the concentration as received from the supplier (Ci/mmol) and the concentrations tested (nM).

11. Stock Solution Preparation

200 mM EDTA Stock Solution:

- For example, 7.444 g disodium EDTA will be added to 100 ml purified H_2O. The solution will be stored at approximately 4°C.

1M Tris Buffer:

- For example, 147.24 g Tris-HCl and 8.0 g Tris base will be added to 800 ml purified H_2O. The final volume will be brought to 1 Liter. The pH will be adjusted to approximately 7.4 and stored at approximately 4°C for up to 12 months.

Preparation of 2X TEG Buffer (20 mM Tris, 3 mM EDTA, 20% glycerol, pH ~7.4):

For example, to make 100 ml of 2X TEG Buffer, the following will be added in this order:

- 70 ml purified H_2O
- 2.0 ml 1M Tris Buffer
- 20 ml glycerol
- 1.5 ml 200 mM EDTA

The buffer will be cooled to approximately 4°C before the pH is adjusted to ~7.4, and the volume brought to 100 ml with purified H^2O and stored at approximately 4°C for up to 3 months.

Preparation of Working Assay Buffer (10 mM Tris, 1.5 mM EDTA (Ethylenediaminetetraacetic acid), 1 mM DTT(Dithiothreitol), 0.5% Protease Inhibitor (with PMSF)(v/v), 10% glycerol, pH 7.4) [TEDG + PI]:

This will be prepared daily as needed. For example to make 100 ml, the following will be added in this order:

- 50 ml 2X TEG buffer (prepared as above and cooled to approximately 4°C)
- 15.43 mg DTT (added immediately before use)
- 1.0 ml Protease Inhibitor added immediately before use
- Brought to 100 ml with ice cold purified H_2O.

Any unused 1X buffer will be discarded

Preparation of 60% hydroxyapatite (HAP) slurry:

- HAP will be mixed gently to resuspend and approximately 25 ml of slurry will be added to a 100 ml graduated cylinder for washing.
- TEDG + PI buffer will be added to a final volume of approximately 100 ml, the container capped, mixed by inversion and refrigerated for at least 2 hours.
- The supernatant will be aspirated or decanted and the HAP resuspended in fresh TEDG + PI buffer (~4°C) to 100 ml. This will be mixed gently. The HAP will be allowed to settle for ~2 hours at ~4°C and the wash step repeated.
- After the last wash, the HAP slurry will be left to settle overnight (at least 8 to 10 hours at ~4°C).
- On the next day (the day of use), the volume of HAP will be noted on the graduated cylinder. The supernatant will be aspirated or decanted and the HAP resuspended to a final volume of 60% HAP and 40% cold TEDG + PI. The HAP slurry should be well-suspended and ice-cold when used in the separation procedure.

Preparation of [^{3}H]-17β-estradiol Stock Solutions

- For example the original stock of [^{3}H]–17β-estradiol will be diluted to 50 nM (i.e., 5 X 10-^{8} M) by pipetting 1 µl of the stock solution for every specific activity unit (Ci/mmol) and diluting this to 20.0 ml with TEDG + PI. Store the [^{3}H]–R1881 stock solution and dilutions at approximately -20°C.
- A copy of the Certificate of Analysis for [^{3}H]–17β-estradiol will be maintained with the study records.

Preparation of 100X Radioinert 17β-estradiol Solutions

- A 50 mM solution of 17β-estradiol will be prepared in DMSO. For example, 5.00 mg of radioinert 17β-estradiol will be weighed in a tared amber vial and 367.1 µl solvent added. The 50 mM stock will be diluted 1:10 in DMSO to get 5 mM stock.
- The 500 µM radioinert 17β-estradiol stock will be prepared by diluting the 5 mM stock 1:10 in an amber vial. This will be the 500 µM radioinert 17β-estradiol stock.
- The 50 µM radioinert 17β-estradiol stock will be prepared by pipetting the 500 µM stock 1:10 in an amber vial. This will be the 50 µM radioinert 17β-estradiol stock.

12. Competitive Radioligand Binding Assay

The competitive binding assay will be performed a minimum of three times. The optimal amount of cytosolic protein added contains enough receptor to bind 10 - 15% of the radiolabeled estradiol that has been added to the tube.

