Estrogen Receptor Binding (Rat Uterine Cytosol)

Final Report

DATA REQUIREMENT(S): OPPTS 890.1250 (2009)

AUTHOR(S): [redacted]

STUDY COMPLETION DATE: January 27, 2012

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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study Number: 9070-100107ERB

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

27 Jan 2012

Date

FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Title: Estrogen Receptor Binding (Rat Uterine Cytosol)

Study Number: 9070-100107ERB

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

Date(s) of Inspection/Audit	Inspection/Audit	Date(s) reported to Study Director	Date(s) reported to Management
27Jun11	Draft protocol audit	27Jun11	27Jun11
01Aug11 and 02Aug11	In-process assay audit	03Aug11	03Aug11
16Dec11	Data binder audit	16Dec11	16Dec11
27Jan12	Draft report audit	27Jan12	27Jan12

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

[redacted]

27 Jan 2012
Date

Quality Assurance Auditor
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GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
[redacted]	Study Director
	Director of Laboratory Operations
	Senior Scientist/Endocrine Group Leader
	Scientist
	Scientist
	Director of Project Management

Study Dates

Study initiation date: June 29, 2011
Experimental start date: July 23, 2011
Experimental termination date: August 04, 2011
Study termination date: January 27, 2012

Deviations from the Protocol

See Appendix 3. There were six deviations however they did not impact the integrity of the data in this report.

Other

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

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

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

The objective of this study was to evaluate the ability of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene to interact with the estrogen receptors (ERs) isolated from rat uteri.

Preliminary assessments of precipitation were conducted in order to identify a suitable top concentration of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene for use in the binding assays.

The final concentrations of the test articles 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 the first independent run (25-July-2011) and 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} for the second and third independent runs (01-August-2011 and 03-August-2011). The high concentration of each test article was lowered for the second and third independent runs from 10^{-3} M to 10^{-4} M because of precipitation at 10^{-3} M.

Three independent runs of the ER binding assay were conducted. 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 oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene for use in the binding assays was 10^{-4} M. There was precipitation observed with each test article at 10^{-3} M (every run) and with 10^{-4} M octocrylene in the third valid independent run.

In all three valid independent runs, the mean specific binding was > 84% for all concentrations of the negative control octyltriethoxysilane except for 10^{-3} M on 01-August-2011 and 03-August-2011, where the mean specific binding was 45.8% and 48.2%, respectively. We have observed this phenomenon at the highest concentration of octyltriethoxysilane before, though usually it is accompanied by precipitation (visual

assessment). Although precipitation was not specifically observed and recorded, the control and test substances are prepared at ambient room temperature, and the assay is performed at 4ºC, so precipitate could form and go undetected. The reference and test substances are added to the cytosol preparation containing ERs (an opaque protein slurry) making identification of precipitation difficult to assess after the compound is added. Additionally, it has been shown that when the competitive binding curve drops sharply over a single log increase in test substance concentration, as exhibited by octyltriethoxysilane, followup K_i assays show that the test substance is typically not a true competitive inhibitor (Laws et al, 2006).

In the first independent run (25-July-2011), the mean specific binding was > 75% at every soluble concentration tested for oxybenzone, octylmethoxycinnamate and octocrylene, classifying them as "non-interacting" for this run. The mean specific binding was 74.9% for octylsalate at 10^{-4} M classifying it as "equivocal" 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 -9.0 M.

In the second independent run (01-August-2011), the mean specific binding was > 75% at every concentration tested for oxybenzone, octylmethoxycinnamate and octocrylene, classifying them as "non-interacting" for this run. The mean specific binding was 68.7% for octylsalate at 10^{-4} M classifying it as "equivocal" 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 -9.0 M.

Finally, in the third independent run (03-August-2011), the mean specific binding was > 75% for octylmethoxycinnamate, octocrylene and oxybenzone, classifying them as "non-interacting" for this run. The mean specific binding was 69.7% for octylsalate at 10^{-4} M, classifying it as "equivocal" 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 -8.8 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

Oxybenzone, octylmethoxycinnamate and octocrylene were classified as "non-interacting" in all three independent runs and thus have a final classification of "non-interacting." Octylsalate was classified as "equivocal" in all three independent runs and thus has a final classification of "equivocal."

2.0 INTRODUCTION

2.1 Purpose

The objective of this study was to evaluate the ability of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene 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 system. Results of the Tier 1 screening battery, along with other scientifically relevant information, are to be used in a weight-of-evidence determination of a substance's potential to interact with these systems. The fact that a substance may interact with a hormone system does not mean that when the substance is used, it will cause adverse effects in humans or ecological systems. The Tier 1 battery is intended for screening purposes only and should not be used for endocrine classification or risk assessment.

2.2 Regulatory Citations

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 (25-July-2011, 01-August-2011 and 03-August-2011) only.

3.1 Test Substance

3.1.1 Test substance details

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

A certificate of analysis for the test substance is presented in Appendix 4.

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

A certificate of analysis for the test substance is presented in Appendix 4.

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

A certificate of analysis for the test substance is presented in Appendix 4.

Test Substance Name:	2-ethylhexyl-2-cyano-3,3-diphenylacrylate (Octocrylene)
Test Substance Manufacturer:	Sigma-Aldrich
CAS Number:	6197-30-4
Description:	Yellow viscous liquid
Solvent Used:	DMSO
Batch/Lot Number:	01697MJ
Expiry Date:	15-July-2011
Purity:	99.2%
Molecular Formula:	$C_{24}H_{27}NO_2$
Molecular Weight:	361.48
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 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 044K10.

The negative control octyltriethoxysilane (CAS# 2943-75-1) was purchased from Sigma-Aldrich (St. Louis, MO) and was 99.34% pure. The catalog number was 440213 and the lot number was 2499KK.

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 had a specific activity (SA) of 130.2 Ci/mmol on the certification date (06-May-2011). The $SA_{adjusted}$ was 128.6 Ci/mmol for the first independent run (25-July-2011), 128.5 Ci/mmol for the second independent run (01-August-2011) and 128.4 Ci/mmol for the third independent run (03-August-2011).

3.1.2 Vehicle selection

Dimethyl sulfoxide (DMSO) is one of the recommended solvents according to the EPA guideline (OPPTS 890.1250) and was selected as a suitable vehicle for oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene. Therefore, test article solutions with a concentration of up to 10^{-4} M (the limit concentration for the assay) can be 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 July 25, 2011 for use in the first independent run and on August 01, 2011 for use in the second and third independent runs. Oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene were prepared in DMSO on July 25, 2011 for use in the first independent run and prepared August 01, 2011 for use in the second and third 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 substance. 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 oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene were kept at approximately 2% final concentration. The dose concentrations of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene 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 oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene.

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
TC1	Use 500 µl of stock test chemical (100 mM)	500 µl	1 ml	5×10^{-2} M	1×10^{-3} M
TC2	Use 100 µl of dilution TC1 (50 mM)	900 µl	1 ml	5×10^{-3} M	1×10^{-4} M
TC3	Use 100 µl of dilution TC2 (5 mM)	900 µl	1 ml	5×10^{-4} M	1×10^{-5} M
TC4	Use 100 µl of dilution TC3 (500 µM)	900 µl	1 ml	5×10^{-5} M	1×10^{-6} M
TC5	Use 100 µl of dilution TC4 (50 µM)	900 µl	1 ml	5×10^{-6} M	1×10^{-7} M
TC6	Use 100 µl of dilution TC5 (5 µM)	900 µl	1 ml	5×10^{-7} M	1×10^{-8} M
TC7	Use 100 µl of dilution TC6 (500 nM)	900 µl	1 ml	5×10^{-8} M	1×10^{-9} M
TC8	Use 100 µl of dilution TC7 (50 nM)	900 µl	1 ml	5×10^{-9} M	1×10^{-10} M

*Final concentration of test chemical in assay tube when 10 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 (10 mM)	400 µl	800 µl	5 x 10^{-3} M	1 x 10^{-4} M
P2	Use 150 µl of stock positive control (10 mM)	800 µl	950 µl	1.58 x 10^{-3} M	3.16 x 10^{-5} M
P3	Use 100 µl of P2 (1.58 mM)	900 µl	1 ml	1.58 x 10^{-4} M	3.16 x 10^{-6} M
Intermed	Use 100 µl of P1 (5 mM)	900 µl	1 ml	5 x 10^{-4} M	Not used
P4	Use 100 µl of Intermed (500 µM)	900 µl	1 ml	5 x 10^{-5} M	1 x 10^{-6} M
P5	Use 100 µl of P3 (158 µM)	900 µl	1 ml	1.58 x 10^{-5} M	3.16 x 10^{-7} M
P6	Use 100 µl of P4 (50 µM)	900 µl	1 ml	5 x 10^{-6} M	1 x 10^{-7} M
P7	Use 100 µl of P5 (15.8 µM)	900 µl	1 ml	1.58 x 10^{-6} M	3.16 x 10^{-8} M
P8	Use 100 µl of P7 (1.58 µM)	900 µl	1 ml	1.58 x 10^{-7} M	3.16 x 10^{-9} M

The positive contol, 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 (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

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

Cytosol was collected, processed, and validated per EPA guideline and CeeTox SOP 2057 for use on this study. Related data was 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 4±2°C. A 1 M Tris buffer was prepared and the pH was adjusted to 7.4. The buffer can be stored at 4±2°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 4±2°C before adjusting to pH 7.4 and stored at 4±2°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 4±2°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 (4±2°C). The slurry was mixed gently and allowed to settle for approximately 2 hours at 4±2°C. After the second wash, the HAP slurry settled overnight (at least 8 to 10 hours at 4±2°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±2°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 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 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	380	48.72	155	6	58.9	0.292	59.192
Diluted [3H]-17β-estradiol (50 nM)	10	1.28	155	6	1.55	0.008	1.558
Total	390	50			60.45	0.3	60.75

3.5.4 Individual Tubes

For the assay tubes, 390 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 14 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. Ten µl 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 4±2°C prior to the addition of the cytosol. The assay tubes were vortexed after additions and incubated at 4±2°C for 16 to 20 hours on a rotator.

Competitive Binding Assay Additions

Volume (µl)	Component
10	Unlabeled 17β-estradiol, weak positive control, negative control, or test substance
390	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 (4±2°C) TEDG + PI buffer was added, quickly vortexed, and centrifuged at 4±2°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 4±2°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 14 ml scintillation cocktail (Perkin Elmer Opti-Fluor, cat# 6013199, lot# 47-11091). 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

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:	$0.5 \leq$ 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 3.2. The final concentrations of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene to assess precipitation were 10^{-5}, 10^{-4} and 10^{-3} M.

The suitable top concentration of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene for use in the binding assays was 10^{-4} M and the final concentrations of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene in the binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M for the first independent run and 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M for the second and third independent runs.

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 3.6.1 with the following exceptions:

- In the second run of the assay, the top plateau level for 17β-estradiol was marginally greater than the specified range (top plateau level = 93%; compared to the specified range of 94% ~ 111%)
- In the second run of the assay, the bottom plateau level for 19-norethindrone was less than the specified range (bottom plateau level = -8%; compared to the specified range of -5% ~ 1%)
- In the third run of the assay, the bottom plateau level for 17β-estradiol was marginally less than the specified range (bottom plateau level = -5%; compared to the specified range of -4% ~ 1%)

These deviations were minor and not considered to reflect true deviation from the suggested ranges outlined in the OPPTS guideline. Therefore, both independent runs of the assay were considered to have met the assay acceptance criteria and were considered to be definitive.

No data were excluded from either evaluation or interpretation due to excessive precipitation with addition of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene in any independent run of the assay.

4.3 Results

The suitable top concentration of oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene for use in the binding assays was 10^{-4} M. There was precipitation observed with each test article at 10^{-3} M (every run) and with 10^{-4} M octocrylene in the third valid independent run.

In all three valid independent runs, the mean specific binding was > 84% for all concentrations of the negative control octyltriethoxysilane except for 10^{-3} M on 01-August-2011 and 03-August-2011, where the mean specific binding was 45.8% and 48.2%, respectively. We have observed this phenomenon at the highest concentration of octyltriethoxysilane before, though usually it is accompanied by precipitation (visual assessment). Although precipitation was not specifically observed and recorded, the control and test substances are prepared at ambient room temperature, and the assay is performed at 4ºC, so precipitate could form and go undetected. The reference and test substances are added to the cytosol preparation containing ERs (an opaque protein slurry) making identification of precipitation difficult to assess after the compound is added. Additionally, it has been shown that when the competitive binding curve drops sharply over a single log increase in test substance concentration, as exhibited by octyltriethoxysilane, followup K_i assays show that the test substance is typically not a true competitive inhibitor (Laws et al, 2006).

In the first independent run (25-July-2011), the mean specific binding was > 75% at every soluble concentration tested for oxybenzone, octylmethoxycinnamate and octocrylene, classifying them as "non-interacting" for this run. The mean specific binding was 74.9% for octylsalate at 10^{-4} M classifying it as "equivocal" 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 -9.0 M.

In the second independent run (01-August-2011), the mean specific binding was > 75% at every concentration tested for oxybenzone, octylmethoxycinnamate and octocrylene, classifying them as "non-interacting" for this run. The mean specific binding was 68.7% for octylsalate at 10^{-4} M classifying it as "equivocal" 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 -9.0 M.

Finally, in the third independent run (03-August-2011), the mean specific binding was > 75% for octylmethoxycinnamate, octocrylene and oxybenzone, classifying them as "non-interacting" for this run. The mean specific binding was 69.7% for octylsalate at 10^{-4} M, classifying it as "equivocal" 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 -8.8 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

Oxybenzone, octylmethoxycinnamate and octocrylene were classified as "non-interacting" in all three independent runs and thus have a final classification of "non-interacting." Octylsalate was classified as "equivocal" in all three independent runs and thus has a final classification of "equivocal."

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.

Laws, S.C., Yavanhxay, S., Cooper, R.L. and Eldridge, J.C. (2006) Nature of the Binding Interaction for 50 Structurally Diverse Chemicals with Rat Estrogen Receptors. *Toxicological Sciences* **94**(1), 46-56.

TABLES SECTION

TABLE 1 Results of 1st Valid Binding Assay – Controls – July 25, 2011

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Estradiol (NSB)	-7	0.0	0.7	0.4	3.2E+17
	-8	7.7	0.3	0.2	4.0
	-8.5	24.9	1.1	0.6	4.3
	-9	47.7	2.3	1.3	4.8
	-9.5	75.4	1.8	1.0	2.3
	-10	89.3	4.1	2.3	4.6
	-11	92.8	1.3	0.7	1.4
19-Norethindrone	-4	0.9	0.2	0.1	21.6
	-4.5	6.7	0.4	0.2	5.9
	-5.5	44.0	2.0	1.1	4.5
	-6	72.1	0.4	0.2	0.5
	-6.5	84.2	2.1	1.2	2.5
	-7	90.7	1.4	0.8	1.5
	-7.5	94.0	5.9	3.4	6.3
	-8.5	92.2	1.9	1.1	2.0
Octyltriethoxysilane	-3	84.9	1.8	1.1	2.2
	-4	95.5	3.3	1.9	3.5
	-5	95.6	0.5	0.3	0.5
	-6	94.0	3.1	1.8	3.3
	-7	96.3	3.2	1.8	3.3
	-8	94.7	1.7	1.0	1.8
	-9	98.0	3.2	1.8	3.2
	-10	96.6	5.9	3.4	6.1

TABLE 2 Results of 1st Valid Binding Assay – Test Articles – July 25, 2011

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Oxybenzone	-3	70.0	13.5	7.8	19.3
	-4	83.2	1.6	0.9	2.0
	-5	96.0	2.2	1.3	2.3
	-6	95.1	3.1	1.8	3.2
	-7	95.3	2.8	1.6	2.9
	-8	95.8	3.0	1.8	3.2
	-9	94.9	5.8	3.3	6.1
	-10	95.0	4.8	2.8	5.1
Octyl-methoxycinnamate	-3	84.0	3.1	1.8	3.7
	-4	89.2	5.0	2.9	5.6
	-5	90.7	7.0	4.1	7.8
	-6	98.3	3.4	2.0	3.4
	-7	97.8	2.4	1.4	2.5
	-8	97.5	1.6	0.9	1.7
	-9	97.0	1.6	0.9	1.6
	-10	97.0	1.8	1.0	1.8
Octylsalate	-3	60.3	1.2	0.7	1.9
	-4	74.9	3.8	2.2	5.1
	-5	88.8	0.8	0.4	0.9
	-6	90.7	0.5	0.3	0.6
	-7	93.2	1.8	1.0	1.9
	-8	96.2	2.5	1.4	2.6
	-9	92.4	0.4	0.2	0.4
	-10	91.8	0.2	0.1	0.2
Octocrylene	-3	90.7	2.2	1.3	2.5
	-4	102.4	2.0	1.1	1.9
	-5	103.9	1.2	0.7	1.2
	-6	101.7	1.1	0.7	1.1
	-7	130.9	56.8	32.8	43.3
	-8	103.4	1.4	0.8	1.4
	-9	97.0	4.7	2.7	4.9
	-10	94.1	2.0	1.2	2.1

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 – July 25, 2011

Parameter	Unit	17β-estradiol	19-norethindrone
$Log_e(S_{yx})$	--	0.84	0.89
Bottom Plateau Level	% binding	0	-1
Top Plateau Level	% binding	95	93
Hill Slope	$Log_{10}(M)^{-1}$	-1.1	-1.1

TABLE 4 Results of 2nd Valid Binding Assay – Controls – August 01, 2011

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Estradiol (NSB)	-7	0.0	0.2	0.1	2.0E+17
	-8	7.7	0.5	0.3	6.3
	-8.5	26.2	2.1	1.2	7.9
	-9	44.4	0.8	0.4	1.7
	-9.5	70.8	1.9	1.1	2.7
	-10	85.5	4.7	2.7	5.5
	-11	90.2	4.3	2.5	4.8
19-Norethindrone	-4	0.8	0.1	0.1	12.5
	-4.5	6.3	1.0	0.6	16.1
	-5.5	43.4	3.3	1.9	7.7
	-6	65.1	4.0	2.3	6.2
	-6.5	79.2	3.3	1.9	4.2
	-7	88.7	3.8	2.2	4.3
	-7.5	90.1	0.8	0.5	0.9
	-8.5	98.4	1.7	1.0	1.7
Octyltriethoxysilane	-3	45.8	0.2	0.1	0.5
	-4	88.5	2.5	1.4	2.8
	-5	98.4	1.1	0.6	1.1
	-6	92.3	4.3	2.5	4.6
	-7	89.9	2.0	1.1	2.2
	-8	87.3	2.3	1.3	2.6
	-9	93.0	3.0	1.7	3.2
	-10	89.9	1.4	0.8	1.5

TABLE 5 Results of 2nd Valid Binding Assay – Test Articles – August 01, 2011

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Oxybenzone	-4	76.4	0.9	0.5	1.2
	-5	93.4	2.0	1.2	2.2
	-6	91.2	0.5	0.3	0.5
	-7	95.5	4.4	2.5	4.6
	-8	95.9	5.1	2.9	5.3
	-9	101.2	3.2	1.8	3.1
	-10	97.1	3.0	1.7	3.0
	-11	96.6	2.3	1.3	2.4
Octyl-methoxycinnamate	-4	93.4	1.4	0.8	1.5
	-5	97.3	0.4	0.2	0.4
	-6	94.2	0.8	0.5	0.9
	-7	89.4	5.9	3.4	6.6
	-8	91.0	1.4	0.8	1.5
	-9	89.5	1.9	1.1	2.2
	-10	90.9	0.4	0.2	0.5
	-11	90.1	2.5	1.5	2.8
Octylsalate	-4	68.7	0.5	0.3	0.8
	-5	83.2	2.2	1.3	2.7
	-6	88.8	2.9	1.6	3.2
	-7	91.2	2.3	1.3	2.5
	-8	91.3	1.5	0.8	1.6
	-9	89.6	6.0	3.5	6.7
	-10	89.2	4.8	2.8	5.4
	-11	89.0	1.0	0.6	1.2
Octocrylene	-4	81.6	2.0	1.2	2.4
	-5	90.3	2.4	1.4	2.7
	-6	92.4	2.1	1.2	2.2
	-7	92.1	2.8	1.6	3.0
	-8	93.0	1.3	0.7	1.4
	-9	91.8	2.5	1.5	2.7
	-10	93.8	4.8	2.7	5.1
	-11	92.7	1.8	1.0	1.9

TABLE 6 Results of 2nd Valid Binding Assay - Upper and Lower Parameters in Competitive Assay Binding Curves for the Standards – August 01, 2011

Parameter	Unit	17β-estradiol	19-norethindrone
$Log_e(S_{yx})$	--	1.14	1.08
Bottom Plateau Level	% binding	-1	-8
Top Plateau Level	% binding	93	97
Hill Slope	$Log_{10}(M)^{-1}$	-1.0	-0.7

TABLE 7 Results of 3rd Valid Binding Assay - Controls – August 03, 2011

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Estradiol (NSB)	-7	0.0	0.1	0.1	N/A
	-8	9.3	1.3	0.8	14.3
	-8.5	35.3	5.7	3.3	16.0
	-9	56.9	8.1	4.7	14.3
	-9.5	73.1	2.9	1.7	4.0
	-10	85.9	3.0	1.7	3.5
	-11	95.5	2.9	1.7	3.0
19-Norethindrone	-4	1.6	1.0	0.6	62.1
	-4.5	6.9	0.7	0.4	9.7
	-5.5	41.7	1.3	0.7	3.0
	-6	68.8	0.7	0.4	1.1
	-6.5	87.3	2.6	1.5	3.0
	-7	94.2	5.1	2.9	5.4
	-7.5	99.1	2.0	1.2	2.1
	-8.5	100.3	1.6	0.9	1.6
Octyltriethoxysilane	-3	48.2	2.6	1.5	5.4
	-4	90.9	2.8	1.6	3.1
	-5	101.4	2.0	1.2	2.0
	-6	105.1	1.7	1.0	1.6
	-7	101.7	3.0	1.8	3.0
	-8	98.1	0.4	0.2	0.4
	-9	93.9	2.1	1.2	2.2
	-10	93.3	1.5	0.9	1.7