Preparation of test substance stock solutions

Test substances will be prepared at **25X** the desired final concentration (listed in Table 2). Initial stocks will be prepared in TEDG assay buffer at a concentration of **25 mM (or the highest allowed by solubility)**.

Table 5. Summary of Assay Conditions

		Competitive Binding Assay Protocol
Source of receptor		Rat uterine cytosol
Concentration of radioligand		1 nM
Concentration of receptor		Sufficient to bind 10-15% of radioligand
Concentration of test substance (as serial dilutions)		100 pM to 1 mM
Temperature		~4°C
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH ~7.4)
	EDTA	1.5 mM
	Glycerol	10% (v/v)
	Protease Inhibitor	0.5% (v/v)
	DTT	1 mM

The specific activity (SA) of [^{3}H]–17β-estradiol will be adjusted for decay over time. The SA will be calculated on the day of the assay using the following equation:

$SA_{adjusted} = SA * e^{-Kdecay*Time}$

$SA_{adjusted}/SA$ = Fraction Isotope Remaining (FIR)

Where:

SA is the specific activity on the packaging date (both SA and the packaging date are printed on the stock bottle from the manufacturer).

K_{decay} is the decay constant for tritium (equal to 1.54×10^{-4}/day)

Time = number of days since the printer date on the stock bottle from the manufacturer

The [^{3}H]-17β-estradiol will be diluted with TEDG + PI buffer

A stock dilution in TEDG + PI buffer will be prepared.

To calculate the amount of stock [^{3}H]-17β-estradiol to add to the dilution (for example having a final concentration of 1 nM in 500 µl assay tube volume) the following steps will be used:

The SA from Ci/mmole will be converted to nM. If SA = X Ci/mmole, and Y = concentration of radiolabel, then X Ci/mmole is converted to nM and the SA activity adjusted for decay over time by the following conversion:

(Y mCi/ml / X Ci/mmole) * 1 Ci/1000 mCi * 10^6 nmole/mmole * 1000 ml/L = (Y/X) * 10^6 nM

A 50 nM diluted stock of the [^{3}H]-17β-estradiol will be prepared for a final concentration of 1 nM.

The 50 nM [^{3}H]-17β-estradiol will be kept on ice until standards, test substances, and assay tubes are prepared.

Assay Preparations

12 x 75 mm (or appropriately sized) siliconized glass tubes will be used for the assay. A master mixture of radioligand and buffer will be prepared to be used for the assay. An example is shown below; 155 tubes are required for a run that includes the solvent control, three standards, and three unknowns. Trace tubes are also required. Trace tubes are 50 µl TEDG Buffer + PI with diluted [3H]-17β-estradiol. The following table describes the example preparation of a master mixture for 155 tubes.

Table 6. Master Mixture for Competitive Binding Assay

Substance	Target Volume/Tube (µl)		# of Tubes		Total Volume Needed (ml)		Master Mix Volumes (ml)
	Assay Tubes	Trace Tubes	Assay Tubes	Trace Tubes	Assay Tubes	Trace Tubes	
TEDG Buffer + PI	**370**	48.72	155	6	**57.35**	0.292	59.192
Diluted [^{3}H]-17β-estradiol (50 nM)	10	1.28	155	6	1.55	0.008	1.558
Total	**380**	50			**58.9**	0.3	60.75

Individual Tubes

For the assay individual tubes, approximately **380 µl** of the master mixture above will be added to each assay tube and kept on ice. For the trace tubes, approximately 50 µl will be added directly to approximately 10 ml of scintillation fluid in scintillation vials and counted immediately. The standard, weak positive, negative and test substances will be prepared as described in section 10 and added to the assay tubes. **Twenty** microliters of **reference control and test** substance will be added by pipette per tube. After all substances have been added to the tubes, approximately 100 µl of cytosol will be added to each tube for a final volume of approximately 500 µl. Assay tubes will be vortexed after additions and incubated at approximately 4°C for approximately 16 to 20 hours on a rotary mixer.

Signatures

[redacted]

Chief, Toxicology Branch
National Toxicology Program, NIEHS

1/22/13
Date

[redacted]

Contract Office Technical Representative
National Toxicology Program, NIEHS

1/23/13
Date

[redacted]

Integrated Laboratory Systems, Inc
Study Monitor

22 Jan 2013
Date

[redacted]

Study Director

31 Jan 13
Date