TABLE 8 Results of 3rd Valid Binding Assay – Test Articles – August 03, 2011

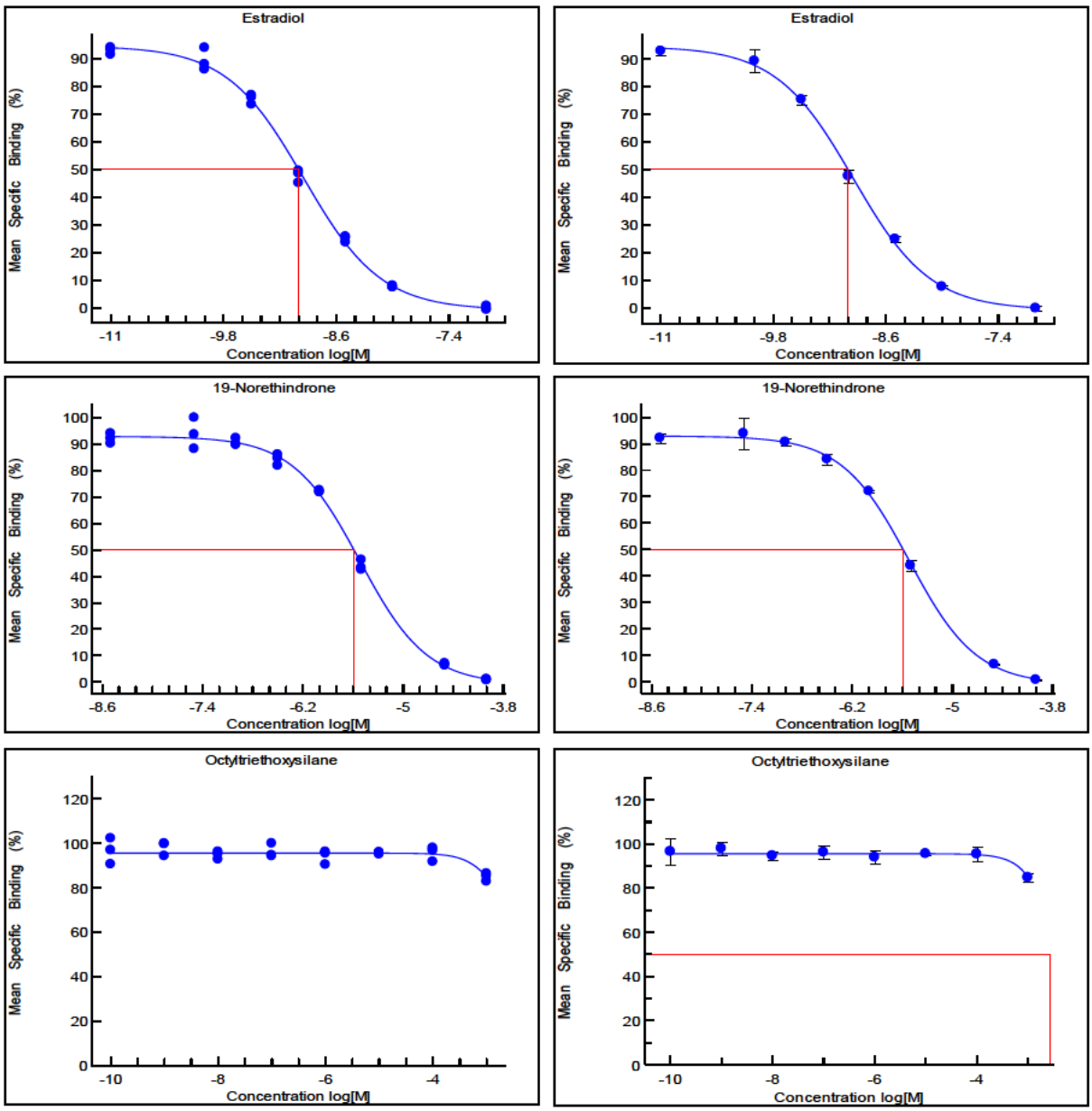
Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Oxybenzone	-4	77.2	0.7	0.4	0.9
	-5	97.7	0.9	0.5	1.0
	-6	98.1	2.0	1.2	2.0
	-7	98.3	3.1	1.8	3.2
	-8	100.2	1.6	0.9	1.6
	-9	97.6	1.0	0.6	1.0
	-10	99.7	2.9	1.6	2.9
	-11	97.5	1.0	0.6	1.1
Octyl-methoxycinnamate	-4	93.1	3.6	2.1	3.9
	-5	101.2	0.5	0.3	0.5
	-6	101.9	2.7	1.6	2.7
	-7	98.8	0.7	0.4	0.8
	-8	93.7	2.5	1.4	2.7
	-9	96.8	0.8	0.5	0.8
	-10	97.7	2.0	1.1	2.0
	-11	100.6	2.1	1.2	2.1
Octylsalate	-4	69.7	2.9	1.7	4.2
	-5	89.9	2.7	1.6	3.0
	-6	95.5	1.6	0.9	1.7
	-7	97.0	3.2	1.9	3.3
	-8	96.8	4.3	2.5	4.5
	-9	92.9	3.0	1.7	3.2
	-10	96.1	1.6	0.9	1.7
	-11	95.6	4.9	2.8	5.1
Octocrylene	-4	83.3	8.1	4.7	9.7
	-5	94.4	3.1	1.8	3.3
	-6	101.1	5.3	3.1	5.3
	-7	101.2	0.9	0.5	0.9
	-8	102.9	2.2	1.3	2.1
	-9	103.9	0.6	0.3	0.6
	-10	100.4	3.1	1.8	3.1
	-11	95.5	0.9	0.5	0.9

TABLE 9 Results of 3rd Valid Binding Assay - Upper and Lower Parameters in Competitive Assay Binding Curves for the Standards – August 03, 2011

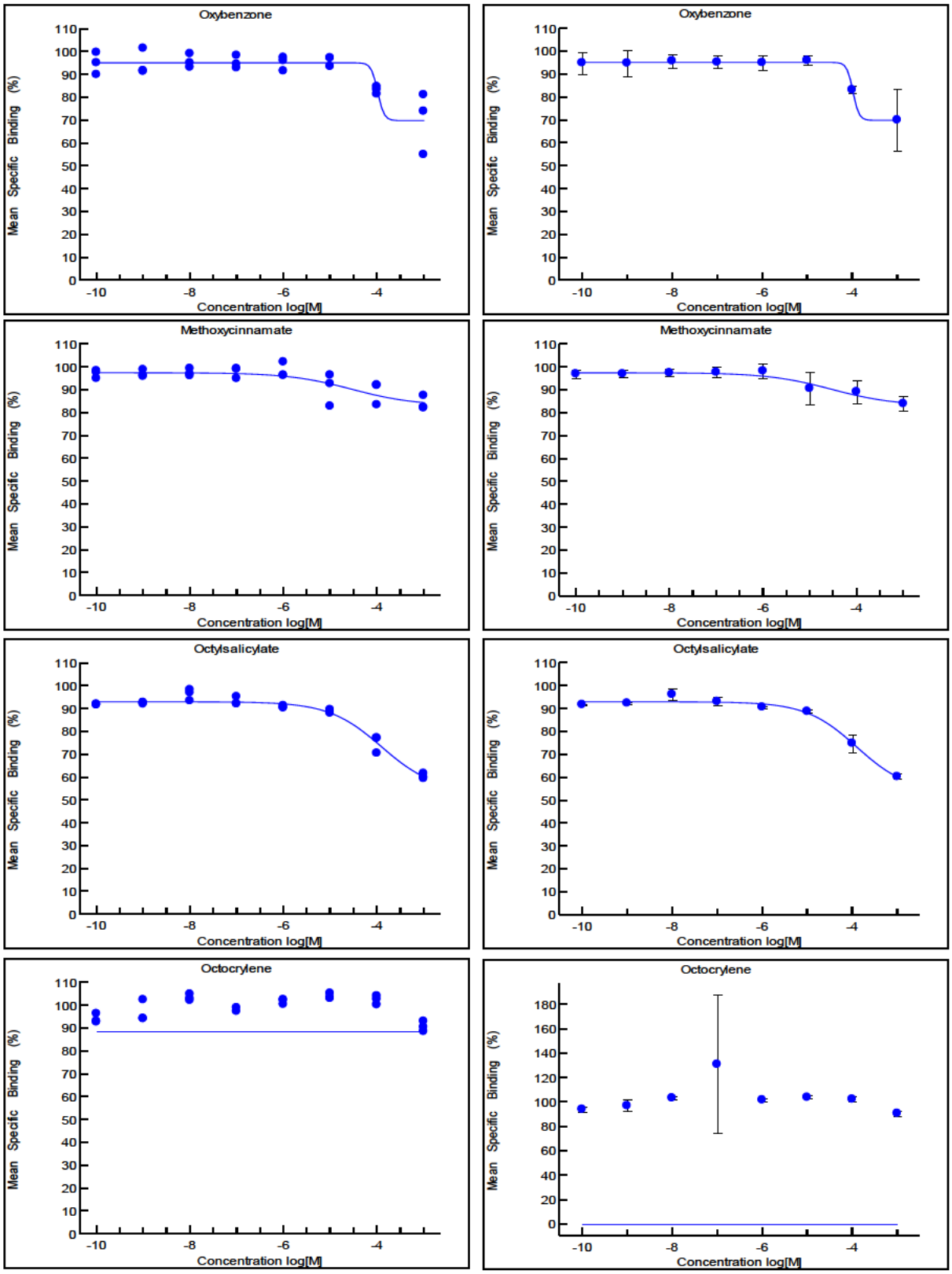
Parameter	Unit	17β-estradiol	19-norethindrone
$Log_e(S_{yx})$	--	1.54	0.76
Bottom Plateau Level	% binding	-5	-1
Top Plateau Level	% binding	96	100
Hill Slope	$Log_{10}(M)^{-1}$	-0.8	-0.9

FIGURES SECTION

FIGURE 1 Specific Binding for 1st Valid Run - Controls and Test Articles – July 25, 2011

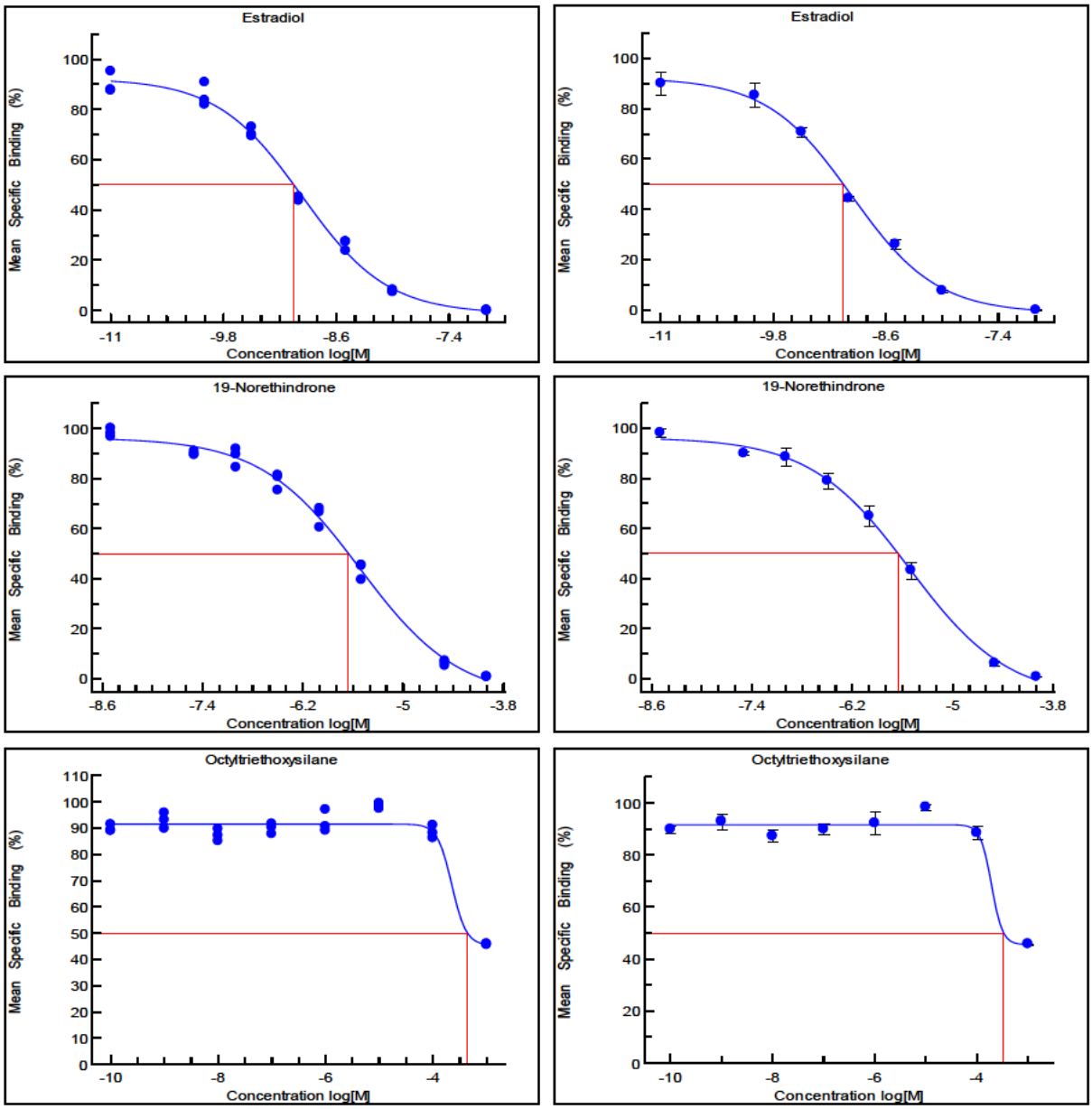


The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the first independent run of the assay (n=3).

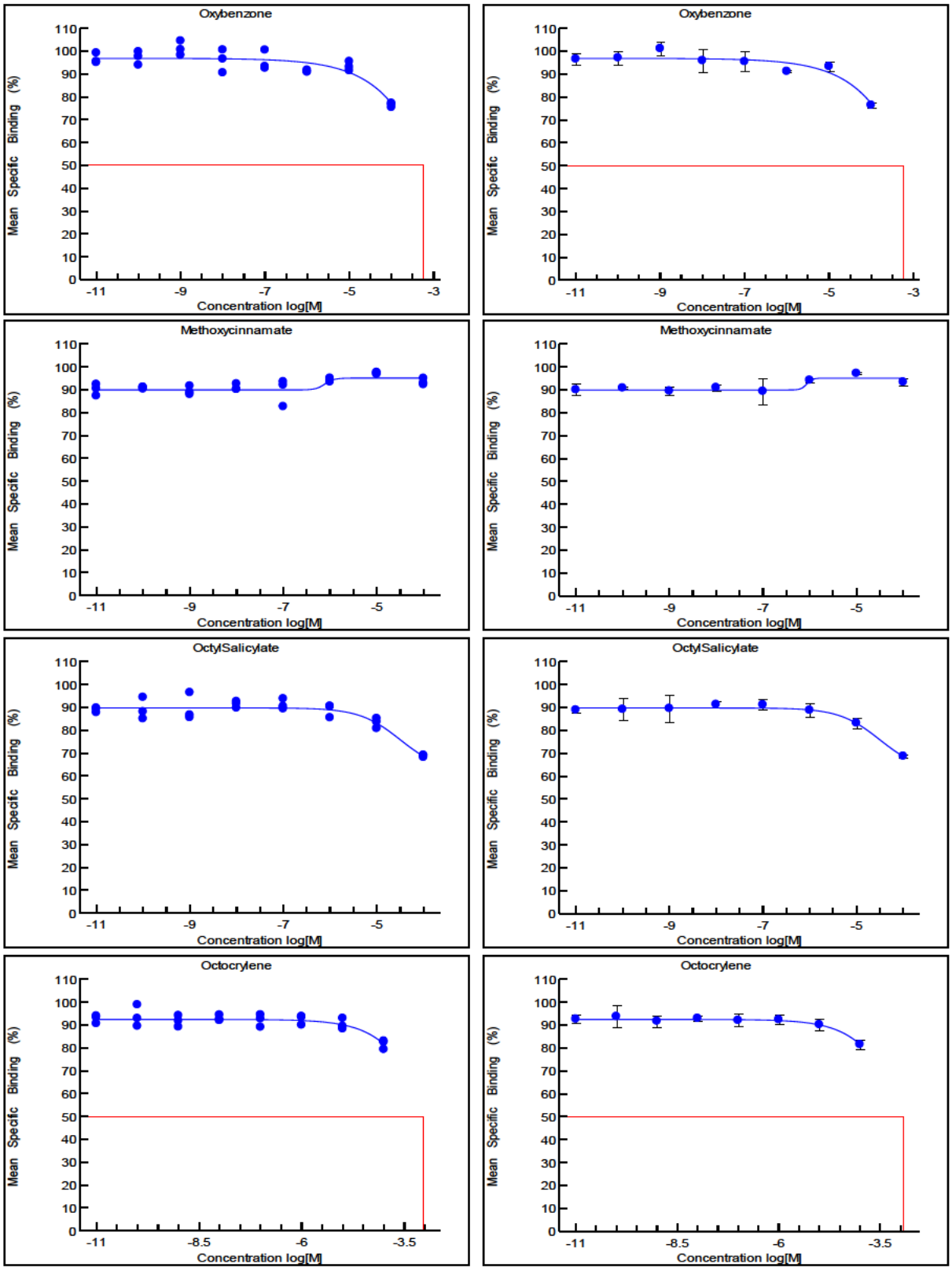


The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the first independent run of the assay (n=3).

FIGURE 2 Specific Binding for 2nd Valid Run - Controls and Test Articles – August 01, 2011

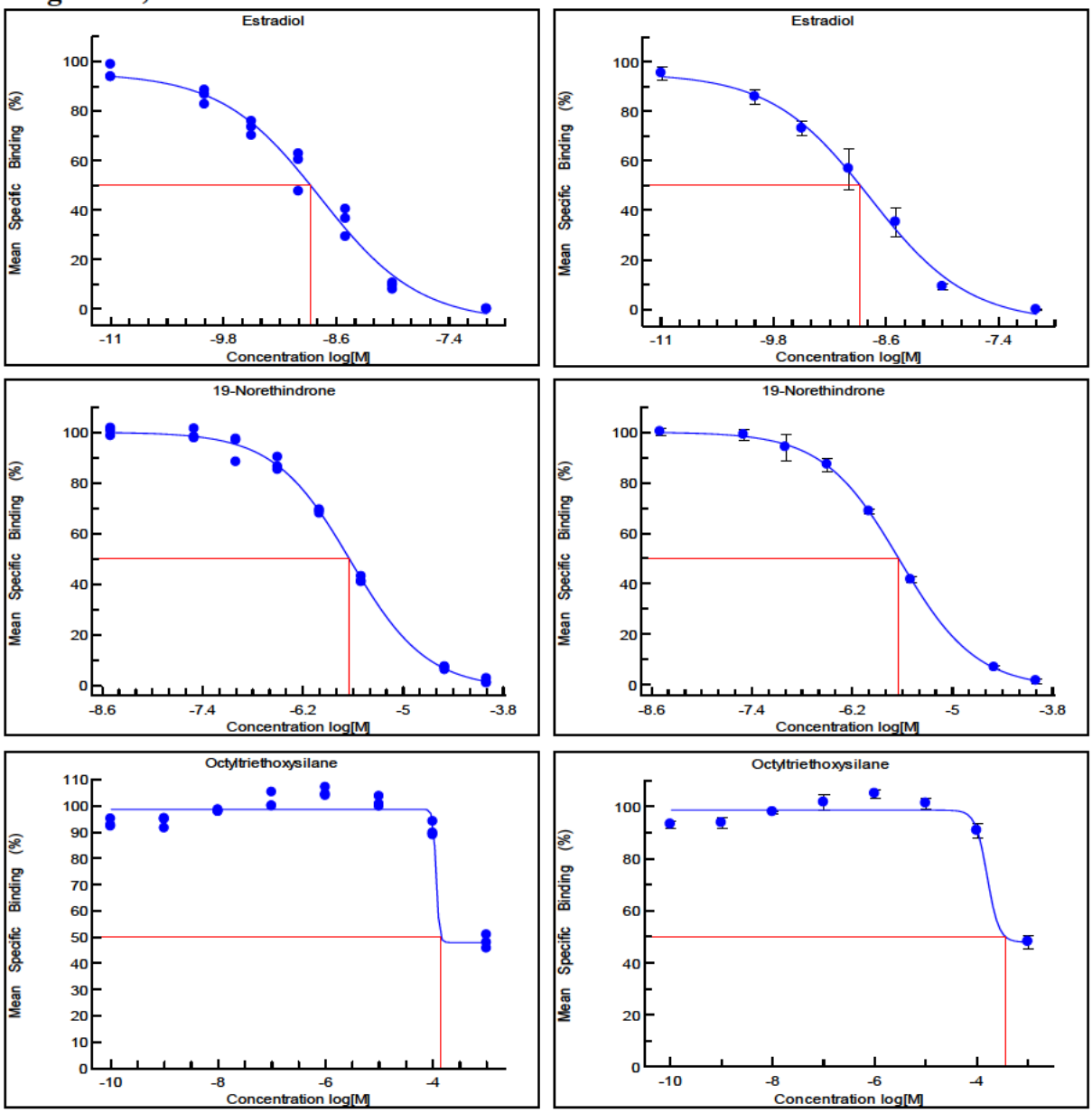


The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the second independent run of the assay (n=3).

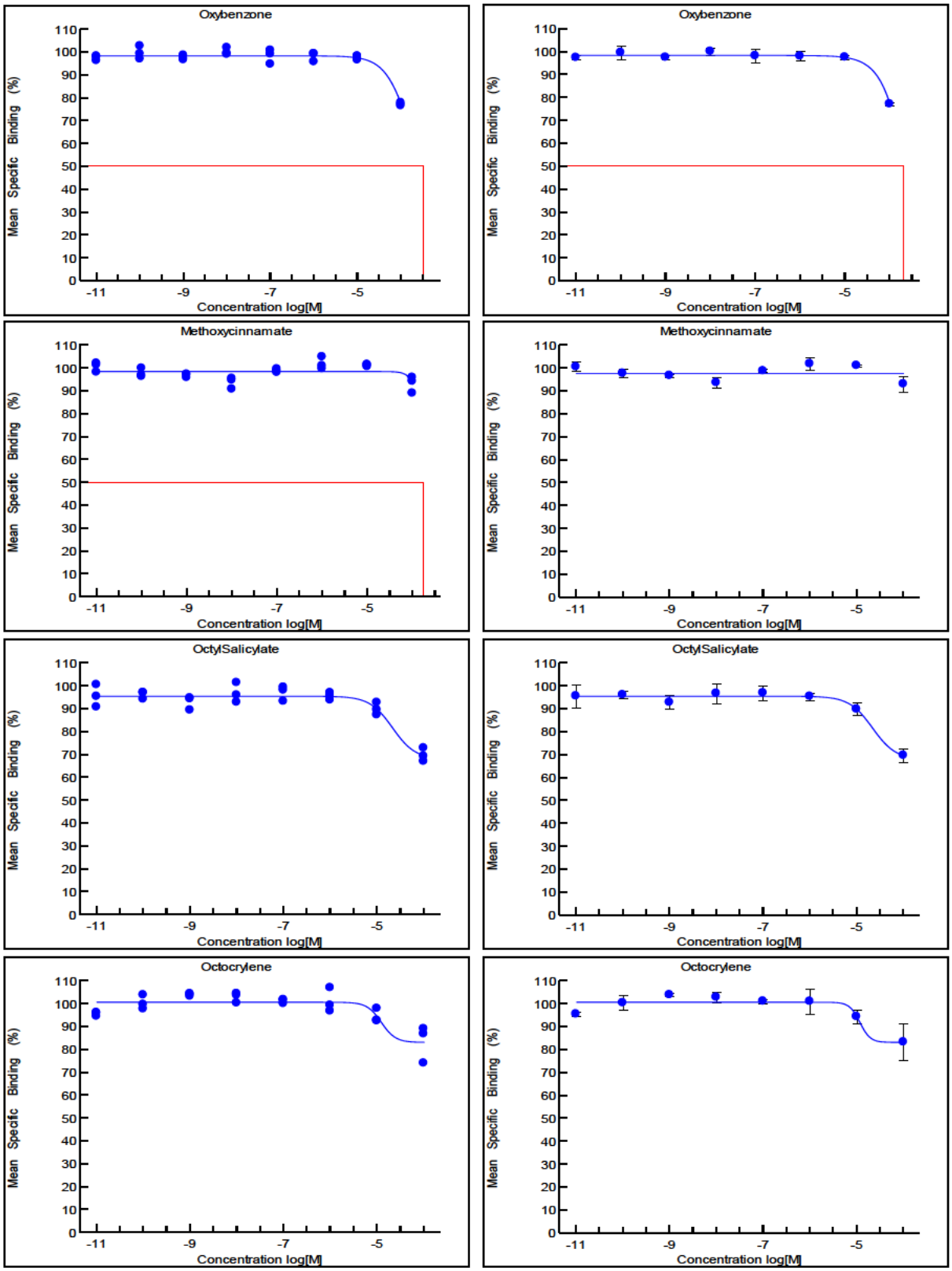


The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the third independent run of the assay (n=3).

FIGURE 3 Specific Binding for 3rd Valid Run - Controls and Test Articles – August 03, 2011



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

APPENDICES SECTION

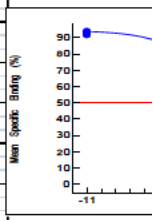
APPENDIX 1 Raw and Normalized Data 1st Valid Run – July 25, 2011

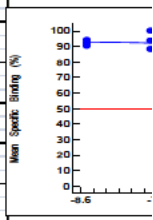
Experiment Date:	25-Jul-11	Study Number:	9070-100107ERB	Assays Conducted by:	[redacted]
Test substance:	Oxybenzone				
12/6/2011 17:42					

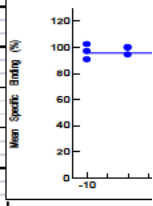
10 uL of 50 nM E2=	___	DPM and 0.5 x10-12 moles
Therefore there are	___	DPM/mole
ug protein/assay tube =	80.0	

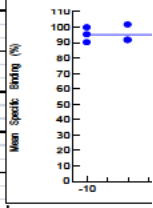
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17709	–	138130.2	138465.6
2		17865	–	139347.0	
3		17769	–	138598.2	
4		17719	–	138208.2	
5		17718	–	138200.4	
6		17732	–	138309.6	
7	Total Binding (Solvent Control)	22497	21933.3	32900.0	32687.5
8		22817	22253.3	33380.0	
9		21752	21188.3	31782.5	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
513.0	10	Estradiol (NSB)	-7	-50.7	-76.0	-0.2	-0.6	0.3	0.0	0.7	0.4	3.2E+17	0.6
439.0	11		-7	-124.7	-187.0	-0.6	-0.9	0.9					0.5
739.0	12		-7	175.3	263.0	0.8	0.5	0.2					0.8
2328.0	13	Estradiol	-8	1764.3	2646.5	8.1	0.1	0.0	7.7	0.3	0.2	4.0	2.5
2216.0	14		-8	1652.3	2478.5	7.6	-0.4	0.2					2.4
2206.0	15		-8	1642.3	2463.5	7.5	-0.5	0.2					2.4
5737.0	16	Estradiol	-8.5	5173.3	7760.0	23.7	0.6	0.3	24.9	1.1	0.6	4.3	6.2
6080.0	17		-8.5	5516.3	8274.5	25.3	2.1	4.6					6.6
6180.0	18		-8.5	5616.3	8424.5	25.8	2.6	6.7					6.7
10397.0	19	Estradiol	-9	9833.3	14750.0	45.1	-4.6	21.2	47.7	2.3	1.3	4.8	11.3
11152.0	20		-9	10588.3	15882.5	48.6	-1.1	1.3					12.1
11345.0	21		-9	10781.3	16172.0	49.5	-0.3	0.1					12.3
17285.0	22	Estradiol	-9.5	16721.3	25082.0	76.7	2.0	3.9	75.4	1.8	1.0	2.3	18.7
16557.0	23		-9.5	15993.3	23990.0	73.4	-1.4	1.8					17.9
17119.0	24		-9.5	16555.3	24833.0	76.0	1.2	1.5					18.5
21014.0	25	Estradiol	-10	20450.3	30675.5	93.8	6.1	36.8	89.3	4.1	2.3	4.6	22.8
19720.0	26		-10	19156.3	28734.5	87.9	0.1	0.0					21.4
19318.0	27		-10	18754.3	28131.5	86.1	-1.7	2.9					20.9
21033.0	28	Estradiol	-11	20469.3	30704.0	93.9	-0.1	0.0	92.8	1.3	0.7	1.4	22.8
20859.0	29		-11	20295.3	30443.0	93.1	-0.9	0.9					22.6
20483.0	30		-11	19919.3	29879.0	91.4	-2.7	7.0					22.2
805.0	31	19-Norethindrone	-4	241.3	362.0	1.1	-0.2	0.1	0.9	0.2	0.1	21.6	0.9
722.0	32		-4	158.3	237.5	0.7	-0.6	0.4					0.8
750.0	33		-4	186.3	279.5	0.9	-0.5	0.3					0.8
2032.0	34	19-Norethindrone	-4.5	1468.3	2202.5	6.7	0.7	0.4	6.7	0.4	0.2	5.9	2.2
2109.0	35		-4.5	1545.3	2318.0	7.1	1.0	1.0					2.3
1937.0	36		-4.5	1373.3	2060.0	6.3	0.2	0.1					2.1
9984.0	37	19-Norethindrone	-5.5	9420.3	14130.5	43.2	-1.4	1.9	44.0	2.0	1.1	4.5	10.8
10634.0	38		-5.5	10070.3	15105.5	46.2	1.6	2.6					11.5
9825.0	39		-5.5	9261.3	13892.0	42.5	-2.1	4.4					10.6
16245.0	40	19-Norethindrone	-6	15681.3	23522.0	72.0	1.0	1.0	72.1	0.4	0.2	0.5	17.6
16378.0	41		-6	15814.3	23721.5	72.6	1.6	2.6					17.7
16223.0	42		-6	15659.3	23489.0	71.9	0.9	0.8					17.6
18403.0	43	19-Norethindrone	-6.5	17839.3	26759.0	81.9	-3.6	12.7	84.2	2.1	1.2	2.5	19.9
19313.0	44		-6.5	18749.3	28124.0	86.0	0.6	0.4					20.9
19010.0	45		-6.5	18446.3	27669.5	84.6	-0.8	0.6					20.6
20117.0	46	19-Norethindrone	-7	19553.3	29330.0	89.7	-1.0	1.1	90.7	1.4	0.8	1.5	21.8
20187.0	47		-7	19623.3	29435.0	90.0	-0.7	0.5					21.9
20664.0	48		-7	20100.3	30150.5	92.2	1.5	2.2					22.4
22360.0	49	19-Norethindrone	-7.5	21796.3	32694.5	100.0	7.6	57.7	94.0	5.9	3.4	6.3	24.2
20967.0	50		-7.5	20403.3	30605.0	93.6	1.2	1.4					22.7
19786.0	51		-7.5	19222.3	28833.5	88.2	-4.2	17.8					21.4
20235.0	52	19-Norethindrone	-8.5	19671.3	29507.0	90.3	-2.8	7.8	92.2	1.9	1.1	2.0	21.9
21057.0	53		-8.5	20493.3	30740.0	94.0	1.0	1.0					22.8
20647.0	54		-8.5	20083.3	30125.0	92.2	-0.9	0.8					22.4
19400.0	55	Octyltriethoxysilane	-3	18836.3	28254.5	86.4	1.6	2.5	84.9	1.8	1.1	2.2	21.0
19152.0	56		-3	18588.3	27882.5	85.3	0.4	0.2					20.7
18614.0	57		-3	18050.3	27075.5	82.8	-2.0	4.1					20.2
20554.0	58	Octyltriethoxysilane	-4	19990.3	29985.5	91.7	-3.7	13.8	95.5	3.3	1.9	3.5	22.3
21637.0	59		-4	21073.3	31610.0	96.7	1.3	1.6					23.4
21914.0	60		-4	21350.3	32025.5	98.0	2.5	6.4					23.7
21469.0	61	Octyltriethoxysilane	-5	20905.3	31358.0	95.9	0.1	0.0	95.6	0.5	0.3	0.5	23.3
21281.0	62		-5	20717.3	31076.0	95.1	-0.8	0.6					23.1
21463.0	63		-5	20899.3	31349.0	95.9	0.0	0.0					23.3
21521.0	64	Octyltriethoxysilane	-6	20957.3	31436.0	96.2	0.3	0.1	94.0	3.1	1.8	3.3	23.3
21370.0	65		-6	20806.3	31209.5	95.5	-0.4	0.2					23.2
20276.0	66		-6	19712.3	29568.5	90.5	-5.4	29.5					22.0
22349.0	67	Octyltriethoxysilane	-7	21785.3	32678.0	100.0	4.1	16.7	96.3	3.2	1.8	3.3	24.2
21196.0	68		-7	20632.3	30948.5	94.7	-1.2	1.5					23.0
21110.0	69		-7	20546.3	30819.5	94.3	-1.6	2.6					22.9
21533.0	70	Octyltriethoxysilane	-8	20969.3	31454.0	96.2	0.3	0.1	94.7	1.7	1.0	1.8	23.3
21291.0	71		-8	20727.3	31091.0	95.1	-0.8	0.6					23.1
20786.0	72		-8	20222.3	30333.5	92.8	-3.1	9.5					22.5
22344.0	73	Octyltriethoxysilane	-9	21780.3	32670.5	99.9	4.1	16.5	98.0	3.2	1.8	3.2	24.2
22286.0	74		-9	21722.3	32583.5	99.7	3.8	14.4					24.1
21123.0	75		-9	20559.3	30839.0	94.3	-1.5	2.4					22.9
21688.0	76	Octyltriethoxysilane	-10	21124.3	31686.5	96.9	1.0	1.1	96.6	5.9	3.4	6.1	23.5
22858.0	77		-10	22294.3	33441.5	102.3	6.4	41.2					24.8
20303.0	78		-10	19739.3	29609.0	90.6	-5.3	28.2					22.0
12540	79	Oxybenzone	-3	11976.3	17964.5	55.0	-15.1	226.8	70.0	13.5	7.8	19.3	13.6
16677	80		-3	16113.3	24170.0	73.9	3.9	15.4					18.1
18249	81		-3	17685.3	26528.0	81.2	11.1	124.0					19.8
19026	82	Oxybenzone	-4	18462.3	27693.5	84.7	1.5	2.3	83.2	1.6	0.9	2.0	20.6
18315	83		-4	17751.3	26627.0	81.5	-1.7	3.0					19.8
18741	84		-4	18177.3	27266.0	83.4	0.2	0.0					20.3
21755	85	Oxybenzone	-5	21191.3	31787.0	97.2	1.9	3.6	96.0	2.2	1.3	2.3	23.6
20944	86		-5	20380.3	30570.5	93.5	-1.8	3.3					22.7
21768	87		-5	21204.3	31806.5	97.3	2.0	3.9					23.6
21503	88	Oxybenzone	-6	20939.3	31409.0	96.1	0.8	0.6	95.1	3.1	1.8	3.2	23.3
20523	89		-6	19959.3	29939.0	91.6	-3.7	14.0					22.2
21806	90		-6	21242.3	31863.5	97.5	2.1	4.6					23.6
21171	91	Oxybenzone	-7	20607.3	30911.0	94.6	-0.8	0.6	95.3	2.8	1.6	2.9	22.9
20813	92		-7	20249.3	30374.0	92.9	-2.4	5.8					22.5
22005	93		-7	21441.3	32162.0	98.4	3.1	9.3					23.8
20876	94	Oxybenzone	-8	20312.3	30468.5	93.2	-2.1	4.5	95.8	3.0	1.8	3.2	22.6
21290	95		-8	20726.3	31089.5	95.1	-0.2	0.1					23.1
22170	96		-8	21606.3	32409.5	99.1	3.8	14.5					24.0
20482	97	Oxybenzone	-9	19918.3	29877.5	91.4	-3.9	15.5	94.9	5.8	3.3	6.1	22.2
20546	98		-9	19982.3	29973.5	91.7	-3.6	13.3					22.3
22688	99		-9	22124.3	33186.5	101.5	6.2	38.3					24.6
21305	100	Oxybenzone	-10	20741.3	31112.0	95.2	-0.2	0.0	95.0	4.8	2.8	5.1	23.1
20178	101		-10	19614.3	29421.5	90.0	-5.3	28.4					21.9
22286	102		-10	21722.3	32583.5	99.7	4.3	18.9					24.1







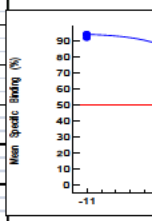


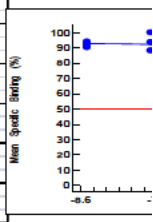
APPENDIX 1 Raw and Normalized Data 1st Valid Run (continued) – July 25, 2011

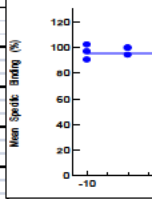
Experiment Date:	25-Jul-11	Study Number:	9070-100107ERB	Assays Conducted by:	[redacted]
Test substance:	Methoxycinnamate				
12/6/2011 17:42					
	10 uL of 50 nM E2=	___	DPM and 0.5 x10-12 moles		
	Therefore there are	___	DPM/mole		
	ug protein/assay tube =	80.0			

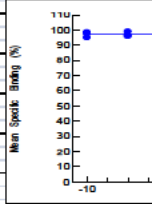
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17709	–	138130.2	138465.6
2		17865	–	139347.0	
3		17769	–	138598.2	
4		17719	–	138208.2	
5		17718	–	138200.4	
6		17732	–	138309.6	
7	Total Binding (Solvent Control)	22497	21933.3	32900.0	32687.5
8		22817	22253.3	33380.0	
9		21752	21188.3	31782.5	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
513.0	10	Estradiol (NSB)	-7	-50.7	-76.0	-0.2	-0.6	0.3	0.0	0.7	0.4	3.2E+17	0.6
439.0	11		-7	-124.7	-187.0	-0.6	-0.9	0.9					0.5
739.0	12		-7	175.3	263.0	0.8	0.5	0.2					0.8
2328.0	13	Estradiol	-8	1764.3	2646.5	8.1	0.1	0.0	7.7	0.3	0.2	4.0	2.5
2216.0	14		-8	1652.3	2478.5	7.6	-0.4	0.2					2.4
2206.0	15		-8	1642.3	2463.5	7.5	-0.5	0.2					2.4
5737.0	16	Estradiol	-8.5	5173.3	7760.0	23.7	0.6	0.3	24.9	1.1	0.6	4.3	6.2
6080.0	17		-8.5	5516.3	8274.5	25.3	2.1	4.6					6.6
6180.0	18		-8.5	5616.3	8424.5	25.8	2.6	6.7					6.7
10397.0	19	Estradiol	-9	9833.3	14750.0	45.1	-4.6	21.2	47.7	2.3	1.3	4.8	11.3
11152.0	20		-9	10588.3	15882.5	48.6	-1.1	1.3					12.1
11345.0	21		-9	10781.3	16172.0	49.5	-0.3	0.1					12.3
17285.0	22	Estradiol	-9.5	16721.3	25082.0	76.7	2.0	3.9	75.4	1.8	1.0	2.3	18.7
16557.0	23		-9.5	15993.3	23990.0	73.4	-1.4	1.8					17.9
17119.0	24		-9.5	16555.3	24833.0	76.0	1.2	1.5					18.5
21014.0	25	Estradiol	-10	20450.3	30675.5	93.8	6.1	36.8	89.3	4.1	2.3	4.6	22.8
19720.0	26		-10	19156.3	28734.5	87.9	0.1	0.0					21.4
19318.0	27		-10	18754.3	28131.5	86.1	-1.7	2.9					20.9
21033.0	28	Estradiol	-11	20469.3	30704.0	93.9	-0.1	0.0	92.8	1.3	0.7	1.4	22.8
20859.0	29		-11	20295.3	30443.0	93.1	-0.9	0.9					22.6
20483.0	30		-11	19919.3	29879.0	91.4	-2.7	7.0					22.2
805.0	31	19-Norethindrone	-4	241.3	362.0	1.1	-0.2	0.1	0.9	0.2	0.1	21.6	0.9
722.0	32		-4	158.3	237.5	0.7	-0.6	0.4					0.8
750.0	33		-4	186.3	279.5	0.9	-0.5	0.3					0.8
2032.0	34	19-Norethindrone	-4.5	1468.3	2202.5	6.7	0.7	0.4	6.7	0.4	0.2	5.9	2.2
2109.0	35		-4.5	1545.3	2318.0	7.1	1.0	1.0					2.3
1937.0	36		-4.5	1373.3	2060.0	6.3	0.2	0.1					2.1
9984.0	37	19-Norethindrone	-5.5	9420.3	14130.5	43.2	-1.4	1.9	44.0	2.0	1.1	4.5	10.8
10634.0	38		-5.5	10070.3	15105.5	46.2	1.6	2.6					11.5
9825.0	39		-5.5	9261.3	13892.0	42.5	-2.1	4.4					10.6
16245.0	40	19-Norethindrone	-6	15681.3	23522.0	72.0	1.0	1.0	72.1	0.4	0.2	0.5	17.6
16378.0	41		-6	15814.3	23721.5	72.6	1.6	2.6					17.7
16223.0	42		-6	15659.3	23489.0	71.9	0.9	0.8					17.6
18403.0	43	19-Norethindrone	-6.5	17839.3	26759.0	81.9	-3.6	12.7	84.2	2.1	1.2	2.5	19.9
19313.0	44		-6.5	18749.3	28124.0	86.0	0.6	0.4					20.9
19010.0	45		-6.5	18446.3	27669.5	84.6	-0.8	0.6					20.6
20117.0	46	19-Norethindrone	-7	19553.3	29330.0	89.7	-1.0	1.1	90.7	1.4	0.8	1.5	21.8
20187.0	47		-7	19623.3	29435.0	90.0	-0.7	0.5					21.9
20664.0	48		-7	20100.3	30150.5	92.2	1.5	2.2					22.4
22360.0	49	19-Norethindrone	-7.5	21796.3	32694.5	100.0	7.6	57.7	94.0	5.9	3.4	6.3	24.2
20967.0	50		-7.5	20403.3	30605.0	93.6	1.2	1.4					22.7
19786.0	51		-7.5	19222.3	28833.5	88.2	-4.2	17.8					21.4
20235.0	52	19-Norethindrone	-8.5	19671.3	29507.0	90.3	-2.8	7.8	92.2	1.9	1.1	2.0	21.9
21057.0	53		-8.5	20493.3	30740.0	94.0	1.0	1.0					22.8
20647.0	54		-8.5	20083.3	30125.0	92.2	-0.9	0.8					22.4
19400.0	55	Octyltriethoxysilane	-3	18836.3	28254.5	86.4	1.6	2.5	84.9	1.8	1.1	2.2	21.0
19152.0	56		-3	18588.3	27882.5	85.3	0.4	0.2					20.7
18614.0	57		-3	18050.3	27075.5	82.8	-2.0	4.1					20.2
20554.0	58	Octyltriethoxysilane	-4	19990.3	29985.5	91.7	-3.7	13.8	95.5	3.3	1.9	3.5	22.3
21637.0	59		-4	21073.3	31610.0	96.7	1.3	1.6					23.4
21914.0	60		-4	21350.3	32025.5	98.0	2.5	6.4					23.7
21469.0	61	Octyltriethoxysilane	-5	20905.3	31358.0	95.9	0.1	0.0	95.6	0.5	0.3	0.5	23.3
21281.0	62		-5	20717.3	31076.0	95.1	-0.8	0.6					23.1
21463.0	63		-5	20899.3	31349.0	95.9	0.0	0.0					23.3
21521.0	64	Octyltriethoxysilane	-6	20957.3	31436.0	96.2	0.3	0.1	94.0	3.1	1.8	3.3	23.3
21370.0	65		-6	20806.3	31209.5	95.5	-0.4	0.2					23.2
20276.0	66		-6	19712.3	29568.5	90.5	-5.4	29.5					22.0
22349.0	67	Octyltriethoxysilane	-7	21785.3	32678.0	100.0	4.1	16.7	96.3	3.2	1.8	3.3	24.2
21196.0	68		-7	20632.3	30948.5	94.7	-1.2	1.5					23.0
21110.0	69		-7	20546.3	30819.5	94.3	-1.6	2.6					22.9
21533.0	70	Octyltriethoxysilane	-8	20969.3	31454.0	96.2	0.3	0.1	94.7	1.7	1.0	1.8	23.3
21291.0	71		-8	20727.3	31091.0	95.1	-0.8	0.6					23.1
20786.0	72		-8	20222.3	30333.5	92.8	-3.1	9.5					22.5
22344.0	73	Octyltriethoxysilane	-9	21780.3	32670.5	99.9	4.1	16.5	98.0	3.2	1.8	3.2	24.2
22286.0	74		-9	21722.3	32583.5	99.7	3.8	14.4					24.1
21123.0	75		-9	20559.3	30839.0	94.3	-1.5	2.4					22.9
21688.0	76	Octyltriethoxysilane	-10	21124.3	31686.5	96.9	1.0	1.1	96.6	5.9	3.4	6.1	23.5
22858.0	77		-10	22294.3	33441.5	102.3	6.4	41.2					24.8
20303.0	78		-10	19739.3	29609.0	90.6	-5.3	28.2					22.0
19642	79	Methoxycinnamate	-3	19078.3	28617.5	87.5	2.9	8.6	84.0	3.1	1.8	3.7	21.3
18451	80		-3	17887.3	26831.0	82.1	-2.5	6.4					20.0
18517	81		-3	17953.3	26930.0	82.4	-2.2	4.9					20.1
20648	82	Methoxycinnamate	-4	20084.3	30126.5	92.2	4.5	19.8	89.2	5.0	2.9	5.6	22.4
18746	83		-4	18182.3	27273.5	83.4	-4.3	18.3					20.3
20601	84		-4	20037.3	30056.0	91.9	4.2	17.9					22.3
21591	85	Methoxycinnamate	-5	21027.3	31541.0	96.5	3.7	13.8	90.7	7.0	4.1	7.8	23.4
20766	86		-5	20202.3	30303.5	92.7	-0.1	0.0					22.5
18619	87		-5	18055.3	27083.0	82.9	-9.9	98.5					20.2
21546	88	Methoxycinnamate	-6	20982.3	31473.5	96.3	0.1	0.0	98.3	3.4	2.0	3.4	23.3
21578	89		-6	21014.3	31521.5	96.4	0.3	0.1					23.4
22840	90		-6	22276.3	33414.5	102.2	6.1	36.9					24.7
21260	91	Methoxycinnamate	-7	20696.3	31044.5	95.0	-2.3	5.4	97.8	2.4	1.4	2.5	23.0
22200	92		-7	21636.3	32454.5	99.3	2.0	4.0					24.0
22142	93		-7	21578.3	32367.5	99.0	1.7	3.0					24.0
22206	94	Methoxycinnamate	-8	21642.3	32463.5	99.3	1.7	3.0	97.5	1.6	0.9	1.7	24.1
21697	95		-8	21133.3	31700.0	97.0	-0.6	0.4					23.5
21520	96		-8	20956.3	31434.5	96.2	-1.4	2.0					23.3
21464	97	Methoxycinnamate	-9	20900.3	31350.5	95.9	-1.7	3.1	97.0	1.6	0.9	1.6	23.3
22098	98		-9	21534.3	32301.5	98.8	1.2	1.4					23.9
21549	99		-9	20985.3	31478.0	96.3	-1.4	1.8					23.3
22000	100	Methoxycinnamate	-10	21436.3	32154.5	98.4	0.7	0.5	97.0	1.8	1.0	1.8	23.8
21855	101		-10	21291.3	31937.0	97.7	0.0	0.0					23.7
21262	102		-10	20698.3	31047.5	95.0	-2.7	7.2					23.0









Experiment Date:	25-Jul-11	Study Number:	9070-100107ERB	Assays Conducted by:	[redacted]
Test substance:	Octylsalicylate				
12/6/2011 17:42					
	10 uL of 50 nM E2=	____	DPM and 0.5 x10-12 moles		
	Therefore there are	____	DPM/mole		
	ug protein/assay tube =	80.0			

Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17709	--	138130.2	138465.6
2		17865	--	139347.0	
3		17769	--	138598.2	
4		17719	--	138208.2	
5		17718	--	138200.4	
6		17732	--	138309.6	
7	Total Binding (Solvent Control)	22497	21933.3	32900.0	32687.5
8		22817	22253.3	33380.0	
9		21752	21188.3	31782.5	

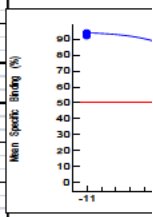
DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
513.0	10	Estradiol (NSB)	-7	-50.7	-76.0	-0.2	-0.6	0.3	0.0	0.7	0.4	3.2E+17	0.6
439.0	11		-7	-124.7	-187.0	-0.6	-0.9	0.9					0.5
739.0	12		-7	175.3	263.0	0.8	0.5	0.2					0.8
2328.0	13	Estradiol	-8	1764.3	2646.5	8.1	0.1	0.0	7.7	0.3	0.2	4.0	2.5
2216.0	14		-8	1652.3	2478.5	7.6	-0.4	0.2					2.4
2206.0	15		-8	1642.3	2463.5	7.5	-0.5	0.2					2.4
5737.0	16	Estradiol	-8.5	5173.3	7760.0	23.7	0.6	0.3	24.9	1.1	0.6	4.3	6.2
6080.0	17		-8.5	5516.3	8274.5	25.3	2.1	4.6					6.6
6180.0	18		-8.5	5616.3	8424.5	25.8	2.6	6.7					6.7
10397.0	19	Estradiol	-9	9833.3	14750.0	45.1	-4.6	21.2	47.7	2.3	1.3	4.8	11.3
11152.0	20		-9	10588.3	15882.5	48.6	-1.1	1.3					12.1
11345.0	21		-9	10781.3	16172.0	49.5	-0.3	0.1					12.3
17285.0	22	Estradiol	-9.5	16721.3	25082.0	76.7	2.0	3.9	75.4	1.8	1.0	2.3	18.7
16557.0	23		-9.5	15993.3	23990.0	73.4	-1.4	1.8					17.9
17119.0	24		-9.5	16555.3	24833.0	76.0	1.2	1.5					18.5
21014.0	25	Estradiol	-10	20450.3	30675.5	93.8	6.1	36.8	89.3	4.1	2.3	4.6	22.8
19720.0	26		-10	19156.3	28734.5	87.9	0.1	0.0					21.4
19318.0	27		-10	18754.3	28131.5	86.1	-1.7	2.9					20.9
21033.0	28	Estradiol	-11	20469.3	30704.0	93.9	-0.1	0.0	92.8	1.3	0.7	1.4	22.8
20859.0	29		-11	20295.3	30443.0	93.1	-0.9	0.9					22.6
20483.0	30		-11	19919.3	29879.0	91.4	-2.7	7.0					22.2
805.0	31	19-Norethindrone	-4	241.3	362.0	1.1	-0.2	0.1	0.9	0.2	0.1	21.6	0.9
722.0	32		-4	158.3	237.5	0.7	-0.6	0.4					0.8
750.0	33		-4	186.3	279.5	0.9	-0.5	0.3					0.8
2032.0	34	19-Norethindrone	-4.5	1468.3	2202.5	6.7	0.7	0.4	6.7	0.4	0.2	5.9	2.2
2109.0	35		-4.5	1545.3	2318.0	7.1	1.0	1.0					2.3
1937.0	36		-4.5	1373.3	2060.0	6.3	0.2	0.1					2.1
9984.0	37	19-Norethindrone	-5.5	9420.3	14130.5	43.2	-1.4	1.9	44.0	2.0	1.1	4.5	10.8
10634.0	38		-5.5	10070.3	15105.5	46.2	1.6	2.6					11.5
9825.0	39		-5.5	9261.3	13892.0	42.5	-2.1	4.4					10.6
16245.0	40	19-Norethindrone	-6	15681.3	23522.0	72.0	1.0	1.0	72.1	0.4	0.2	0.5	17.6
16378.0	41		-6	15814.3	23721.5	72.6	1.6	2.6					17.7
16223.0	42		-6	15659.3	23489.0	71.9	0.9	0.8					17.6
18403.0	43	19-Norethindrone	-6.5	17839.3	26759.0	81.9	-3.6	12.7	84.2	2.1	1.2	2.5	19.9
19313.0	44		-6.5	18749.3	28124.0	86.0	0.6	0.4					20.9
19010.0	45		-6.5	18446.3	27669.5	84.6	-0.8	0.6					20.6
20117.0	46	19-Norethindrone	-7	19553.3	29330.0	89.7	-1.0	1.1	90.7	1.4	0.8	1.5	21.8
20187.0	47		-7	19623.3	29435.0	90.0	-0.7	0.5					21.9
20664.0	48		-7	20100.3	30150.5	92.2	1.5	2.2					22.4
22360.0	49	19-Norethindrone	-7.5	21796.3	32694.5	100.0	7.6	57.7	94.0	5.9	3.4	6.3	24.2
20967.0	50		-7.5	20403.3	30605.0	93.6	1.2	1.4					22.7
19786.0	51		-7.5	19222.3	28833.5	88.2	-4.2	17.8					21.4
20235.0	52	19-Norethindrone	-8.5	19671.3	29507.0	90.3	-2.8	7.8	92.2	1.9	1.1	2.0	21.9
21057.0	53		-8.5	20493.3	30740.0	94.0	1.0	1.0					22.8
20647.0	54		-8.5	20083.3	30125.0	92.2	-0.9	0.8					22.4
19400.0	55	Octyltriethoxysilane	-3	18836.3	28254.5	86.4	1.6	2.5	84.9	1.8	1.1	2.2	21.0
19152.0	56		-3	18588.3	27882.5	85.3	0.4	0.2					20.7
18614.0	57		-3	18050.3	27075.5	82.8	-2.0	4.1					20.2
20554.0	58	Octyltriethoxysilane	-4	19990.3	29985.5	91.7	-3.7	13.8	95.5	3.3	1.9	3.5	22.3
21637.0	59		-4	21073.3	31610.0	96.7	1.3	1.6					23.4
21914.0	60		-4	21350.3	32025.5	98.0	2.5	6.4					23.7
21469.0	61	Octyltriethoxysilane	-5	20905.3	31358.0	95.9	0.1	0.0	95.6	0.5	0.3	0.5	23.3
21281.0	62		-5	20717.3	31076.0	95.1	-0.8	0.6					23.1
21463.0	63		-5	20899.3	31349.0	95.9	0.0	0.0					23.3
21521.0	64	Octyltriethoxysilane	-6	20957.3	31436.0	96.2	0.3	0.1	94.0	3.1	1.8	3.3	23.3
21370.0	65		-6	20806.3	31209.5	95.5	-0.4	0.2					23.2
20276.0	66		-6	19712.3	29568.5	90.5	-5.4	29.5					22.0
22349.0	67	Octyltriethoxysilane	-7	21785.3	32678.0	100.0	4.1	16.7	96.3	3.2	1.8	3.3	24.2
21196.0	68		-7	20632.3	30948.5	94.7	-1.2	1.5					23.0
21110.0	69		-7	20546.3	30819.5	94.3	-1.6	2.6					22.9
21533.0	70	Octyltriethoxysilane	-8	20969.3	31454.0	96.2	0.3	0.1	94.7	1.7	1.0	1.8	23.3
21291.0	71		-8	20727.3	31091.0	95.1	-0.8	0.6					23.1
20786.0	72		-8	20222.3	30333.5	92.8	-3.1	9.5					22.5
22344.0	73	Octyltriethoxysilane	-9	21780.3	32670.5	99.9	4.1	16.5	98.0	3.2	1.8	3.2	24.2
22286.0	74		-9	21722.3	32583.5	99.7	3.8	14.4					24.1
21123.0	75		-9	20559.3	30839.0	94.3	-1.5	2.4					22.9
21688.0	76	Octyltriethoxysilane	-10	21124.3	31686.5	96.9	1.0	1.1	96.6	5.9	3.4	6.1	23.5
22858.0	77		-10	22294.3	33441.5	102.3	6.4	41.2					24.8
20303.0	78		-10	19739.3	29609.0	90.6	-5.3	28.2					22.0
13505	79	Octylsalicylate	-3	12941.3	19412.0	59.4	-0.9	0.9	60.3	1.2	0.7	1.9	14.6
13643	80		-3	13079.3	19619.0	60.0	-0.3	0.1					14.8
13993	81		-3	13429.3	20144.0	61.6	1.3	1.7					15.2
15923	82	Octylsalicylate	-4	15359.3	23039.0	70.5	-4.6	20.8	74.9	3.8	2.2	5.1	17.2
17337	83		-4	16773.3	25160.0	77.0	1.9	3.7					18.8
17389	84		-4	16825.3	25238.0	77.2	2.2	4.7					18.8
19745	85	Octylsalicylate	-5	19181.3	28772.0	88.0	-0.2	0.0	88.8	0.8	0.4	0.9	21.4
19933	86		-5	19369.3	29054.0	88.9	0.7	0.4					21.6
20080	87		-5	19516.3	29274.5	89.6	1.3	1.8					21.8
20460	88	Octylsalicylate	-6	19896.3	29844.5	91.3	-0.9	0.9	90.7	0.5	0.3	0.6	22.2
20253	89		-6	19689.3	29534.0	90.4	-1.9	3.6					21.9
20273	90		-6	19709.3	29564.0	90.4	-1.8	3.2					22.0
20636	91	Octylsalicylate	-7	20072.3	30108.5	92.1	-0.9	0.8	93.2	1.8	1.0	1.9	22.4
21318	92		-7	20754.3	31131.5	95.2	2.2	4.9					23.1
20638	93		-7	20074.3	30111.5	92.1	-0.9	0.8					22.4
20924	94	Octylsalicylate	-8	20360.3	30540.5	93.4	0.3	0.1	96.2	2.5	1.4	2.6	22.7
21691	95		-8	21127.3	31691.0	97.0	3.8	14.4					23.5
21982	96		-8	21418.3	32127.5	98.3	5.1	26.4					23.8
20737	97	Octylsalicylate	-9	20173.3	30260.0	92.6	-0.6	0.4	92.4	0.4	0.2	0.4	22.5
20758	98		-9	20194.3	30291.5	92.7	-0.5	0.3					22.5
20615	99		-9	20051.3	30077.0	92.0	-1.2	1.3					22.3
20530	100	Octylsalicylate	-10	19966.3	29949.5	91.6	-1.6	2.4	91.8	0.2	0.1	0.2	22.2
20609	101		-10	20045.3	30068.0	92.0	-1.2	1.4					22.3
20549	102		-10	19985.3	29978.0	91.7	-1.5	2.1					22.3

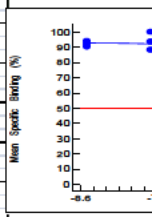
APPENDIX 1 Raw and Normalized Data 1st Valid Run (continued) – July 25, 2011

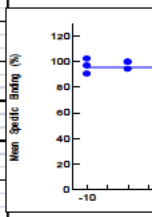
Experiment Date:	25-Jul-11		Study Number:	9070-100107ERB		Assays Conducted by: [redacted]
Test substance:	Octocrylene					
12/6/2011 17:42						
		10 uL of 50 nM E2=	___	DPM and 0.5 x10-12 moles		
		Therefore there are	___	DPM/mole		
		ug protein/assay tube =	80.0			

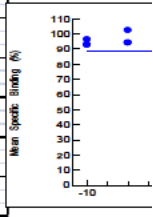
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17709	–	138130.2	138465.6
2		17865	–	139347.0	
3		17769	–	138598.2	
4		17719	–	138208.2	
5		17718	–	138200.4	
6		17732	–	138309.6	
7	Total Binding (Solvent Control)	22497	21933.3	32900.0	32687.5
8		22817	22253.3	33380.0	
9		21752	21188.3	31782.5	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
513.0	10	Estradiol (NSB)	-7	-50.7	-76.0	-0.2	-0.6	0.3	0.0	0.7	0.4	3.2E+17	0.6
439.0	11		-7	-124.7	-187.0	-0.6	-0.9	0.9					0.5
739.0	12		-7	175.3	263.0	0.8	0.5	0.2					0.8
2328.0	13	Estradiol	-8	1764.3	2646.5	8.1	0.1	0.0	7.7	0.3	0.2	4.0	2.5
2216.0	14		-8	1652.3	2478.5	7.6	-0.4	0.2					2.4
2206.0	15		-8	1642.3	2463.5	7.5	-0.5	0.2					2.4
5737.0	16	Estradiol	-8.5	5173.3	7760.0	23.7	0.6	0.3	24.9	1.1	0.6	4.3	6.2
6080.0	17		-8.5	5516.3	8274.5	25.3	2.1	4.6					6.6
6180.0	18		-8.5	5616.3	8424.5	25.8	2.6	6.7					6.7
10397.0	19	Estradiol	-9	9833.3	14750.0	45.1	-4.6	21.2	47.7	2.3	1.3	4.8	11.3
11152.0	20		-9	10588.3	15882.5	48.6	-1.1	1.3					12.1
11345.0	21		-9	10781.3	16172.0	49.5	-0.3	0.1					12.3
17285.0	22	Estradiol	-9.5	16721.3	25082.0	76.7	2.0	3.9	75.4	1.8	1.0	2.3	18.7
16557.0	23		-9.5	15993.3	23990.0	73.4	-1.4	1.8					17.9
17119.0	24		-9.5	16555.3	24833.0	76.0	1.2	1.5					18.5
21014.0	25	Estradiol	-10	20450.3	30675.5	93.8	6.1	36.8	89.3	4.1	2.3	4.6	22.8
19720.0	26		-10	19156.3	28734.5	87.9	0.1	0.0					21.4
19318.0	27		-10	18754.3	28131.5	86.1	-1.7	2.9					20.9
21033.0	28	Estradiol	-11	20469.3	30704.0	93.9	-0.1	0.0	92.8	1.3	0.7	1.4	22.8
20859.0	29		-11	20295.3	30443.0	93.1	-0.9	0.9					22.6
20483.0	30		-11	19919.3	29879.0	91.4	-2.7	7.0					22.2
805.0	31	19-Norethindrone	-4	241.3	362.0	1.1	-0.2	0.1	0.9	0.2	0.1	21.6	0.9
722.0	32		-4	158.3	237.5	0.7	-0.6	0.4					0.8
750.0	33		-4	186.3	279.5	0.9	-0.5	0.3					0.8
2032.0	34	19-Norethindrone	-4.5	1468.3	2202.5	6.7	0.7	0.4	6.7	0.4	0.2	5.9	2.2
2109.0	35		-4.5	1545.3	2318.0	7.1	1.0	1.0					2.3
1937.0	36		-4.5	1373.3	2060.0	6.3	0.2	0.1					2.1
9984.0	37	19-Norethindrone	-5.5	9420.3	14130.5	43.2	-1.4	1.9	44.0	2.0	1.1	4.5	10.8
10634.0	38		-5.5	10070.3	15105.5	46.2	1.6	2.6					11.5
9825.0	39		-5.5	9261.3	13892.0	42.5	-2.1	4.4					10.6
16245.0	40	19-Norethindrone	-6	15681.3	23522.0	72.0	1.0	1.0	72.1	0.4	0.2	0.5	17.6
16378.0	41		-6	15814.3	23721.5	72.6	1.6	2.6					17.7
16223.0	42		-6	15659.3	23489.0	71.9	0.9	0.8					17.6
18403.0	43	19-Norethindrone	-6.5	17839.3	26759.0	81.9	-3.6	12.7	84.2	2.1	1.2	2.5	19.9
19313.0	44		-6.5	18749.3	28124.0	86.0	0.6	0.4					20.9
19010.0	45		-6.5	18446.3	27669.5	84.6	-0.8	0.6					20.6
20117.0	46	19-Norethindrone	-7	19553.3	29330.0	89.7	-1.0	1.1	90.7	1.4	0.8	1.5	21.8
20187.0	47		-7	19623.3	29435.0	90.0	-0.7	0.5					21.9
20664.0	48		-7	20100.3	30150.5	92.2	1.5	2.2					22.4
22360.0	49	19-Norethindrone	-7.5	21796.3	32694.5	100.0	7.6	57.7	94.0	5.9	3.4	6.3	24.2
20967.0	50		-7.5	20403.3	30605.0	93.6	1.2	1.4					22.7
19786.0	51		-7.5	19222.3	28833.5	88.2	-4.2	17.8					21.4
20235.0	52	19-Norethindrone	-8.5	19671.3	29507.0	90.3	-2.8	7.8	92.2	1.9	1.1	2.0	21.9
21057.0	53		-8.5	20493.3	30740.0	94.0	1.0	1.0					22.8
20647.0	54		-8.5	20083.3	30125.0	92.2	-0.9	0.8					22.4
19400.0	55	Octyltriethoxysilane	-3	18836.3	28254.5	86.4	1.6	2.5	84.9	1.8	1.1	2.2	21.0
19152.0	56		-3	18588.3	27882.5	85.3	0.4	0.2					20.7
18614.0	57		-3	18050.3	27075.5	82.8	-2.0	4.1					20.2
20554.0	58	Octyltriethoxysilane	-4	19990.3	29985.5	91.7	-3.7	13.8	95.5	3.3	1.9	3.5	22.3
21637.0	59		-4	21073.3	31610.0	96.7	1.3	1.6					23.4
21914.0	60		-4	21350.3	32025.5	98.0	2.5	6.4					23.7
21469.0	61	Octyltriethoxysilane	-5	20905.3	31358.0	95.9	0.1	0.0	95.6	0.5	0.3	0.5	23.3
21281.0	62		-5	20717.3	31076.0	95.1	-0.8	0.6					23.1
21463.0	63		-5	20899.3	31349.0	95.9	0.0	0.0					23.3
21521.0	64	Octyltriethoxysilane	-6	20957.3	31436.0	96.2	0.3	0.1	94.0	3.1	1.8	3.3	23.3
21370.0	65		-6	20806.3	31209.5	95.5	-0.4	0.2					23.2
20276.0	66		-6	19712.3	29568.5	90.5	-5.4	29.5					22.0
22349.0	67	Octyltriethoxysilane	-7	21785.3	32678.0	100.0	4.1	16.7	96.3	3.2	1.8	3.3	24.2
21196.0	68		-7	20632.3	30948.5	94.7	-1.2	1.5					23.0
21110.0	69		-7	20546.3	30819.5	94.3	-1.6	2.6					22.9
21533.0	70	Octyltriethoxysilane	-8	20969.3	31454.0	96.2	0.3	0.1	94.7	1.7	1.0	1.8	23.3
21291.0	71		-8	20727.3	31091.0	95.1	-0.8	0.6					23.1
20786.0	72		-8	20222.3	30333.5	92.8	-3.1	9.5					22.5
22344.0	73	Octyltriethoxysilane	-9	21780.3	32670.5	99.9	4.1	16.5	98.0	3.2	1.8	3.2	24.2
22286.0	74		-9	21722.3	32583.5	99.7	3.8	14.4					24.1
21123.0	75		-9	20559.3	30839.0	94.3	-1.5	2.4					22.9
21688.0	76	Octyltriethoxysilane	-10	21124.3	31686.5	96.9	1.1	1.1	96.6	5.9	3.4	6.1	23.5
22858.0	77		-10	22294.3	33441.5	102.3	6.4	41.2					24.8
20303.0	78		-10	19739.3	29609.0	90.6	-5.3	28.2					22.0
19873	79	Octocrylene	-3	19309.3	28964.0	88.6	0.0	0.0	90.7	2.2	1.3	2.5	21.5
20289	80		-3	19725.3	29588.0	90.5	1.9	3.6					22.0
20845	81		-3	20281.3	30422.0	93.1	4.5	19.9					22.6
22416	82	Octocrylene	-4	21852.3	32778.5	100.3	11.7	136.2	102.4	2.0	1.1	1.9	24.3
22945	83		-4	22381.3	33572.0	102.7	14.1	198.7					24.9
23263	84		-4	22699.3	34049.0	104.2	15.6	242.0					25.2
23088	85	Octocrylene	-5	22524.3	33786.5	103.4	14.8	217.7	103.9	1.2	0.7	1.2	25.0
23018	86		-5	22454.3	33681.5	103.0	14.4	208.3					24.9
23511	87		-5	22947.3	34421.0	105.3	16.7	278.7					25.5
22848	88	Octocrylene	-6	22284.3	33426.5	102.3	13.7	186.4	101.7	1.1	0.7	1.1	24.8
22449	89		-6	21885.3	32828.0	100.4	11.8	139.7					24.3
22903	90		-6	22339.3	33509.0	102.5	13.9	193.3					24.8
21786	91	Octocrylene	-7	21222.3	31833.5	97.4	8.8	77.1	130.9	56.8	32.8	43.3	23.6
22132	92		-7	21568.3	32352.5	99.0	10.4	107.5					24.0
43379	93		-7	42815.3	64223.0	196.5	107.9	11635.3					47.0
23018	94	Octocrylene	-8	22454.3	33681.5	103.0	14.4	208.3	103.4	1.4	0.8	1.4	24.9
22833	95		-8	22269.3	33404.0	102.2	13.6	184.5					24.7
23435	96		-8	22871.3	34307.0	105.0	16.3	267.2					25.4
21097	97	Octocrylene	-9	20533.3	30800.0	94.2	5.6	31.5	97.0	4.7	2.7	4.9	22.9
21105	98		-9	20541.3	30812.0	94.3	5.7	32.0					22.9
22891	99		-9	22327.3	33491.0	102.5	13.8	191.8					24.8
20768	100	Octocrylene	-10	20204.3	30306.5	92.7	4.1	16.9	94.1	2.0	1.2	2.1	22.5
20856	101		-10	20292.3	30438.5	93.1	4.5	20.3					22.6
21570	102		-10	21006.3	31509.5	96.4	7.8	60.6					23.4







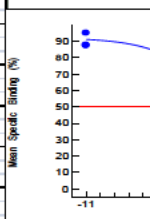


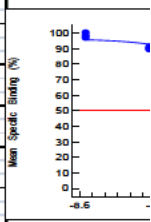
APPENDIX 1 Raw and Normalized Data 2nd Valid Run – August 01, 2011

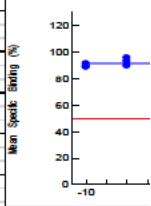
Experiment Date:	1-Aug-11		Study Number:	9070-100107ERB					Assays Conducted by:	[redacted]		
Test substance:	Oxybenzone											
1/27/2012 16:13												
		10 uL of 50 nM E2=	----	DPM and 0.5 x10-12 moles								
		Therefore there are	---	DPM/mole								
		ug protein/assay tube =	80.0									

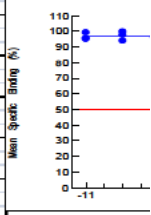
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17533	--	136757.4	136909.5
2		17478	--	136328.4	
3		17405	--	135759.0	
4		17532	--	136749.6	
5		17605	--	137319.0	
6		17762	--	138543.6	
7	Total Binding (Solvent Control)	17487	17114.7	25672.0	24693.0
8		15894	15521.7	23282.5	
9		17122	16749.7	25124.5	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
414.0	10	Estradiol (NSB)	-7	41.7	62.5	0.3	0.1	0.0	0.0	0.2	0.1	2.0E+17	0.5
356.0	11		-7	-16.3	-24.5	-0.1	-0.2	0.0					0.4
347.0	12		-7	-25.3	-38.0	-0.2	-0.3	0.1					0.4
1728.0	13	Estradiol	-8	1355.7	2033.5	8.2	-0.5	0.3	7.7	0.5	0.3	6.3	1.9
1626.0	14		-8	1253.7	1880.5	7.6	-1.1	1.3					1.8
1570.0	15		-8	1197.7	1796.5	7.3	-1.5	2.2					1.7
4889.0	16	Estradiol	-8.5	4516.7	6775.0	27.4	4.1	16.7	26.2	2.1	1.2	7.9	5.4
4289.0	17		-8.5	3916.7	5875.0	23.8	0.4	0.2					4.7
4870.0	18		-8.5	4497.7	6746.5	27.3	4.0	15.7					5.3
7687.0	19	Estradiol	-9	7314.7	10972.0	44.4	-2.7	7.5	44.4	0.8	0.4	1.7	8.4
7813.0	20		-9	7440.7	11161.0	45.2	-2.0	3.9					8.6
7563.0	21		-9	7190.7	10786.0	43.7	-3.5	12.2					8.3
12394.0	22	Estradiol	-9.5	12021.7	18032.5	73.0	2.8	7.6	70.8	1.9	1.1	2.7	13.6
11797.0	23		-9.5	11424.7	17137.0	69.4	-0.9	0.8					12.9
11911.0	24		-9.5	11538.7	17308.0	70.1	-0.2	0.0					13.0
15329.0	25	Estradiol	-10	14956.7	22435.0	90.9	7.1	50.7	85.5	4.7	2.7	5.5	16.8
14149.0	26		-10	13776.7	20665.0	83.7	0.0	0.0					15.5
13855.0	27		-10	13482.7	20224.0	81.9	-1.8	3.4					15.2
16046.0	28	Estradiol	-11	15673.7	23510.5	95.2	3.8	14.1	90.2	4.3	2.5	4.8	17.6
14839.0	29		-11	14466.7	21700.0	87.9	-3.6	12.8					16.3
14779.0	30		-11	14406.7	21610.0	87.5	-3.9	15.5					16.2
526.0	31	19-Norethindrone	-4	153.7	230.5	0.9	1.3	1.7	0.8	0.1	0.1	12.5	0.6
514.0	32		-4	141.7	212.5	0.9	1.2	1.5					0.6
492.0	33		-4	119.7	179.5	0.7	1.1	1.2					0.5
1226.0	34	19-Norethindrone	-4.5	853.7	1280.5	5.2	-2.7	7.5	6.3	1.0	0.6	16.1	1.3
1434.0	35		-4.5	1061.7	1592.5	6.4	-1.5	2.2					1.6
1554.0	36		-4.5	1181.7	1772.5	7.2	-0.7	0.6					1.7
6886.0	37	19-Norethindrone	-5.5	6513.7	9770.5	39.6	-3.6	13.0	43.4	3.3	1.9	7.7	7.5
7807.0	38		-5.5	7434.7	11152.0	45.2	2.0	3.9					8.6
7863.0	39		-5.5	7490.7	11236.0	45.5	2.3	5.4					8.6
10332.0	40	19-Norethindrone	-6	9959.7	14939.5	60.5	-3.6	13.2	65.1	4.0	2.3	6.2	11.3
11344.0	41		-6	10971.7	16457.5	66.6	2.5	6.3					12.4
11584.0	42		-6	11211.7	16817.5	68.1	4.0	15.8					12.7
13652.0	43	19-Norethindrone	-6.5	13279.7	19919.5	80.7	1.1	1.1	79.2	3.3	1.9	4.2	15.0
12780.0	44		-6.5	12407.7	18611.5	75.4	-4.2	18.0					14.0
13776.0	45		-6.5	13403.7	20105.5	81.4	1.8	3.3					15.1
15139.0	46	19-Norethindrone	-7	14766.7	22150.0	89.7	1.2	1.3	88.7	3.8	2.2	4.3	16.6
15498.0	47		-7	15125.7	22688.5	91.9	3.3	11.2					17.0
14283.0	48		-7	13910.7	20866.0	84.5	-4.0	16.3					15.6
15099.0	49	19-Norethindrone	-7.5	14726.7	22090.0	89.5	-3.5	12.4	90.1	0.8	0.5	0.9	16.5
15142.0	50		-7.5	14769.7	22154.5	89.7	-3.3	10.6					16.6
15355.0	51		-7.5	14982.7	22474.0	91.0	-2.0	3.9					16.8
16305.0	52	19-Norethindrone	-8.5	15932.7	23899.0	96.8	0.9	0.7	98.4	1.7	1.0	1.7	17.9
16530.0	53		-8.5	16157.7	24236.5	98.2	2.2	5.0					18.1
16866.0	54		-8.5	16493.7	24740.5	100.2	4.3	18.2					18.5
7933.0	55	Octyltriethoxysilane	-3	7560.7	11341.0	45.9	0.0	0.0	45.8	0.2	0.1	0.5	8.7
7938.0	56		-3	7565.7	11348.5	46.0	0.0	0.0					8.7
7874.0	57		-3	7501.7	11252.5	45.6	-0.4	0.2					8.6
14573.0	58	Octyltriethoxysilane	-4	14200.7	21301.0	86.3	-2.2	4.8	88.5	2.5	1.4	2.8	16.0
14889.0	59		-4	14516.7	21775.0	88.2	-0.3	0.1					16.3
15381.0	60		-4	15008.7	22513.0	91.2	2.7	7.4					16.9
16416.0	61	Octyltriethoxysilane	-5	16043.7	24065.5	97.5	5.6	31.9	98.4	1.1	0.6	1.1	18.0
16553.0	62		-5	16180.7	24271.0	98.3	6.5	42.0					18.1
16763.0	63		-5	16390.7	24586.0	99.6	7.8	60.1					18.4
15302.0	64	Octyltriethoxysilane	-6	14929.7	22394.5	90.7	-1.1	1.3	92.3	4.3	2.5	4.6	16.8
15039.0	65		-6	14666.7	22000.0	89.1	-2.7	7.4					16.5
16361.0	66		-6	15988.7	23983.0	97.1	5.3	28.2					17.9
15466.0	67	Octyltriethoxysilane	-7	15093.7	22640.5	91.7	-0.1	0.0	89.9	2.0	1.1	2.2	16.9
15246.0	68		-7	14873.7	22310.5	90.4	-1.5	2.1					16.7
14825.0	69		-7	14452.7	21679.0	87.8	-4.0	16.2					16.2
15139.0	70	Octyltriethoxysilane	-8	14766.7	22150.0	89.7	-2.1	4.5	87.3	2.3	1.3	2.6	16.6
14731.0	71		-8	14358.7	21538.0	87.2	-4.6	21.1					16.1
14382.0	72		-8	14009.7	21014.5	85.1	-6.7	45.0					15.8
16156.0	73	Octyltriethoxysilane	-9	15783.7	23675.5	95.9	4.1	16.5	93.0	3.0	1.7	3.2	17.7
15710.0	74		-9	15337.7	23006.5	93.2	1.4	1.8					17.2
15166.0	75		-9	14793.7	22190.5	89.9	-1.9	3.8					16.6
15027.0	76	Octyltriethoxysilane	-10	14654.7	21982.0	89.0	-2.8	7.8	89.9	1.4	0.8	1.5	16.5
15433.0	77		-10	15060.7	22591.0	91.5	-0.3	0.1					16.9
15056.0	78		-10	14683.7	22025.5	89.2	-2.6	6.8					16.5
12787	79	Oxybenzone	-4	12414.7	18622.0	75.4	-1.6	2.6	76.4	0.9	0.5	1.2	14.0
13091	80		-4	12718.7	19078.0	77.3	0.2	0.0					14.3
12994	81		-4	12621.7	18932.5	76.7	-0.4	0.1					14.2
15434	82	Oxybenzone	-5	15061.7	22592.5	91.5	0.7	0.5	93.4	2.0	1.2	2.2	16.9
15695	83		-5	15322.7	22984.0	93.1	2.3	5.3					17.2
16091	84		-5	15718.7	23578.0	95.5	4.7	22.1					17.6
15372	85	Oxybenzone	-6	14999.7	22499.5	91.1	-4.0	16.2	91.2	0.5	0.3	0.5	16.8
15475	86		-6	15102.7	22654.0	91.7	-3.4	11.5					17.0
15325	87		-6	14952.7	22429.0	90.8	-4.3	18.5					16.8
15618	88	Oxybenzone	-7	15245.7	22868.5	92.6	-3.9	15.0	95.5	4.4	2.5	4.6	17.1
15729	89		-7	15356.7	23035.0	93.3	-3.2	10.3					17.2
16919	90		-7	16546.7	24820.0	100.5	4.0	16.2					18.5
16930	91	Oxybenzone	-8	16557.7	24836.5	100.6	3.7	13.5	95.9	5.1	2.9	5.3	18.5
15279	92		-8	14906.7	22360.0	90.6	-6.4	40.4					16.7
16276	93		-8	15903.7	23855.5	96.6	-0.3	0.1					17.8
16948	94	Oxybenzone	-9	16575.7	24863.5	100.7	3.7	13.3	101.2	3.2	1.8	3.1	18.6
17582	95		-9	17209.7	25814.5	104.5	7.5	56.3					19.3
16550	96		-9	16177.7	24266.5	98.3	1.2	1.5					18.1
15838	97	Oxybenzone	-10	15465.7	23198.5	93.9	-3.1	9.8	97.1	3.0	1.7	3.0	17.4
16449	98		-10	16076.7	24115.0	97.7	0.6	0.3					18.0
16799	99		-10	16426.7	24640.0	99.8	2.7	7.3					18.4
16092	100	Oxybenzone	-11	15719.7	23579.5	95.5	-1.6	2.6	96.6	2.3	1.3	2.4	17.6
16022	101		-11	15649.7	23474.5	95.1	-2.0	4.1					17.6
16712	102		-11	16339.7	24509.5	99.3	2.2	4.7					18.3







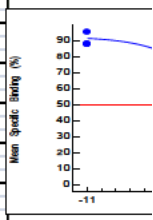


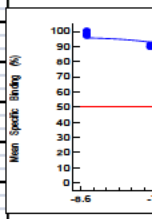
APPENDIX 1 Raw and Normalized Data 2nd Valid Run (continued) – August 01, 2011

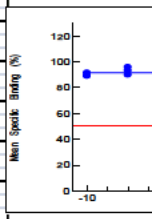
Experiment Date:	1-Aug-11	Study Number:	9070-100107ERB	Assays Conducted by:	[redacted]
Test substance:	Methoxycinnamate				
1/27/2012 16:14					
	10 uL of 50 nM E2=	___	DPM and 0.5 x10-12 moles		
	Therefore there are	___	DPM/mole		
	ug protein/assay tube =	80.0			

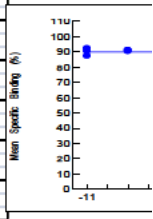
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17533	–	136757.4	136909.5
2		17478	–	136328.4	
3		17405	–	135759.0	
4		17532	–	136749.6	
5		17605	–	137319.0	
6		17762	–	138543.6	
7	Total Binding (Solvent Control)	17487	17114.7	25672.0	24693.0
8		15894	15521.7	23282.5	
9		17122	16749.7	25124.5	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
414.0	10	Estradiol (NSB)	-7	41.7	62.5	0.3	0.1	0.0	0.0	0.2	0.1	2.0E+17	0.5
356.0	11		-7	-16.3	-24.5	-0.1	-0.2	0.0					0.4
347.0	12		-7	-25.3	-38.0	-0.2	-0.3	0.1					0.4
1728.0	13	Estradiol	-8	1355.7	2033.5	8.2	-0.5	0.3	7.7	0.5	0.3	6.3	1.9
1626.0	14		-8	1253.7	1880.5	7.6	-1.1	1.3					1.8
1570.0	15		-8	1197.7	1796.5	7.3	-1.5	2.2					1.7
4889.0	16	Estradiol	-8.5	4516.7	6775.0	27.4	4.1	16.7	26.2	2.1	1.2	7.9	5.4
4289.0	17		-8.5	3916.7	5875.0	23.8	0.4	0.2					4.7
4870.0	18		-8.5	4497.7	6746.5	27.3	4.0	15.7					5.3
7687.0	19	Estradiol	-9	7314.7	10972.0	44.4	-2.7	7.5	44.4	0.8	0.4	1.7	8.4
7813.0	20		-9	7440.7	11161.0	45.2	-2.0	3.9					8.6
7563.0	21		-9	7190.7	10786.0	43.7	-3.5	12.2					8.3
12394.0	22	Estradiol	-9.5	12021.7	18032.5	73.0	2.8	7.6	70.8	1.9	1.1	2.7	13.6
11797.0	23		-9.5	11424.7	17137.0	69.4	-0.9	0.8					12.9
11911.0	24		-9.5	11538.7	17308.0	70.1	-0.2	0.0					13.0
15329.0	25	Estradiol	-10	14956.7	22435.0	90.9	7.1	50.7	85.5	4.7	2.7	5.5	16.8
14149.0	26		-10	13776.7	20665.0	83.7	0.0	0.0					15.5
13855.0	27		-10	13482.7	20224.0	81.9	-1.8	3.4					15.2
16046.0	28	Estradiol	-11	15673.7	23510.5	95.2	3.8	14.1	90.2	4.3	2.5	4.8	17.6
14839.0	29		-11	14466.7	21700.0	87.9	-3.6	12.8					16.3
14779.0	30		-11	14406.7	21610.0	87.5	-3.9	15.5					16.2
526.0	31	19-Norethindrone	-4	153.7	230.5	0.9	1.3	1.7	0.8	0.1	0.1	12.5	0.6
514.0	32		-4	141.7	212.5	0.9	1.2	1.5					0.6
492.0	33		-4	119.7	179.5	0.7	1.1	1.2					0.5
1226.0	34	19-Norethindrone	-4.5	853.7	1280.5	5.2	-2.7	7.5	6.3	1.0	0.6	16.1	1.3
1434.0	35		-4.5	1061.7	1592.5	6.4	-1.5	2.2					1.6
1554.0	36		-4.5	1181.7	1772.5	7.2	-0.7	0.6					1.7
6886.0	37	19-Norethindrone	-5.5	6513.7	9770.5	39.6	-3.6	13.0	43.4	3.3	1.9	7.7	7.5
7807.0	38		-5.5	7434.7	11152.0	45.2	2.0	3.9					8.6
7863.0	39		-5.5	7490.7	11236.0	45.5	2.3	5.4					8.6
10332.0	40	19-Norethindrone	-6	9959.7	14939.5	60.5	-3.6	13.2	65.1	4.0	2.3	6.2	11.3
11344.0	41		-6	10971.7	16457.5	66.6	2.5	6.3					12.4
11584.0	42		-6	11211.7	16817.5	68.1	4.0	15.8					12.7
13652.0	43	19-Norethindrone	-6.5	13279.7	19919.5	80.7	1.1	1.1	79.2	3.3	1.9	4.2	15.0
12780.0	44		-6.5	12407.7	18611.5	75.4	-4.2	18.0					14.0
13776.0	45		-6.5	13403.7	20105.5	81.4	1.8	3.3					15.1
15139.0	46	19-Norethindrone	-7	14766.7	22150.0	89.7	1.2	1.3	88.7	3.8	2.2	4.3	16.6
15498.0	47		-7	15125.7	22688.5	91.9	3.3	11.2					17.0
14283.0	48		-7	13910.7	20866.0	84.5	-4.0	16.3					15.6
15099.0	49	19-Norethindrone	-7.5	14726.7	22090.0	89.5	-3.5	12.4	90.1	0.8	0.5	0.9	16.5
15142.0	50		-7.5	14769.7	22154.5	89.7	-3.3	10.6					16.6
15355.0	51		-7.5	14982.7	22474.0	91.0	-2.0	3.9					16.8
16305.0	52	19-Norethindrone	-8.5	15932.7	23899.0	96.8	0.9	0.7	98.4	1.7	1.0	1.7	17.9
16530.0	53		-8.5	16157.7	24236.5	98.2	2.2	5.0					18.1
16866.0	54		-8.5	16493.7	24740.5	100.2	4.3	18.2					18.5
7933.0	55	Octyltriethoxysilane	-3	7560.7	11341.0	45.9	0.0	0.0	45.8	0.2	0.1	0.5	8.7
7938.0	56		-3	7565.7	11348.5	46.0	0.0	0.0					8.7
7874.0	57		-3	7501.7	11252.5	45.6	-0.4	0.2					8.6
14573.0	58	Octyltriethoxysilane	-4	14200.7	21301.0	86.3	-2.2	4.8	88.5	2.5	1.4	2.8	16.0
14889.0	59		-4	14516.7	21775.0	88.2	-0.3	0.1					16.3
15381.0	60		-4	15008.7	22513.0	91.2	2.7	7.4					16.9
16416.0	61	Octyltriethoxysilane	-5	16043.7	24065.5	97.5	5.6	31.9	98.4	1.1	0.6	1.1	18.0
16553.0	62		-5	16180.7	24271.0	98.3	6.5	42.0					18.1
16763.0	63		-5	16390.7	24586.0	99.6	7.8	60.1					18.4
15302.0	64	Octyltriethoxysilane	-6	14929.7	22394.5	90.7	-1.1	1.3	92.3	4.3	2.5	4.6	16.8
15039.0	65		-6	14666.7	22000.0	89.1	-2.7	7.4					16.5
16361.0	66		-6	15988.7	23983.0	97.1	5.3	28.2					17.9
15466.0	67	Octyltriethoxysilane	-7	15093.7	22640.5	91.7	-0.1	0.0	89.9	2.0	1.1	2.2	16.9
15246.0	68		-7	14873.7	22310.5	90.4	-1.5	2.1					16.7
14825.0	69		-7	14452.7	21679.0	87.8	-4.0	16.2					16.2
15139.0	70	Octyltriethoxysilane	-8	14766.7	22150.0	89.7	-2.1	4.5	87.3	2.3	1.3	2.6	16.6
14731.0	71		-8	14358.7	21538.0	87.2	-4.6	21.1					16.1
14382.0	72		-8	14009.7	21014.5	85.1	-6.7	45.0					15.8
16156.0	73	Octyltriethoxysilane	-9	15783.7	23675.5	95.9	4.1	16.5	93.0	3.0	1.7	3.2	17.7
15710.0	74		-9	15337.7	23006.5	93.2	1.4	1.8					17.2
15166.0	75		-9	14793.7	22190.5	89.9	-1.9	3.8					16.6
15027.0	76	Octyltriethoxysilane	-10	14654.7	21982.0	89.0	-2.8	7.8	89.9	1.4	0.8	1.5	16.5
15433.0	77		-10	15060.7	22591.0	91.5	-0.3	0.1					16.9
15056.0	78		-10	14683.7	22025.5	89.2	-2.6	6.8					16.5
16017	79	Methoxycinnamate	-4	15644.7	23467.0	95.0	-0.3	0.1	93.4	1.4	0.8	1.5	17.5
15679	80		-4	15306.7	22960.0	93.0	-2.4	5.6					17.2
15565	81		-4	15192.7	22789.0	92.3	-3.1	9.3					17.1
16396	82	Methoxycinnamate	-5	16023.7	24035.5	97.3	2.0	4.0	97.3	0.4	0.2	0.4	18.0
16305	83		-5	15932.7	23899.0	96.8	1.4	2.1					17.9
16444	84		-5	16071.7	24107.5	97.6	2.3	5.2					18.0
16025	85	Methoxycinnamate	-6	15652.7	23479.0	95.1	0.9	0.7	94.2	0.8	0.5	0.9	17.6
15866	86		-6	15493.7	23240.5	94.1	-0.1	0.0					17.4
15759	87		-6	15386.7	23080.0	93.5	-0.8	0.6					17.3
15530	88	Methoxycinnamate	-7	15157.7	22736.5	92.1	1.9	3.6	89.4	5.9	3.4	6.6	17.0
15767	89		-7	15394.7	23092.0	93.5	3.3	11.1					17.3
13978	90		-7	13605.7	20408.5	82.6	-7.5	56.7					15.3
15221	91	Methoxycinnamate	-8	14848.7	22273.0	90.2	0.0	0.0	91.0	1.4	0.8	1.5	16.7
15234	92		-8	14861.7	22292.5	90.3	0.1	0.0					16.7
15620	93		-8	15247.7	22871.5	92.6	2.4	6.0					17.1
14856	94	Methoxycinnamate	-9	14483.7	21725.5	88.0	-2.2	4.8	89.5	1.9	1.1	2.2	16.3
15003	95		-9	14630.7	21946.0	88.9	-1.3	1.7					16.4
15467	96		-9	15094.7	22642.0	91.7	1.5	2.3					16.9
15249	97	Methoxycinnamate	-10	14876.7	22315.0	90.4	0.2	0.0	90.9	0.4	0.2	0.5	16.7
15353	98		-10	14980.7	22471.0	91.0	0.8	0.7					16.8
15383	99		-10	15010.7	22516.0	91.2	1.0	1.0					16.9
15270	100	Methoxycinnamate	-11	14897.7	22346.5	90.5	0.3	0.1	90.1	2.5	1.5	2.8	16.7
14752	101		-11	14379.7	21569.5	87.4	-2.8	8.0					16.2
15581	102		-11	15208.7	22813.0	92.4	2.2	4.9					17.1







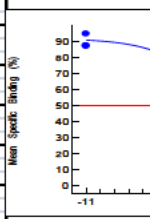


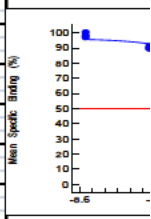
APPENDIX 1 Raw and Normalized Data 2nd Valid Run (continued) – August 01, 2011

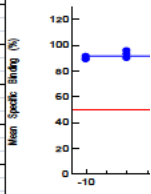
Experiment Date:	1-Aug-11	Study Number:	9070-100107ERB	Assays Conducted by:	[redacted]
Test substance:	OctylSalicylate				
1/27/2012 16:15					
	10 uL of 50 nM E2=	____	DPM and 0.5 x10-12 moles		
	Therefore there are	____	DPM/mole		
	ug protein/assay tube =	80.0			

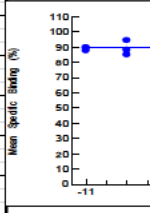
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17533	--	136757.4	136909.5
2		17478	--	136328.4	
3		17405	--	135759.0	
4		17532	--	136749.6	
5		17605	--	137319.0	
6		17762	--	138543.6	
7	Total Binding (Solvent Control)	17487	17114.7	25672.0	24693.0
8		15894	15521.7	23282.5	
9		17122	16749.7	25124.5	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
414.0	10	Estradiol (NSB)	-7	41.7	62.5	0.3	0.1	0.0	0.0	0.2	0.1	2.0E+17	0.5
356.0	11		-7	-16.3	-24.5	-0.1	-0.2	0.0					0.4
347.0	12		-7	-25.3	-38.0	-0.2	-0.3	0.1					0.4
1728.0	13	Estradiol	-8	1355.7	2033.5	8.2	-0.5	0.3	7.7	0.5	0.3	6.3	1.9
1626.0	14		-8	1253.7	1880.5	7.6	-1.1	1.3					1.8
1570.0	15		-8	1197.7	1796.5	7.3	-1.5	2.2					1.7
4889.0	16	Estradiol	-8.5	4516.7	6775.0	27.4	4.1	16.7	26.2	2.1	1.2	7.9	5.4
4289.0	17		-8.5	3916.7	5875.0	23.8	0.4	0.2					4.7
4870.0	18		-8.5	4497.7	6746.5	27.3	4.0	15.7					5.3
7687.0	19	Estradiol	-9	7314.7	10972.0	44.4	-2.7	7.5	44.4	0.8	0.4	1.7	8.4
7813.0	20		-9	7440.7	11161.0	45.2	-2.0	3.9					8.6
7563.0	21		-9	7190.7	10786.0	43.7	-3.5	12.2					8.3
12394.0	22	Estradiol	-9.5	12021.7	18032.5	73.0	2.8	7.6	70.8	1.9	1.1	2.7	13.6
11797.0	23		-9.5	11424.7	17137.0	69.4	-0.9	0.8					12.9
11911.0	24		-9.5	11538.7	17308.0	70.1	-0.2	0.0					13.0
15329.0	25	Estradiol	-10	14956.7	22435.0	90.9	7.1	50.7	85.5	4.7	2.7	5.5	16.8
14149.0	26		-10	13776.7	20665.0	83.7	0.0	0.0					15.5
13855.0	27		-10	13482.7	20224.0	81.9	-1.8	3.4					15.2
16046.0	28	Estradiol	-11	15673.7	23510.5	95.2	3.8	14.1	90.2	4.3	2.5	4.8	17.6
14839.0	29		-11	14466.7	21700.0	87.9	-3.6	12.8					16.3
14779.0	30		-11	14406.7	21610.0	87.5	-3.9	15.5					16.2
526.0	31	19-Norethindrone	-4	153.7	230.5	0.9	1.3	1.7	0.8	0.1	0.1	12.5	0.6
514.0	32		-4	141.7	212.5	0.9	1.2	1.5					0.6
492.0	33		-4	119.7	179.5	0.7	1.1	1.2					0.5
1226.0	34	19-Norethindrone	-4.5	853.7	1280.5	5.2	-2.7	7.5	6.3	1.0	0.6	16.1	1.3
1434.0	35		-4.5	1061.7	1592.5	6.4	-1.5	2.2					1.6
1554.0	36		-4.5	1181.7	1772.5	7.2	-0.7	0.6					1.7
6886.0	37	19-Norethindrone	-5.5	6513.7	9770.5	39.6	-3.6	13.0	43.4	3.3	1.9	7.7	7.5
7807.0	38		-5.5	7434.7	11152.0	45.2	2.0	3.9					8.6
7863.0	39		-5.5	7490.7	11236.0	45.5	2.3	5.4					8.6
10332.0	40	19-Norethindrone	-6	9959.7	14939.5	60.5	-3.6	13.2	65.1	4.0	2.3	6.2	11.3
11344.0	41		-6	10971.7	16457.5	66.6	2.5	6.3					12.4
11584.0	42		-6	11211.7	16817.5	68.1	4.0	15.8					12.7
13652.0	43	19-Norethindrone	-6.5	13279.7	19919.5	80.7	1.1	1.1	79.2	3.3	1.9	4.2	15.0
12780.0	44		-6.5	12407.7	18611.5	75.4	-4.2	18.0					14.0
13776.0	45		-6.5	13403.7	20105.5	81.4	1.8	3.3					15.1
15139.0	46	19-Norethindrone	-7	14766.7	22150.0	89.7	1.2	1.3	88.7	3.8	2.2	4.3	16.6
15498.0	47		-7	15125.7	22688.5	91.9	3.3	11.2					17.0
14283.0	48		-7	13910.7	20866.0	84.5	-4.0	16.3					15.6
15099.0	49	19-Norethindrone	-7.5	14726.7	22090.0	89.5	-3.5	12.4	90.1	0.8	0.5	0.9	16.5
15142.0	50		-7.5	14769.7	22154.5	89.7	-3.3	10.6					16.6
15355.0	51		-7.5	14982.7	22474.0	91.0	-2.0	3.9					16.8
16305.0	52	19-Norethindrone	-8.5	15932.7	23899.0	96.8	0.9	0.7	98.4	1.7	1.0	1.7	17.9
16530.0	53		-8.5	16157.7	24236.5	98.2	2.2	5.0					18.1
16866.0	54		-8.5	16493.7	24740.5	100.2	4.3	18.2					18.5
7933.0	55	Octyltriethoxysilane	-3	7560.7	11341.0	45.9	0.0	0.0	45.8	0.2	0.1	0.5	8.7
7938.0	56		-3	7565.7	11348.5	46.0	0.0	0.0					8.7
7874.0	57		-3	7501.7	11252.5	45.6	-0.4	0.2					8.6
14573.0	58	Octyltriethoxysilane	-4	14200.7	21301.0	86.3	-2.2	4.8	88.5	2.5	1.4	2.8	16.0
14889.0	59		-4	14516.7	21775.0	88.2	-0.3	0.1					16.3
15381.0	60		-4	15008.7	22513.0	91.2	2.7	7.4					16.9
16416.0	61	Octyltriethoxysilane	-5	16043.7	24065.5	97.5	5.6	31.9	98.4	1.1	0.6	1.1	18.0
16553.0	62		-5	16180.7	24271.0	98.3	6.5	42.0					18.1
16763.0	63		-5	16390.7	24586.0	99.6	7.8	60.1					18.4
15302.0	64	Octyltriethoxysilane	-6	14929.7	22394.5	90.7	-1.1	1.3	92.3	4.3	2.5	4.6	16.8
15039.0	65		-6	14666.7	22000.0	89.1	-2.7	7.4					16.5
16361.0	66		-6	15988.7	23983.0	97.1	5.3	28.2					17.9
15466.0	67	Octyltriethoxysilane	-7	15093.7	22640.5	91.7	-0.1	0.0	89.9	2.0	1.1	2.2	16.9
15246.0	68		-7	14873.7	22310.5	90.4	-1.5	2.1					16.7
14825.0	69		-7	14452.7	21679.0	87.8	-4.0	16.2					16.2
15139.0	70	Octyltriethoxysilane	-8	14766.7	22150.0	89.7	-2.1	4.5	87.3	2.3	1.3	2.6	16.6
14731.0	71		-8	14358.7	21538.0	87.2	-4.6	21.1					16.1
14382.0	72		-8	14009.7	21014.5	85.1	-6.7	45.0					15.8
16156.0	73	Octyltriethoxysilane	-9	15783.7	23675.5	95.9	4.1	16.5	93.0	3.0	1.7	3.2	17.7
15710.0	74		-9	15337.7	23006.5	93.2	1.4	1.8					17.2
15166.0	75		-9	14793.7	22190.5	89.9	-1.9	3.8					16.6
15027.0	76	Octyltriethoxysilane	-10	14654.7	21982.0	89.0	-2.8	7.8	89.9	1.4	0.8	1.5	16.5
15433.0	77		-10	15060.7	22591.0	91.5	-0.3	0.1					16.9
15056.0	78		-10	14683.7	22025.5	89.2	-2.6	6.8					16.5
11592	79	OctylSalicylate	-4	11219.7	16829.5	68.2	-0.6	0.4	68.7	0.5	0.3	0.8	12.7
11746	80		-4	11373.7	17060.5	69.1	0.3	0.1					12.9
11731	81		-4	11358.7	17038.0	69.0	0.2	0.1					12.9
13671	82	OctylSalicylate	-5	13298.7	19948.0	80.8	-2.4	5.8	83.2	2.2	1.3	2.7	15.0
14148	83		-5	13775.7	20663.5	83.7	0.5	0.2					15.5
14397	84		-5	14024.7	21037.0	85.2	2.0	4.0					15.8
14451	85	OctylSalicylate	-6	14078.7	21118.0	85.5	-3.6	13.1	88.8	2.9	1.6	3.2	15.8
15246	86		-6	14873.7	22310.5	90.4	1.2	1.5					16.7
15284	87		-6	14911.7	22367.5	90.6	1.4	2.1					16.7
15812	88	OctylSalicylate	-7	15439.7	23159.5	93.8	3.9	14.8	91.2	2.3	1.3	2.5	17.3
15259	89		-7	14886.7	22330.0	90.4	0.5	0.2					16.7
15091	90		-7	14718.7	22078.0	89.4	-0.5	0.3					16.5
15459	91	OctylSalicylate	-8	15086.7	22630.0	91.6	1.6	2.6	91.3	1.5	0.8	1.6	16.9
15148	92		-8	14775.7	22163.5	89.8	-0.3	0.1					16.6
15619	93		-8	15246.7	22870.0	92.6	2.6	6.8					17.1
14459	94	OctylSalicylate	-9	14086.7	21130.0	85.6	-4.5	19.9	89.6	6.0	3.5	6.7	15.8
14646	95		-9	14273.7	21410.5	86.7	-3.3	11.0					16.0
16268	96		-9	15895.7	23843.5	96.6	6.5	42.7					17.8
14882	97	OctylSalicylate	-10	14509.7	21764.5	88.1	-1.9	3.6	89.2	4.8	2.8	5.4	16.3
14377	98		-10	14004.7	21007.0	85.1	-5.0	24.6					15.8
15919	99		-10	15546.7	23320.0	94.4	4.4	19.5					17.4
14822	100	OctylSalicylate	-11	14449.7	21674.5	87.8	-2.3	5.1	89.0	1.0	0.6	1.2	16.2
15078	101		-11	14705.7	22058.5	89.3	-0.7	0.5					16.5
15149	102		-11	14776.7	22165.0	89.8	-0.3	0.1					16.6







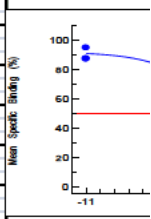


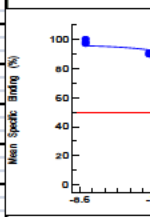
APPENDIX 1 Raw and Normalized Data 2nd Valid Run (continued) – August 01, 2011

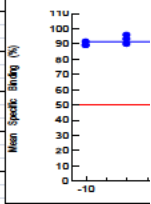
Experiment Date:	1-Aug-11	Study Number:	9070-100107ERB	Assays Conducted by:	[redacted]
Test substance:	Octocrylene				
1/27/2012 16:16					
	10 uL of 50 nM E2=	____	DPM and 0.5 x10-12 moles		
	Therefore there are	____	DPM/mole		
	ug protein/assay tube =	80.0			

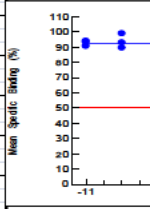
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17533	--	136757.4	136909.5
2		17478	--	136328.4	
3		17405	--	135759.0	
4		17532	--	136749.6	
5		17605	--	137319.0	
6		17762	--	138543.6	
7	Total Binding (Solvent Control)	17487	17114.7	25672.0	24693.0
8		15894	15521.7	23282.5	
9		17122	16749.7	25124.5	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
414.0	10	Estradiol (NSB)	-7	41.7	62.5	0.3	0.1	0.0	0.0	0.2	0.1	2.0E+17	0.5
356.0	11		-7	-16.3	-24.5	-0.1	-0.2	0.0					0.4
347.0	12		-7	-25.3	-38.0	-0.2	-0.3	0.1					0.4
1728.0	13	Estradiol	-8	1355.7	2033.5	8.2	-0.5	0.3	7.7	0.5	0.3	6.3	1.9
1626.0	14		-8	1253.7	1880.5	7.6	-1.1	1.3					1.8
1570.0	15		-8	1197.7	1796.5	7.3	-1.5	2.2					1.7
4889.0	16	Estradiol	-8.5	4516.7	6775.0	27.4	4.1	16.7	26.2	2.1	1.2	7.9	5.4
4289.0	17		-8.5	3916.7	5875.0	23.8	0.4	0.2					4.7
4870.0	18		-8.5	4497.7	6746.5	27.3	4.0	15.7					5.3
7687.0	19	Estradiol	-9	7314.7	10972.0	44.4	-2.7	7.5	44.4	0.8	0.4	1.7	8.4
7813.0	20		-9	7440.7	11161.0	45.2	-2.0	3.9					8.6
7563.0	21		-9	7190.7	10786.0	43.7	-3.5	12.2					8.3
12394.0	22	Estradiol	-9.5	12021.7	18032.5	73.0	2.8	7.6	70.8	1.9	1.1	2.7	13.6
11797.0	23		-9.5	11424.7	17137.0	69.4	-0.9	0.8					12.9
11911.0	24		-9.5	11538.7	17308.0	70.1	-0.2	0.0					13.0
15329.0	25	Estradiol	-10	14956.7	22435.0	90.9	7.1	50.7	85.5	4.7	2.7	5.5	16.8
14149.0	26		-10	13776.7	20665.0	83.7	0.0	0.0					15.5
13855.0	27		-10	13482.7	20224.0	81.9	-1.8	3.4					15.2
16046.0	28	Estradiol	-11	15673.7	23510.5	95.2	3.8	14.1	90.2	4.3	2.5	4.8	17.6
14839.0	29		-11	14466.7	21700.0	87.9	-3.6	12.8					16.3
14779.0	30		-11	14406.7	21610.0	87.5	-3.9	15.5					16.2
526.0	31	19-Norethindrone	-4	153.7	230.5	0.9	1.3	1.7	0.8	0.1	0.1	12.5	0.6
514.0	32		-4	141.7	212.5	0.9	1.2	1.5					0.6
492.0	33		-4	119.7	179.5	0.7	1.1	1.2					0.5
1226.0	34	19-Norethindrone	-4.5	853.7	1280.5	5.2	-2.7	7.5	6.3	1.0	0.6	16.1	1.3
1434.0	35		-4.5	1061.7	1592.5	6.4	-1.5	2.2					1.6
1554.0	36		-4.5	1181.7	1772.5	7.2	-0.7	0.6					1.7
6886.0	37	19-Norethindrone	-5.5	6513.7	9770.5	39.6	-3.6	13.0	43.4	3.3	1.9	7.7	7.5
7807.0	38		-5.5	7434.7	11152.0	45.2	2.0	3.9					8.6
7863.0	39		-5.5	7490.7	11236.0	45.5	2.3	5.4					8.6
10332.0	40	19-Norethindrone	-6	9959.7	14939.5	60.5	-3.6	13.2	65.1	4.0	2.3	6.2	11.3
11344.0	41		-6	10971.7	16457.5	66.6	2.5	6.3					12.4
11584.0	42		-6	11211.7	16817.5	68.1	4.0	15.8					12.7
13652.0	43	19-Norethindrone	-6.5	13279.7	19919.5	80.7	1.1	1.1	79.2	3.3	1.9	4.2	15.0
12780.0	44		-6.5	12407.7	18611.5	75.4	-4.2	18.0					14.0
13776.0	45		-6.5	13403.7	20105.5	81.4	1.8	3.3					15.1
15139.0	46	19-Norethindrone	-7	14766.7	22150.0	89.7	1.2	1.3	88.7	3.8	2.2	4.3	16.6
15498.0	47		-7	15125.7	22688.5	91.9	3.3	11.2					17.0
14283.0	48		-7	13910.7	20866.0	84.5	-4.0	16.3					15.6
15099.0	49	19-Norethindrone	-7.5	14726.7	22090.0	89.5	-3.5	12.4	90.1	0.8	0.5	0.9	16.5
15142.0	50		-7.5	14769.7	22154.5	89.7	-3.3	10.6					16.6
15355.0	51		-7.5	14982.7	22474.0	91.0	-2.0	3.9					16.8
16305.0	52	19-Norethindrone	-8.5	15932.7	23899.0	96.8	0.9	0.7	98.4	1.7	1.0	1.7	17.9
16530.0	53		-8.5	16157.7	24236.5	98.2	2.2	5.0					18.1
16866.0	54		-8.5	16493.7	24740.5	100.2	4.3	18.2					18.5
7933.0	55	Octyltriethoxysilane	-3	7560.7	11341.0	45.9	0.0	0.0	45.8	0.2	0.1	0.5	8.7
7938.0	56		-3	7565.7	11348.5	46.0	0.0	0.0					8.7
7874.0	57		-3	7501.7	11252.5	45.6	-0.4	0.2					8.6
14573.0	58	Octyltriethoxysilane	-4	14200.7	21301.0	86.3	-2.2	4.8	88.5	2.5	1.4	2.8	16.0
14889.0	59		-4	14516.7	21775.0	88.2	-0.3	0.1					16.3
15381.0	60		-4	15008.7	22513.0	91.2	2.7	7.4					16.9
16416.0	61	Octyltriethoxysilane	-5	16043.7	24065.5	97.5	5.6	31.9	98.4	1.1	0.6	1.1	18.0
16553.0	62		-5	16180.7	24271.0	98.3	6.5	42.0					18.1
16763.0	63		-5	16390.7	24586.0	99.6	7.8	60.1					18.4
15302.0	64	Octyltriethoxysilane	-6	14929.7	22394.5	90.7	-1.1	1.3	92.3	4.3	2.5	4.6	16.8
15039.0	65		-6	14666.7	22000.0	89.1	-2.7	7.4					16.5
16361.0	66		-6	15988.7	23983.0	97.1	5.3	28.2					17.9
15466.0	67	Octyltriethoxysilane	-7	15093.7	22640.5	91.7	-0.1	0.0	89.9	2.0	1.1	2.2	16.9
15246.0	68		-7	14873.7	22310.5	90.4	-1.5	2.1					16.7
14825.0	69		-7	14452.7	21679.0	87.8	-4.0	16.2					16.2
15139.0	70	Octyltriethoxysilane	-8	14766.7	22150.0	89.7	-2.1	4.5	87.3	2.3	1.3	2.6	16.6
14731.0	71		-8	14358.7	21538.0	87.2	-4.6	21.1					16.1
14382.0	72		-8	14009.7	21014.5	85.1	-6.7	45.0					15.8
16156.0	73	Octyltriethoxysilane	-9	15783.7	23675.5	95.9	4.1	16.5	93.0	3.0	1.7	3.2	17.7
15710.0	74		-9	15337.7	23006.5	93.2	1.4	1.8					17.2
15166.0	75		-9	14793.7	22190.5	89.9	-1.9	3.8					16.6
15027.0	76	Octyltriethoxysilane	-10	14654.7	21982.0	89.0	-2.8	7.8	89.9	1.4	0.8	1.5	16.5
15433.0	77		-10	15060.7	22591.0	91.5	-0.3	0.1					16.9
15056.0	78		-10	14683.7	22025.5	89.2	-2.6	6.8					16.5
13433	79	Octocrylene	-4	13060.7	19591.0	79.3	-2.3	5.2	81.6	2.0	1.2	2.4	14.7
14039	80		-4	13666.7	20500.0	83.0	1.4	2.0					15.4
13954	81		-4	13581.7	20372.5	82.5	0.9	0.8					15.3
15096	82	Octocrylene	-5	14723.7	22085.5	89.4	-0.9	0.7	90.3	2.4	1.4	2.7	16.5
14922	83		-5	14549.7	21824.5	88.4	-1.9	3.7					16.3
15680	84		-5	15307.7	22961.5	93.0	2.7	7.3					17.2
15193	85	Octocrylene	-6	14820.7	22231.0	90.0	-2.2	4.8	92.4	2.1	1.2	2.2	16.6
15816	86		-6	15443.7	23165.5	93.8	1.6	2.6					17.3
15750	87		-6	15377.7	23066.5	93.4	1.2	1.4					17.3
15938	88	Octocrylene	-7	15565.7	23348.5	94.6	1.9	3.7	92.1	2.8	1.6	3.0	17.5
15042	89		-7	14669.7	22004.5	89.1	-3.5	12.3					16.5
15637	90		-7	15264.7	22897.0	92.7	0.1	0.0					17.1
15521	91	Octocrylene	-8	15148.7	22723.0	92.0	-0.7	0.5	93.0	1.3	0.7	1.4	17.0
15922	92		-8	15549.7	23324.5	94.5	1.7	3.1					17.4
15598	93		-8	15225.7	22838.5	92.5	-0.2	0.0					17.1
15501	94	Octocrylene	-9	15128.7	22693.0	91.9	-0.8	0.7	91.8	2.5	1.5	2.7	17.0
15055	95		-9	14682.7	22024.0	89.2	-3.5	12.5					16.5
15882	96		-9	15509.7	23264.5	94.2	1.5	2.2					17.4
16654	97	Octocrylene	-10	16281.7	24422.5	98.9	6.2	38.1	93.8	4.8	2.7	5.1	18.2
15108	98		-10	14735.7	22103.5	89.5	-3.2	10.4					16.6
15668	99		-10	15295.7	22943.5	92.9	0.2	0.0					17.2
15740	100	Octocrylene	-11	15367.7	23051.5	93.4	0.6	0.4	92.7	1.8	1.0	1.9	17.2
15851	101		-11	15478.7	23218.0	94.0	1.3	1.7					17.4
15295	102		-11	14922.7	22384.0	90.6	-2.1	4.3					16.8







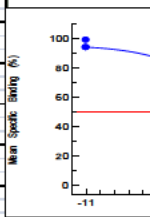


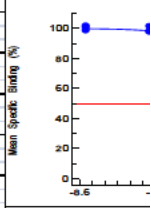
APPENDIX 1 Raw and Normalized Data 3rd Valid Run – August 03, 2011

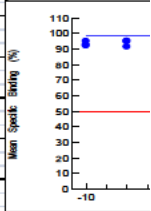
Experiment Date:	3-Aug-11		Study Number:	9070-100107ERB					Assays Conducted by:	[redacted]			
Test substance:	Oxybenzone												
1/27/2012 16:16													
		10 uL of 50 nM E2=	____	DPM and 0.5 x10-12 moles									
		Therefore there are		DPM/mole									
		ug protein/assay tube =	80.0										

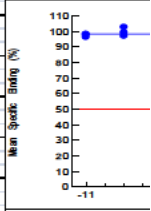
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17691	--	137989.8	137285.2
2		17594	--	137233.2	
3		17342	--	135267.6	
4		17475	--	136305.0	
5		17914	--	139729.2	
6		17588	--	137186.4	
7	Total Binding (Solvent Control)	15791	15438.0	23157.0	23891.5
8		18098	17745.0	26617.5	
9		14953	14600.0	21900.0	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
330.0	10	Estradiol (NSB)	-7	-23.0	-34.5	-0.1	1.6	2.4	0.0	0.1	0.1	#DIV/0!	0.4
369.0	11		-7	16.0	24.0	0.1	1.8	3.2					0.4
360.0	12		-7	7.0	10.5	0.0	1.7	3.0					0.4
1608.0	13	Estradiol	-8	1255.0	1882.5	7.9	-5.8	33.4	9.3	1.3	0.8	14.3	1.8
1869.0	14		-8	1516.0	2274.0	9.5	-4.1	17.1					2.0
2029.0	15		-8	1676.0	2514.0	10.5	-3.1	9.8					2.2
6167.0	16	Estradiol	-8.5	5814.0	8721.0	36.5	4.2	17.8	35.3	5.7	3.3	16.0	6.7
6777.0	17		-8.5	6424.0	9636.0	40.3	8.1	64.8					7.4
5002.0	18		-8.5	4649.0	6973.5	29.2	-3.1	9.6					5.5
7930.0	19	Estradiol	-9	7577.0	11365.5	47.6	-8.1	66.4	56.9	8.1	4.7	14.3	8.7
10344.0	20		-9	9991.0	14986.5	62.7	7.0	49.1					11.3
9954.0	21		-9	9601.0	14401.5	60.3	4.6	20.8					10.9
11512.0	22	Estradiol	-9.5	11159.0	16738.5	70.1	-5.2	26.8	73.1	2.9	1.7	4.0	12.6
12049.0	23		-9.5	11696.0	17544.0	73.4	-1.8	3.3					13.2
12434.0	24		-9.5	12081.0	18121.5	75.8	0.6	0.4					13.6
13520.0	25	Estradiol	-10	13167.0	19750.5	82.7	-4.0	15.8	85.9	3.0	1.7	3.5	14.8
14159.0	26		-10	13806.0	20709.0	86.7	0.0	0.0					15.5
14447.0	27		-10	14094.0	21141.0	88.5	1.8	3.4					15.8
15295.0	28	Estradiol	-11	14942.0	22413.0	93.8	-0.4	0.2	95.5	2.9	1.7	3.0	16.7
15296.0	29		-11	14943.0	22414.5	93.8	-0.4	0.2					16.7
16096.0	30		-11	15743.0	23614.5	98.8	4.6	21.4					17.6
506.0	31	19-Norethindrone	-4	153.0	229.5	1.0	-0.8	0.6	1.6	1.0	0.6	62.1	0.6
801.0	32		-4	448.0	672.0	2.8	1.1	1.1					0.9
536.0	33		-4	183.0	274.5	1.1	-0.6	0.4					0.6
1336.0	34	19-Norethindrone	-4.5	983.0	1474.5	6.2	-0.6	0.3	6.9	0.7	0.4	9.7	1.5
1536.0	35		-4.5	1183.0	1774.5	7.4	0.7	0.4					1.7
1503.0	36		-4.5	1150.0	1725.0	7.2	0.5	0.2					1.6
6890.0	37	19-Norethindrone	-5.5	6537.0	9805.5	41.0	-0.9	0.8	41.7	1.3	0.7	3.0	7.5
7226.0	38		-5.5	6873.0	10309.5	43.2	1.2	1.4					7.9
6871.0	39		-5.5	6518.0	9777.0	40.9	-1.0	1.1					7.5
11340.0	40	19-Norethindrone	-6	10987.0	16480.5	69.0	0.4	0.2	68.8	0.7	0.4	1.1	12.4
11182.0	41		-6	10829.0	16243.5	68.0	-0.6	0.4					12.2
11416.0	42		-6	11063.0	16594.5	69.5	0.9	0.8					12.5
14118.0	43	19-Norethindrone	-6.5	13765.0	20647.5	86.4	-0.4	0.2	87.3	2.6	1.5	3.0	15.4
13932.0	44		-6.5	13579.0	20368.5	85.3	-1.6	2.6					15.2
14721.0	45		-6.5	14368.0	21552.0	90.2	3.3	11.1					16.1
14421.0	46	19-Norethindrone	-7	14068.0	21102.0	88.3	-7.0	49.6	94.2	5.1	2.9	5.4	15.8
15865.0	47		-7	15512.0	23268.0	97.4	2.0	4.1					17.3
15783.0	48		-7	15430.0	23145.0	96.9	1.5	2.3					17.2
16511.0	49	19-Norethindrone	-7.5	16158.0	24237.0	101.4	2.8	8.1	99.1	2.0	1.2	2.1	18.0
15991.0	50		-7.5	15638.0	23457.0	98.2	-0.4	0.2					17.5
15909.0	51		-7.5	15556.0	23334.0	97.7	-0.9	0.9					17.4
16054.0	52	19-Norethindrone	-8.5	15701.0	23551.5	98.6	-1.5	2.4	100.3	1.6	0.9	1.6	17.5
16389.0	53		-8.5	16036.0	24054.0	100.7	0.6	0.3					17.9
16552.0	54		-8.5	16199.0	24298.5	101.7	1.6	2.5					18.1
8461.0	55	Octyltriethoxysilane	-3	8108.0	12162.0	50.9	2.7	7.4	48.2	2.6	1.5	5.4	9.2
7634.0	56		-3	7281.0	10921.5	45.7	-2.5	6.1					8.3
7988.0	57		-3	7635.0	11452.5	47.9	-0.2	0.1					8.7
15334.0	58	Octyltriethoxysilane	-4	14981.0	22471.5	94.1	3.2	10.2	90.9	2.8	1.6	3.1	16.8
14505.0	59		-4	14152.0	21228.0	88.9	-2.0	4.0					15.8
14637.0	60		-4	14284.0	21426.0	89.7	-1.2	1.4					16.0
16252.0	61	Octyltriethoxysilane	-5	15899.0	23848.5	99.8	0.9	0.9	101.4	2.0	1.2	2.0	17.8
16864.0	62		-5	16511.0	24766.5	103.7	4.8	22.7					18.4
16382.0	63		-5	16029.0	24043.5	100.6	1.7	3.0					17.9
16894.0	64	Octyltriethoxysilane	-6	16541.0	24811.5	103.9	5.0	24.6	105.1	1.7	1.0	1.6	18.5
17408.0	65		-6	17055.0	25582.5	107.1	8.2	67.0					19.0
16983.0	66		-6	16630.0	24945.0	104.4	5.5	30.4					18.6
16273.0	67	Octyltriethoxysilane	-7	15920.0	23880.0	100.0	1.1	1.1	101.7	3.0	1.8	3.0	17.8
17111.0	68		-7	16758.0	25137.0	105.2	6.3	39.9					18.7
16274.0	69		-7	15921.0	23881.5	100.0	1.1	1.1					17.8
15932.0	70	Octyltriethoxysilane	-8	15579.0	23368.5	97.8	-1.1	1.2	98.1	0.4	0.2	0.4	17.4
16050.0	71		-8	15697.0	23545.5	98.6	-0.3	0.1					17.5
15932.0	72		-8	15579.0	23368.5	97.8	-1.1	1.2					17.4
14920.0	73	Octyltriethoxysilane	-9	14567.0	21850.5	91.5	-7.4	55.3	93.9	2.1	1.2	2.2	16.3
15460.0	74		-9	15107.0	22660.5	94.8	-4.0	16.4					16.9
15524.0	75		-9	15171.0	22756.5	95.2	-3.6	13.3					17.0
15033.0	76	Octyltriethoxysilane	-10	14680.0	22020.0	92.2	-6.7	45.3	93.3	1.5	0.9	1.7	16.4
15100.0	77		-10	14747.0	22120.5	92.6	-6.3	39.8					16.5
15490.0	78		-10	15137.0	22705.5	95.0	-3.9	14.9					16.9
12761	79	Oxybenzone	-4	12408.0	18612.0	77.9	0.6	0.4	77.2	0.7	0.4	0.9	13.9
12550	80		-4	12197.0	18295.5	76.6	-0.7	0.5					13.7
12643	81		-4	12290.0	18435.0	77.2	-0.1	0.0					13.8
16013	82	Oxybenzone	-5	15660.0	23490.0	98.3	0.7	0.5	97.7	0.9	0.5	1.0	17.5
15736	83		-5	15383.0	23074.5	96.6	-1.1	1.1					17.2
15976	84		-5	15623.0	23434.5	98.1	0.4	0.2					17.5
15613	85	Oxybenzone	-6	15260.0	22890.0	95.8	-2.7	7.4	98.1	2.0	1.2	2.0	17.1
16183	86		-6	15830.0	23745.0	99.4	0.9	0.7					17.7
16150	87		-6	15797.0	23695.5	99.2	0.7	0.4					17.6
15445	88	Oxybenzone	-7	15092.0	22638.0	94.8	-3.8	14.4	98.3	3.1	1.8	3.2	16.9
16413	89		-7	16060.0	24090.0	100.8	2.3	5.2					17.9
16152	90		-7	15799.0	23698.5	99.2	0.6	0.4					17.6
16132	91	Oxybenzone	-8	15779.0	23668.5	99.1	0.5	0.3	100.2	1.6	0.9	1.6	17.6
16597	92		-8	16244.0	24366.0	102.0	3.4	11.8					18.1
16195	93		-8	15842.0	23763.0	99.5	0.9	0.8					17.7
15865	94	Oxybenzone	-9	15512.0	23268.0	97.4	-1.2	1.3	97.6	1.0	0.6	1.0	17.3
16063	95		-9	15710.0	23565.0	98.6	0.1	0.0					17.6
15748	96		-9	15395.0	23092.5	96.7	-1.9	3.6					17.2
16166	97	Oxybenzone	-10	15813.0	23719.5	99.3	0.7	0.5	99.7	2.9	1.6	2.9	17.7
15807	98		-10	15454.0	23181.0	97.0	-1.5	2.3					17.3
16711	99		-10	16358.0	24537.0	102.7	4.2	17.2					18.3
15941	100	Oxybenzone	-11	15588.0	23382.0	97.9	-0.7	0.5	97.5	1.0	0.6	1.1	17.4
15690	101		-11	15337.0	23005.5	96.3	-2.3	5.1					17.1
16002	102		-11	15649.0	23473.5	98.3	-0.3	0.1					17.5







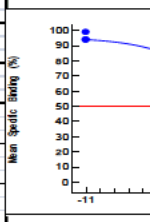


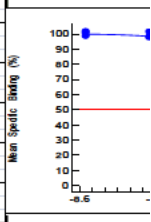
APPENDIX 1 Raw and Normalized Data 3rd Valid Run (continued) – August 03, 2011

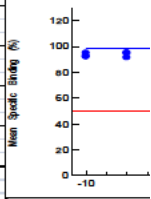
Experiment Date:	3-Aug-11	Study Number:	9070-100107ERB	Assays Conducted by:	[redacted]
Test substance:	Methoxycinnamate				
1/27/2012 16:17					
	10 uL of 50 nM E2=	____	DPM and 0.5 x10-12 moles		
	Therefore there are	____	DPM/mole		
	ug protein/assay tube =	80.0			

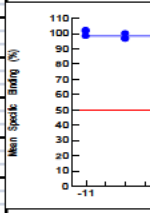
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17691	--	137989.8	137285.2
2		17594	--	137233.2	
3		17342	--	135267.6	
4		17475	--	136305.0	
5		17914	--	139729.2	
6		17588	--	137186.4	
7	Total Binding (Solvent Control)	15791	15438.0	23157.0	23891.5
8		18098	17745.0	26617.5	
9		14953	14600.0	21900.0	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
330.0	10	Estradiol (NSB)	-7	-23.0	-34.5	-0.1	1.6	2.4	0.0	0.1	0.1	#DIV/0!	0.4
369.0	11		-7	16.0	24.0	0.1	1.8	3.2					0.4
360.0	12		-7	7.0	10.5	0.0	1.7	3.0					0.4
1608.0	13	Estradiol	-8	1255.0	1882.5	7.9	-5.8	33.4	9.3	1.3	0.8	14.3	1.8
1869.0	14		-8	1516.0	2274.0	9.5	-4.1	17.1					2.0
2029.0	15		-8	1676.0	2514.0	10.5	-3.1	9.8					2.2
6167.0	16	Estradiol	-8.5	5814.0	8721.0	36.5	4.2	17.8	35.3	5.7	3.3	16.0	6.7
6777.0	17		-8.5	6424.0	9636.0	40.3	8.1	64.8					7.4
5002.0	18		-8.5	4649.0	6973.5	29.2	-3.1	9.6					5.5
7930.0	19	Estradiol	-9	7577.0	11365.5	47.6	-8.1	66.4	56.9	8.1	4.7	14.3	8.7
10344.0	20		-9	9991.0	14986.5	62.7	7.0	49.1					11.3
9954.0	21		-9	9601.0	14401.5	60.3	4.6	20.8					10.9
11512.0	22	Estradiol	-9.5	11159.0	16738.5	70.1	-5.2	26.8	73.1	2.9	1.7	4.0	12.6
12049.0	23		-9.5	11696.0	17544.0	73.4	-1.8	3.3					13.2
12434.0	24		-9.5	12081.0	18121.5	75.8	0.6	0.4					13.6
13520.0	25	Estradiol	-10	13167.0	19750.5	82.7	-4.0	15.8	85.9	3.0	1.7	3.5	14.8
14159.0	26		-10	13806.0	20709.0	86.7	0.0	0.0					15.5
14447.0	27		-10	14094.0	21141.0	88.5	1.8	3.4					15.8
15295.0	28	Estradiol	-11	14942.0	22413.0	93.8	-0.4	0.2	95.5	2.9	1.7	3.0	16.7
15296.0	29		-11	14943.0	22414.5	93.8	-0.4	0.2					16.7
16096.0	30		-11	15743.0	23614.5	98.8	4.6	21.4					17.6
506.0	31	19-Norethindrone	-4	153.0	229.5	1.0	-0.8	0.6	1.6	1.0	0.6	62.1	0.6
801.0	32		-4	448.0	672.0	2.8	1.1	1.1					0.9
536.0	33		-4	183.0	274.5	1.1	-0.6	0.4					0.6
1336.0	34	19-Norethindrone	-4.5	983.0	1474.5	6.2	-0.6	0.3	6.9	0.7	0.4	9.7	1.5
1536.0	35		-4.5	1183.0	1774.5	7.4	0.7	0.4					1.7
1503.0	36		-4.5	1150.0	1725.0	7.2	0.5	0.2					1.6
6890.0	37	19-Norethindrone	-5.5	6537.0	9805.5	41.0	-0.9	0.8	41.7	1.3	0.7	3.0	7.5
7226.0	38		-5.5	6873.0	10309.5	43.2	1.2	1.4					7.9
6871.0	39		-5.5	6518.0	9777.0	40.9	-1.0	1.1					7.5
11340.0	40	19-Norethindrone	-6	10987.0	16480.5	69.0	0.4	0.2	68.8	0.7	0.4	1.1	12.4
11182.0	41		-6	10829.0	16243.5	68.0	-0.6	0.4					12.2
11416.0	42		-6	11063.0	16594.5	69.5	0.9	0.8					12.5
14118.0	43	19-Norethindrone	-6.5	13765.0	20647.5	86.4	-0.4	0.2	87.3	2.6	1.5	3.0	15.4
13932.0	44		-6.5	13579.0	20368.5	85.3	-1.6	2.6					15.2
14721.0	45		-6.5	14368.0	21552.0	90.2	3.3	11.1					16.1
14421.0	46	19-Norethindrone	-7	14068.0	21102.0	88.3	-7.0	49.6	94.2	5.1	2.9	5.4	15.8
15865.0	47		-7	15512.0	23268.0	97.4	2.0	4.1					17.3
15783.0	48		-7	15430.0	23145.0	96.9	1.5	2.3					17.2
16511.0	49	19-Norethindrone	-7.5	16158.0	24237.0	101.4	2.8	8.1	99.1	2.0	1.2	2.1	18.0
15991.0	50		-7.5	15638.0	23457.0	98.2	-0.4	0.2					17.5
15909.0	51		-7.5	15556.0	23334.0	97.7	-0.9	0.9					17.4
16054.0	52	19-Norethindrone	-8.5	15701.0	23551.5	98.6	-1.5	2.4	100.3	1.6	0.9	1.6	17.5
16389.0	53		-8.5	16036.0	24054.0	100.7	0.6	0.3					17.9
16552.0	54		-8.5	16199.0	24298.5	101.7	1.6	2.5					18.1
8461.0	55	Octyltriethoxysilane	-3	8108.0	12162.0	50.9	2.7	7.4	48.2	2.6	1.5	5.4	9.2
7634.0	56		-3	7281.0	10921.5	45.7	-2.5	6.1					8.3
7988.0	57		-3	7635.0	11452.5	47.9	-0.2	0.1					8.7
15334.0	58	Octyltriethoxysilane	-4	14981.0	22471.5	94.1	3.2	10.2	90.9	2.8	1.6	3.1	16.8
14505.0	59		-4	14152.0	21228.0	88.9	-2.0	4.0					15.8
14637.0	60		-4	14284.0	21426.0	89.7	-1.2	1.4					16.0
16252.0	61	Octyltriethoxysilane	-5	15899.0	23848.5	99.8	0.9	0.9	101.4	2.0	1.2	2.0	17.8
16864.0	62		-5	16511.0	24766.5	103.7	4.8	22.7					18.4
16382.0	63		-5	16029.0	24043.5	100.6	1.7	3.0					17.9
16894.0	64	Octyltriethoxysilane	-6	16541.0	24811.5	103.9	5.0	24.6	105.1	1.7	1.0	1.6	18.5
17408.0	65		-6	17055.0	25582.5	107.1	8.2	67.0					19.0
16983.0	66		-6	16630.0	24945.0	104.4	5.5	30.4					18.6
16273.0	67	Octyltriethoxysilane	-7	15920.0	23880.0	100.0	1.1	1.1	101.7	3.0	1.8	3.0	17.8
17111.0	68		-7	16758.0	25137.0	105.2	6.3	39.9					18.7
16274.0	69		-7	15921.0	23881.5	100.0	1.1	1.1					17.8
15932.0	70	Octyltriethoxysilane	-8	15579.0	23368.5	97.8	-1.1	1.2	98.1	0.4	0.2	0.4	17.4
16050.0	71		-8	15697.0	23545.5	98.6	-0.3	0.1					17.5
15932.0	72		-8	15579.0	23368.5	97.8	-1.1	1.2					17.4
14920.0	73	Octyltriethoxysilane	-9	14567.0	21850.5	91.5	-7.4	55.3	93.9	2.1	1.2	2.2	16.3
15460.0	74		-9	15107.0	22660.5	94.8	-4.0	16.4					16.9
15524.0	75		-9	15171.0	22756.5	95.2	-3.6	13.3					17.0
15033.0	76	Octyltriethoxysilane	-10	14680.0	22020.0	92.2	-6.7	45.3	93.3	1.5	0.9	1.7	16.4
15100.0	77		-10	14747.0	22120.5	92.6	-6.3	39.8					16.5
15490.0	78		-10	15137.0	22705.5	95.0	-3.9	14.9					16.9
14534	79	Methoxycinnamate	-4	14181.0	21271.5	89.0	-4.0	16.3	93.1	3.6	2.1	3.9	15.9
15366	80		-4	15013.0	22519.5	94.3	1.2	1.4					16.8
15632	81		-4	15279.0	22918.5	95.9	2.9	8.1					17.1
16534	82	Methoxycinnamate	-5	16181.0	24271.5	101.6	2.9	8.5	101.2	0.5	0.3	0.5	18.1
16381	83		-5	16028.0	24042.0	100.6	2.0	3.8					17.9
16489	84		-5	16136.0	24204.0	101.3	2.6	7.0					18.0
17070	85	Methoxycinnamate	-6	16717.0	25075.5	105.0	6.3	39.5	101.9	2.7	1.6	2.7	18.7
16240	86		-6	15887.0	23830.5	99.7	1.1	1.2					17.7
16449	87		-6	16096.0	24144.0	101.1	2.4	5.7					18.0
16216	88	Methoxycinnamate	-7	15863.0	23794.5	99.6	0.9	0.9	98.8	0.7	0.4	0.8	17.7
16076	89		-7	15723.0	23584.5	98.7	0.0	0.0					17.6
15981	90		-7	15628.0	23442.0	98.1	-0.6	0.3					17.5
15558	91	Methoxycinnamate	-8	15205.0	22807.5	95.5	-3.2	10.3	93.7	2.5	1.4	2.7	17.0
14823	92		-8	14470.0	21705.0	90.8	-7.8	61.2					16.2
15444	93		-8	15091.0	22636.5	94.7	-3.9	15.4					16.9
15823	94	Methoxycinnamate	-9	15470.0	23205.0	97.1	-1.5	2.4	96.8	0.8	0.5	0.8	17.3
15858	95		-9	15505.0	23257.5	97.3	-1.3	1.8					17.3
15622	96		-9	15269.0	22903.5	95.9	-2.8	7.9					17.1
15701	97	Methoxycinnamate	-10	15348.0	23022.0	96.4	-2.3	5.3	97.7	2.0	1.1	2.0	17.2
15785	98		-10	15432.0	23148.0	96.9	-1.8	3.2					17.2
16278	99		-10	15925.0	23887.5	100.0	1.3	1.7					17.8
16506	100	Methoxycinnamate	-11	16153.0	24229.5	101.4	2.7	7.5	100.6	2.1	1.2	2.1	18.0
16000	101		-11	15647.0	23470.5	98.2	-0.4	0.2					17.5
16618	102		-11	16265.0	24397.5	102.1	3.4	11.9					18.2







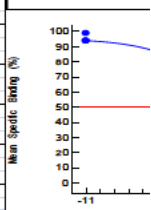


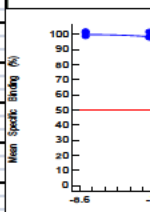
APPENDIX 1 Raw and Normalized Data 3rd Valid Run (continued) – August 03, 2011

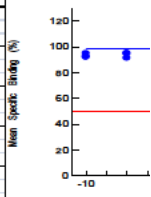
Experiment Date:	3-Aug-11	Study Number:	9070-100107ERB	Assays Conducted by:	[redacted]
Test substance:	OctylSalicylate				
1/27/2012 16:17					
	10 uL of 50 nM E2=	____	DPM and 0.5 x10-12 moles		
	Therefore there are	____	DPM/mole		
	ug protein/assay tube =	80.0			

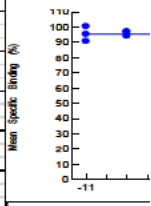
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17691	--	137989.8	137285.2
2		17594	--	137233.2	
3		17342	--	135267.6	
4		17475	--	136305.0	
5		17914	--	139729.2	
6		17588	--	137186.4	
7	Total Binding (Solvent Control)	15791	15438.0	23157.0	23891.5
8		18098	17745.0	26617.5	
9		14953	14600.0	21900.0	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
330.0	10	Estradiol (NSB)	-7	-23.0	-34.5	-0.1	1.6	2.4	0.0	0.1	0.1	#DIV/0!	0.4
369.0	11		-7	16.0	24.0	0.1	1.8	3.2					0.4
360.0	12		-7	7.0	10.5	0.0	1.7	3.0					0.4
1608.0	13	Estradiol	-8	1255.0	1882.5	7.9	-5.8	33.4	9.3	1.3	0.8	14.3	1.8
1869.0	14		-8	1516.0	2274.0	9.5	-4.1	17.1					2.0
2029.0	15		-8	1676.0	2514.0	10.5	-3.1	9.8					2.2
6167.0	16	Estradiol	-8.5	5814.0	8721.0	36.5	4.2	17.8	35.3	5.7	3.3	16.0	6.7
6777.0	17		-8.5	6424.0	9636.0	40.3	8.1	64.8					7.4
5002.0	18		-8.5	4649.0	6973.5	29.2	-3.1	9.6					5.5
7930.0	19	Estradiol	-9	7577.0	11365.5	47.6	-8.1	66.4	56.9	8.1	4.7	14.3	8.7
10344.0	20		-9	9991.0	14986.5	62.7	7.0	49.1					11.3
9954.0	21		-9	9601.0	14401.5	60.3	4.6	20.8					10.9
11512.0	22	Estradiol	-9.5	11159.0	16738.5	70.1	-5.2	26.8	73.1	2.9	1.7	4.0	12.6
12049.0	23		-9.5	11696.0	17544.0	73.4	-1.8	3.3					13.2
12434.0	24		-9.5	12081.0	18121.5	75.8	0.6	0.4					13.6
13520.0	25	Estradiol	-10	13167.0	19750.5	82.7	-4.0	15.8	85.9	3.0	1.7	3.5	14.8
14159.0	26		-10	13806.0	20709.0	86.7	0.0	0.0					15.5
14447.0	27		-10	14094.0	21141.0	88.5	1.8	3.4					15.8
15295.0	28	Estradiol	-11	14942.0	22413.0	93.8	-0.4	0.2	95.5	2.9	1.7	3.0	16.7
15296.0	29		-11	14943.0	22414.5	93.8	-0.4	0.2					16.7
16096.0	30		-11	15743.0	23614.5	98.8	4.6	21.4					17.6
506.0	31	19-Norethindrone	-4	153.0	229.5	1.0	-0.8	0.6	1.6	1.0	0.6	62.1	0.6
801.0	32		-4	448.0	672.0	2.8	1.1	1.1					0.9
536.0	33		-4	183.0	274.5	1.1	-0.6	0.4					0.6
1336.0	34	19-Norethindrone	-4.5	983.0	1474.5	6.2	-0.6	0.3	6.9	0.7	0.4	9.7	1.5
1536.0	35		-4.5	1183.0	1774.5	7.4	0.7	0.4					1.7
1503.0	36		-4.5	1150.0	1725.0	7.2	0.5	0.2					1.6
6890.0	37	19-Norethindrone	-5.5	6537.0	9805.5	41.0	-0.9	0.8	41.7	1.3	0.7	3.0	7.5
7226.0	38		-5.5	6873.0	10309.5	43.2	1.2	1.4					7.9
6871.0	39		-5.5	6518.0	9777.0	40.9	-1.0	1.1					7.5
11340.0	40	19-Norethindrone	-6	10987.0	16480.5	69.0	0.4	0.2	68.8	0.7	0.4	1.1	12.4
11182.0	41		-6	10829.0	16243.5	68.0	-0.6	0.4					12.2
11416.0	42		-6	11063.0	16594.5	69.5	0.9	0.8					12.5
14118.0	43	19-Norethindrone	-6.5	13765.0	20647.5	86.4	-0.4	0.2	87.3	2.6	1.5	3.0	15.4
13932.0	44		-6.5	13579.0	20368.5	85.3	-1.6	2.6					15.2
14721.0	45		-6.5	14368.0	21552.0	90.2	3.3	11.1					16.1
14421.0	46	19-Norethindrone	-7	14068.0	21102.0	88.3	-7.0	49.6	94.2	5.1	2.9	5.4	15.8
15865.0	47		-7	15512.0	23268.0	97.4	2.0	4.1					17.3
15783.0	48		-7	15430.0	23145.0	96.9	1.5	2.3					17.2
16511.0	49	19-Norethindrone	-7.5	16158.0	24237.0	101.4	2.8	8.1	99.1	2.0	1.2	2.1	18.0
15991.0	50		-7.5	15638.0	23457.0	98.2	-0.4	0.2					17.5
15909.0	51		-7.5	15556.0	23334.0	97.7	-0.9	0.9					17.4
16054.0	52	19-Norethindrone	-8.5	15701.0	23551.5	98.6	-1.5	2.4	100.3	1.6	0.9	1.6	17.5
16389.0	53		-8.5	16036.0	24054.0	100.7	0.6	0.3					17.9
16552.0	54		-8.5	16199.0	24298.5	101.7	1.6	2.5					18.1
8461.0	55	Octyltriethoxysilane	-3	8108.0	12162.0	50.9	2.7	7.4	48.2	2.6	1.5	5.4	9.2
7634.0	56		-3	7281.0	10921.5	45.7	-2.5	6.1					8.3
7988.0	57		-3	7635.0	11452.5	47.9	-0.2	0.1					8.7
15334.0	58	Octyltriethoxysilane	-4	14981.0	22471.5	94.1	3.2	10.2	90.9	2.8	1.6	3.1	16.8
14505.0	59		-4	14152.0	21228.0	88.9	-2.0	4.0					15.8
14637.0	60		-4	14284.0	21426.0	89.7	-1.2	1.4					16.0
16252.0	61	Octyltriethoxysilane	-5	15899.0	23848.5	99.8	0.9	0.9	101.4	2.0	1.2	2.0	17.8
16864.0	62		-5	16511.0	24766.5	103.7	4.8	22.7					18.4
16382.0	63		-5	16029.0	24043.5	100.6	1.7	3.0					17.9
16894.0	64	Octyltriethoxysilane	-6	16541.0	24811.5	103.9	5.0	24.6	105.1	1.7	1.0	1.6	18.5
17408.0	65		-6	17055.0	25582.5	107.1	8.2	67.0					19.0
16983.0	66		-6	16630.0	24945.0	104.4	5.5	30.4					18.6
16273.0	67	Octyltriethoxysilane	-7	15920.0	23880.0	100.0	1.1	1.1	101.7	3.0	1.8	3.0	17.8
17111.0	68		-7	16758.0	25137.0	105.2	6.3	39.9					18.7
16274.0	69		-7	15921.0	23881.5	100.0	1.1	1.1					17.8
15932.0	70	Octyltriethoxysilane	-8	15579.0	23368.5	97.8	-1.1	1.2	98.1	0.4	0.2	0.4	17.4
16050.0	71		-8	15697.0	23545.5	98.6	-0.3	0.1					17.5
15932.0	72		-8	15579.0	23368.5	97.8	-1.1	1.2					17.4
14920.0	73	Octyltriethoxysilane	-9	14567.0	21850.5	91.5	-7.4	55.3	93.9	2.1	1.2	2.2	16.3
15460.0	74		-9	15107.0	22660.5	94.8	-4.0	16.4					16.9
15524.0	75		-9	15171.0	22756.5	95.2	-3.6	13.3					17.0
15033.0	76	Octyltriethoxysilane	-10	14680.0	22020.0	92.2	-6.7	45.3	93.3	1.5	0.9	1.7	16.4
15100.0	77		-10	14747.0	22120.5	92.6	-6.3	39.8					16.5
15490.0	78		-10	15137.0	22705.5	95.0	-3.9	14.9					16.9
11386	79	OctylSalicylate	-4	11033.0	16549.5	69.3	-0.4	0.2	69.7	2.9	1.7	4.2	12.4
11028	80		-4	10675.0	16012.5	67.0	-2.7	7.3					12.0
11957	81		-4	11604.0	17406.0	72.9	3.1	9.8					13.1
14262	82	OctylSalicylate	-5	13909.0	20863.5	87.3	-2.6	6.6	89.9	2.7	1.6	3.0	15.6
14622	83		-5	14269.0	21403.5	89.6	-0.3	0.1					16.0
15131	84		-5	14778.0	22167.0	92.8	2.9	8.3					16.5
15302	85	OctylSalicylate	-6	14949.0	22423.5	93.9	-1.7	2.9	95.5	1.6	0.9	1.7	16.7
15810	86		-6	15457.0	23185.5	97.0	1.5	2.2					17.3
15580	87		-6	15227.0	22840.5	95.6	0.1	0.0					17.0
15214	88	OctylSalicylate	-7	14861.0	22291.5	93.3	-2.4	5.6	97.0	3.2	1.9	3.3	16.6
15989	89		-7	15636.0	23454.0	98.2	2.5	6.3					17.5
16192	90		-7	15839.0	23758.5	99.4	3.8	14.3					17.7
16511	91	OctylSalicylate	-8	16158.0	24237.0	101.4	5.8	33.4	96.8	4.3	2.5	4.5	18.0
15643	92		-8	15290.0	22935.0	96.0	0.3	0.1					17.1
15148	93		-8	14795.0	22192.5	92.9	-2.8	7.7					16.6
14600	94	OctylSalicylate	-9	14247.0	21370.5	89.4	-6.2	38.7	92.9	3.0	1.7	3.2	16.0
15385	95		-9	15032.0	22548.0	94.4	-1.3	1.7					16.8
15444	96		-9	15091.0	22636.5	94.7	-0.9	0.8					16.9
15364	97	OctylSalicylate	-10	15011.0	22516.5	94.2	-1.4	2.0	96.1	1.6	0.9	1.7	16.8
15818	98		-10	15465.0	23197.5	97.1	1.4	2.0					17.3
15817	99		-10	15464.0	23196.0	97.1	1.4	2.0					17.3
14817	100	OctylSalicylate	-11	14464.0	21696.0	90.8	-4.9	23.6	95.6	4.9	2.8	5.1	16.2
16376	101		-11	16023.0	24034.5	100.6	4.9	24.3					17.9
15556	102		-11	15203.0	22804.5	95.5	-0.2	0.0					17.0







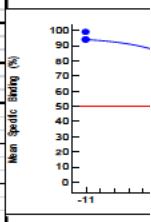


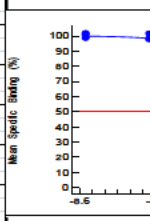
APPENDIX 1 Raw and Normalized Data 3rd Valid Run (continued) – August 03, 2011

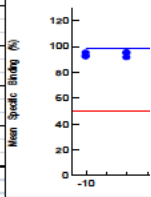
Experiment Date:	3-Aug-11	Study Number:	9070-100107ERB	Assays Conducted by:	[redacted]
Test substance:	Octocrylene				
1/27/2012 16:17					
	10 uL of 50 nM E2=	____	DPM and 0.5 x10-12 moles		
	Therefore there are	____	DPM/mole		
	ug protein/assay tube =	80.0			

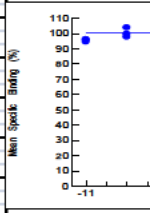
Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Mean
1	Total Activity (Master Mix)	17691	--	137989.8	137285.2
2		17594	--	137233.2	
3		17342	--	135267.6	
4		17475	--	136305.0	
5		17914	--	139729.2	
6		17588	--	137186.4	
7	Total Binding (Solvent Control)	15791	15438.0	23157.0	23891.5
8		18098	17745.0	26617.5	
9		14953	14600.0	21900.0	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
330.0	10	Estradiol (NSB)	-7	-23.0	-34.5	-0.1	1.6	2.4	0.0	0.1	0.1	#DIV/0!	0.4
369.0	11		-7	16.0	24.0	0.1	1.8	3.2					0.4
360.0	12		-7	7.0	10.5	0.0	1.7	3.0					0.4
1608.0	13	Estradiol	-8	1255.0	1882.5	7.9	-5.8	33.4	9.3	1.3	0.8	14.3	1.8
1869.0	14		-8	1516.0	2274.0	9.5	-4.1	17.1					2.0
2029.0	15		-8	1676.0	2514.0	10.5	-3.1	9.8					2.2
6167.0	16	Estradiol	-8.5	5814.0	8721.0	36.5	4.2	17.8	35.3	5.7	3.3	16.0	6.7
6777.0	17		-8.5	6424.0	9636.0	40.3	8.1	64.8					7.4
5002.0	18		-8.5	4649.0	6973.5	29.2	-3.1	9.6					5.5
7930.0	19	Estradiol	-9	7577.0	11365.5	47.6	-8.1	66.4	56.9	8.1	4.7	14.3	8.7
10344.0	20		-9	9991.0	14986.5	62.7	7.0	49.1					11.3
9954.0	21		-9	9601.0	14401.5	60.3	4.6	20.8					10.9
11512.0	22	Estradiol	-9.5	11159.0	16738.5	70.1	-5.2	26.8	73.1	2.9	1.7	4.0	12.6
12049.0	23		-9.5	11696.0	17544.0	73.4	-1.8	3.3					13.2
12434.0	24		-9.5	12081.0	18121.5	75.8	0.6	0.4					13.6
13520.0	25	Estradiol	-10	13167.0	19750.5	82.7	-4.0	15.8	85.9	3.0	1.7	3.5	14.8
14159.0	26		-10	13806.0	20709.0	86.7	0.0	0.0					15.5
14447.0	27		-10	14094.0	21141.0	88.5	1.8	3.4					15.8
15295.0	28	Estradiol	-11	14942.0	22413.0	93.8	-0.4	0.2	95.5	2.9	1.7	3.0	16.7
15296.0	29		-11	14943.0	22414.5	93.8	-0.4	0.2					16.7
16096.0	30		-11	15743.0	23614.5	98.8	4.6	21.4					17.6
506.0	31	19-Norethindrone	-4	153.0	229.5	1.0	-0.8	0.6	1.6	1.0	0.6	62.1	0.6
801.0	32		-4	448.0	672.0	2.8	1.1	1.1					0.9
536.0	33		-4	183.0	274.5	1.1	-0.6	0.4					0.6
1336.0	34	19-Norethindrone	-4.5	983.0	1474.5	6.2	-0.6	0.3	6.9	0.7	0.4	9.7	1.5
1536.0	35		-4.5	1183.0	1774.5	7.4	0.7	0.4					1.7
1503.0	36		-4.5	1150.0	1725.0	7.2	0.5	0.2					1.6
6890.0	37	19-Norethindrone	-5.5	6537.0	9805.5	41.0	-0.9	0.8	41.7	1.3	0.7	3.0	7.5
7226.0	38		-5.5	6873.0	10309.5	43.2	1.2	1.4					7.9
6871.0	39		-5.5	6518.0	9777.0	40.9	-1.0	1.1					7.5
11340.0	40	19-Norethindrone	-6	10987.0	16480.5	69.0	0.4	0.2	68.8	0.7	0.4	1.1	12.4
11182.0	41		-6	10829.0	16243.5	68.0	-0.6	0.4					12.2
11416.0	42		-6	11063.0	16594.5	69.5	0.9	0.8					12.5
14118.0	43	19-Norethindrone	-6.5	13765.0	20647.5	86.4	-0.4	0.2	87.3	2.6	1.5	3.0	15.4
13932.0	44		-6.5	13579.0	20368.5	85.3	-1.6	2.6					15.2
14721.0	45		-6.5	14368.0	21552.0	90.2	3.3	11.1					16.1
14421.0	46	19-Norethindrone	-7	14068.0	21102.0	88.3	-7.0	49.6	94.2	5.1	2.9	5.4	15.8
15865.0	47		-7	15512.0	23268.0	97.4	2.0	4.1					17.3
15783.0	48		-7	15430.0	23145.0	96.9	1.5	2.3					17.2
16511.0	49	19-Norethindrone	-7.5	16158.0	24237.0	101.4	2.8	8.1	99.1	2.0	1.2	2.1	18.0
15991.0	50		-7.5	15638.0	23457.0	98.2	-0.4	0.2					17.5
15909.0	51		-7.5	15556.0	23334.0	97.7	-0.9	0.9					17.4
16054.0	52	19-Norethindrone	-8.5	15701.0	23551.5	98.6	-1.5	2.4	100.3	1.6	0.9	1.6	17.5
16389.0	53		-8.5	16036.0	24054.0	100.7	0.6	0.3					17.9
16552.0	54		-8.5	16199.0	24298.5	101.7	1.6	2.5					18.1
8461.0	55	Octyltriethoxysilane	-3	8108.0	12162.0	50.9	2.7	7.4	48.2	2.6	1.5	5.4	9.2
7634.0	56		-3	7281.0	10921.5	45.7	-2.5	6.1					8.3
7988.0	57		-3	7635.0	11452.5	47.9	-0.2	0.1					8.7
15334.0	58	Octyltriethoxysilane	-4	14981.0	22471.5	94.1	3.2	10.2	90.9	2.8	1.6	3.1	16.8
14505.0	59		-4	14152.0	21228.0	88.9	-2.0	4.0					15.8
14637.0	60		-4	14284.0	21426.0	89.7	-1.2	1.4					16.0
16252.0	61	Octyltriethoxysilane	-5	15899.0	23848.5	99.8	0.9	0.9	101.4	2.0	1.2	2.0	17.8
16864.0	62		-5	16511.0	24766.5	103.7	4.8	22.7					18.4
16382.0	63		-5	16029.0	24043.5	100.6	1.7	3.0					17.9
16894.0	64	Octyltriethoxysilane	-6	16541.0	24811.5	103.9	5.0	24.6	105.1	1.7	1.0	1.6	18.5
17408.0	65		-6	17055.0	25582.5	107.1	8.2	67.0					19.0
16983.0	66		-6	16630.0	24945.0	104.4	5.5	30.4					18.6
16273.0	67	Octyltriethoxysilane	-7	15920.0	23880.0	100.0	1.1	1.1	101.7	3.0	1.8	3.0	17.8
17111.0	68		-7	16758.0	25137.0	105.2	6.3	39.9					18.7
16274.0	69		-7	15921.0	23881.5	100.0	1.1	1.1					17.8
15932.0	70	Octyltriethoxysilane	-8	15579.0	23368.5	97.8	-1.1	1.2	98.1	0.4	0.2	0.4	17.4
16050.0	71		-8	15697.0	23545.5	98.6	-0.3	0.1					17.5
15932.0	72		-8	15579.0	23368.5	97.8	-1.1	1.2					17.4
14920.0	73	Octyltriethoxysilane	-9	14567.0	21850.5	91.5	-7.4	55.3	93.9	2.1	1.2	2.2	16.3
15460.0	74		-9	15107.0	22660.5	94.8	-4.0	16.4					16.9
15524.0	75		-9	15171.0	22756.5	95.2	-3.6	13.3					17.0
15033.0	76	Octyltriethoxysilane	-10	14680.0	22020.0	92.2	-6.7	45.3	93.3	1.5	0.9	1.7	16.4
15100.0	77		-10	14747.0	22120.5	92.6	-6.3	39.8					16.5
15490.0	78		-10	15137.0	22705.5	95.0	-3.9	14.9					16.9
14543	79	Octocrylene	-4	14190.0	21285.0	89.1	5.8	33.2	83.3	8.1	4.7	9.7	15.9
12146	80		-4	11793.0	17689.5	74.0	-9.3	86.2					13.3
14187	81		-4	13834.0	20751.0	86.9	3.5	12.5					15.5
15128	82	Octocrylene	-5	14775.0	22162.5	92.8	-1.7	2.8	94.4	3.1	1.8	3.3	16.5
15097	83		-5	14744.0	22116.0	92.6	-1.9	3.5					16.5
15960	84		-5	15607.0	23410.5	98.0	3.5	12.5					17.4
16172	85	Octocrylene	-6	15819.0	23728.5	99.3	-1.5	2.3	101.1	5.3	3.1	5.3	17.7
15777	86		-6	15424.0	23136.0	96.8	-4.0	15.9					17.2
17404	87		-6	17051.0	25576.5	107.1	6.2	38.7					19.0
16532	88	Octocrylene	-7	16179.0	24268.5	101.6	0.7	0.5	101.2	0.9	0.5	0.9	18.1
16296	89		-7	15943.0	23914.5	100.1	-0.7	0.6					17.8
16570	90		-7	16217.0	24325.5	101.8	1.0	0.9					18.1
16340	91	Octocrylene	-8	15987.0	23980.5	100.4	-0.5	0.2	102.9	2.2	1.3	2.1	17.9
16998	92		-8	16645.0	24967.5	104.5	3.7	13.4					18.6
16878	93		-8	16525.0	24787.5	103.8	2.9	8.5					18.4
16807	94	Octocrylene	-9	16454.0	24681.0	103.3	2.5	6.1	103.9	0.6	0.3	0.6	18.4
16933	95		-9	16580.0	24870.0	104.1	3.3	10.6					18.5
16989	96		-9	16636.0	24954.0	104.4	3.6	13.0					18.6
16233	97	Octocrylene	-10	15880.0	23820.0	99.7	-1.1	1.3	100.4	3.1	1.8	3.1	17.7
15921	98		-10	15568.0	23352.0	97.7	-3.1	9.6					17.4
16902	99		-10	16549.0	24823.5	103.9	3.1	9.4					18.5
15680	100	Octocrylene	-11	15327.0	22990.5	96.2	-4.6	21.3	95.5	0.9	0.5	0.9	17.1
15416	101		-11	15063.0	22594.5	94.6	-6.3	39.3					16.8
15616	102		-11	15263.0	22894.5	95.8	-5.0	25.2					17.1









APPENDIX 2 Rat Uterine Cytosol Preparation and Information

Supplier	Harlan Laboratories
Strain	Sprague-Dawley
Age	12-13 weeks
Days after ovariectomy	7 days
Protein Concentration	1.10 mg/mL
Method of Determination	Bradford Method
Supplier and Product	Bio-Rad Dye Reagent Concentrate
Catalog Number	500-0006
Batch/Lot Number	210007463
Method of Transport	FedEx – priority overnight
Conditions of Transport	Dry Ice

Isolation Procedure

- □ Inspected uterine tissue for signs of residual ovarian tissue after ovariectomy (*e.g.*, uterine imbibition) and discarded tissue that was compromised.

- □ Weighed trimmed uterus, if weights not provided, and placed in ice-cold TEDG buffer + PI at a ratio of 0.1 g of tissue per 1.0 ml TEDG + PI buffer. Homogenize the tissue using a Polytron (PT 35/10) or similar homogenizer for 3 to 5 bursts (~5 seconds per burst).

- □ Transferred homogenate to pre-cooled centrifuge tubes and centrifuged for 10 minutes at 2,500 x g (Sorval RC SS34 rotor at 4500 RPM) at 4°C. The supernatant contains the ER.

- □ Transferred the supernatant to pre-cooled ultracentrifuge tubes and centrifuge at 105,000 x g (Beckman 50.2TI rotor at 34,000 RPM) for 60 minutes at 4°C. Discard the pellet.

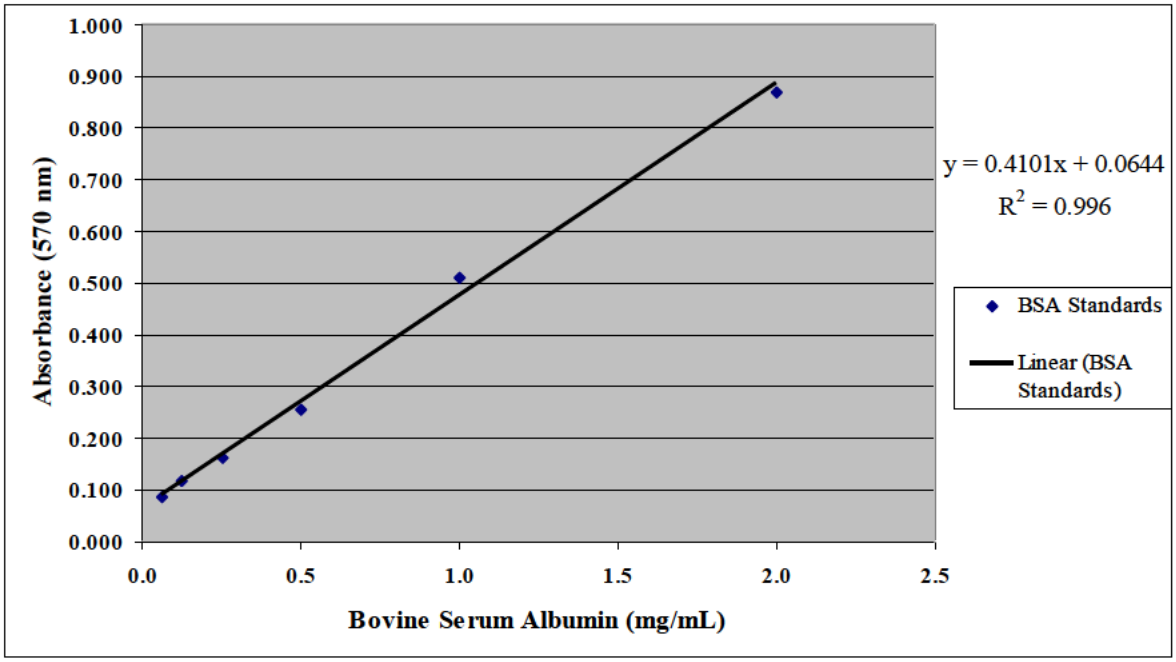
- □ Keeping cytosol ice-cold, pooled the cytosol supernatants containing ER.

- □ Determined protein content for each batch of cytosol using a method that is compatible with buffers that contain DTT. Typical protein values are 1 to 4 mg/ml.

Note: *Some protein kits are not compatible with the DTT in the TEDG buffer. Be sure to use a protein assay that is compatible with DTT (e.g., BioRad Protein Assay Kit).*

- □ Aliquoted cytosol (1 to 6 ml aliquots) either for immediate use in ER binding assay or for storage at -80ºC.

Calibration Curve



Raw Data Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	3x	3x	3x		2mg/mL	2mg/mL	2mg/mL					
B	5x	5x	5x		1mg/mL	1mg/mL	1mg/mL					
C	10x	10x	10x		0.5mg/mL	0.5mg/mL	0.5mg/mL					
D	20x	20x	20x		0.25mg/mL	0.25mg/mL	0.25mg/mL					
E	40x	40x	40x		0.125mg/mL	0.125mg/mL	0.125mg/mL					
F	80x	80x	80x		0.06mg/mL	0.06mg/mL	0.06mg/mL					
G	backgrnd	backgrnd	backgrnd		backgrnd	backgrnd	backgrnd					
H	backgrnd	backgrnd	backgrnd		backgrnd	backgrnd	backgrnd					
	cytosol samples	*cytosol samples*	*cytosol samples*	*blank*	*BSA standards (mg/mL)*	*BSA standards (mg/mL)*	*BSA standards (mg/mL)*					

Raw Data

Plate Seq#: 8306

Comment: Acquired: Tuesday, September 28, 2010 2:38 PM Temperature Min/Max: 0.0/0.0°C

Absorbance-A File Report: MTT_UTERINE CYTOSOL 30 MIN_20100928

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.954	0.939	0.941	0.039	1.244	1.223	1.193	0.039	0.04	0.040	0.041	0.042
B	0.661	0.682	0.667	0.041	0.849	0.895	0.841	0.041	0.042	0.040	0.041	0.042
C	0.527	0.535	0.531	0.043	0.603	0.613	0.598	0.042	0.041	0.041	0.041	0.042
D	0.487	0.488	0.487	0.044	0.521	0.508	0.502	0.042	0.042	0.041	0.042	0.041
E	0.454	0.449	0.449	0.043	0.475	0.466	0.459	0.042	0.041	0.041	0.041	0.042
F	0.429	0.437	0.426	0.050	0.445	0.434	0.438	0.042	0.042	0.041	0.041	0.041
G	0.398	0.396	0.397	0.046	0.400	0.399	0.396	0.052	0.042	0.049	0.042	0.043
H	0.388	0.389	0.389	0.051	0.394	0.389	0.388	0.041	0.043	0.042	0.044	0.046

CeeTox

I verify that the three saturation binding assays performed on the rat uterine batch isolated on 27-September-2010 were acceptable according to the Endocrine Disruptor Screening Program (EDSP) Test Guidelines. *OPPTS 890.1250: Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC)*, EPA 740-C-09-005, October, 2009, for use in the competitive binding assays. This cytosol preparation was also shown to be acceptable per OPPTS 890.~~1150~~ 1250 based upon the reference control results shown in this study report.

[redacted] 27 OCT 2011

[redacted]

27 OCT 2011
Date

Senior Scientist/Endocrine Group Leader
CeeTox, Inc.

[redacted]

27 Oct 2011
Date

Director of Project Management
CeeTox, Inc.

APPENDIX 3 Deviation Forms

Form #: SOP-1003-F-1.0

CeeTox
In vitro models to predict toxicity

Deviation & Investigation

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

Date of Reporting: 22 Jul 2011 Reporting Associate: QA Director in process audit

Date of Occurrence: 20 and 21st Jul 2011 Associate Involved: n/a

Description of Deviation:

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

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

Facility Deviation from SOP-4007

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

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

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

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

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

SD/PI/Test Facility Management

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

COPY

Standard Operating Procedure Page 1 of 1

Form #: SOP-1003-F-1.0

CeeTox
in vitro models to predict toxicity

Deviation & Investigation

Study Number (if applicable): 9070-100107ERB

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

Date of Occurrence: 25 Jul 2011, 01 Aug 2011, 03 Aug 2011 Associate Involved: [redacted]

Description of Deviation:

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

Signature [redacted] (Reporting Associate): Date: 26 Aug 2011

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

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

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

Incorrect lot and C of A provided

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

Ask Sponsor to supply proper C of A

Signature: [redacted] SD/PI/Test Facility Management Date: 26 Aug 2011

Standard Operating Procedure Page 1 of 1

Form #: SOP-1003-F-1.0

CeeTox
In vitro models to predict toxicity

Deviation & Investigation

Study Number (if applicable): 9070-100107ERB

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

Date of Occurrence: 21-Sep-11, 26-Sep-11 and 18-Oct-11 Associate Involved: [redacted]

Description of Deviation:

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

Signature [redacted] Date: 04-Jan-12

(Reporting Associate)

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

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

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

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

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

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

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

SD/PI/Test Facility Management

Standard Operating Procedure Page 1 of 1

Form #: SOP-1003-F-1.0

CeeTox
In vitro models to predict toxicity

Deviation & Investigation

Study Number (if applicable): 9070-100107ERB

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

Date of Occurrence: 25-Jul-11, 01-Aug-11 and 03-Aug-11 Associate Involved: [redacted]

Description of Deviation:

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

Signature [redacted] Date: 04-Jan-12

(Reporting Associate)

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

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

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

Wrong purity was used for methoxycinnamate.

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

None. After dilutions, the difference is negligible.

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

SD/PI/Test Facility Management

Standard Operating Procedure Page 1 of 1

Form #: SOP-1003-F-1.0

CeeTox
In vitro models to predict toxicity

Deviation & Investigation

Study Number (if applicable): ERB002 Batch

Date of Reporting: 08-Nov-11 Reporting Associate: [redacted]

Date of Occurrence: 25-Jul-11, 01-Aug-11 and 03-Aug-11 Associate Involved: [redacted]

Description of Deviation:

Protocol states that PI (protease inhibitor) will be used while the OPPTS guideline states that PMSF will be used specifically.

Signature [redacted] Date: 08-Nov-11

(Reporting Associate)

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

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

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

Stated use of PI in protocol but OPPTS guideline states to use PMSF. PMSF was used in the study.

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

None. PMSF is a protese inhibitor and can be used interchangeably with PI for these studies.

Signature: [redacted] Date: 08-Nov-11

SD/PI/Test Facility Management

Standard Operating Procedure Page 1 of 1

Form #: SOP-1003-F-1.0

CeeTox
In vitro models to predict toxicity

Deviation & Investigation

Study Number (if applicable): ERB002 Batch

Date of Reporting: 08-Nov-11 Reporting Associate: [redacted]

Date of Occurrence: 25-Jul-11, 01-Aug-11 and 03-Aug-11 Associate Involved: [redacted]

Description of Deviation:

Protocol states to use 25 mL of HAP slurry and add TEDG+PI to final volume of 100 mL. Added 100 mL of slurry and TEDG+PI up to 250 mL.

Signature [redacted] Date: 08-Nov-11

(Reporting Associate)

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

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

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

Used incorrect volumes in order to prepare a larger amount of washed HAP in order to perform assay properly.

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

None. Needed to make more HAP for assays than indicated in protocol. Necessary to avoid making HAP multiple times per day.

Signature: [redacted] Date: 08-Nov-11

SD/PI/Test Facility Management

Standard Operating Procedure Page 1 of 1

APPENDIX 4 Certificate of Analysis

IVYCHEM

IVY FINE CHEMICALS

http://www.ivychem.com

CERTICATE OF ANALYSIS

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

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

CERTIFICATE OF ANALYSIS

[redacted]

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

Specifications

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

General Product Data

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

Lot Specific Data for Lot No.: A0293319

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

ACROS ORGANICS

[redacted]

Issued: 10-08-10

[redacted] **Quality Assurance Manager**

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

[redacted]

MRS-N TDVTech 1492

A-1

Certificate of Analysis

SIGMA-ALDRICH

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

TEST	SPECIFICATION	LOT 44696PJ RESULTS
Appearance (Color)	Colorless	Colorless
Appearance (Form)	Liquid	Liquid
Refractive Index at 20 °C	1.500 - 1.504	1.502
Infrared spectrum	Conforms to Structure	Conforms
Purity (GC)	≥99.0 %	99.6 %
Color Test	≤100 APHA	10 APHA
Arsenic (As)	≤3.0 ppm	< 1.0 ppm
Cadmium (Cd)	≤1.0 ppm	< 1.0 ppm
Mercury (Hg)	≤1.0 ppm	< 1.0 ppm
Lead (Pb)	≤10.0 ppm	< 1.0 ppm
Specification Date:		DEC 2008
Date of QC Release:		DEC 2008
Print Date:		DEC 18 2009

[redacted]

[redacted] Supervisor
Quality Control
Milwaukee, Wisconsin USA

Certificate of Analysis

SIGMA-ALDRICH

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

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

[redacted]
[redacted] Supervisor
Quality Control
Milwaukee, Wisconsin USA

APPENDIX 5 Protocol and Protocol Amendments

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

CeeTox®

In vitro models to predict toxicity

FINAL PROTOCOL

Estrogen Receptor Binding (Rat Uterine Cytosol)

Data Requirements: *OPPTS 890.1250*

Author

[redacted]

Study Number:
9070-100107ERB

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

Test Facility:
CeeTox
4717 Campus Drive
Kalamazoo, MI 49008

TEST PROTOCOL

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

NIEHS/NTP Investigator

[redacted]
Telephone No.:
Facsimile No.:
E-mail:

Contract Office Technical Representative

[redacted]
(Contract No. HHSN273200900005C; NIEHS Control No. N01-ES-00005)

Study Monitor

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

Table of Contents

Signatures

[redacted] NIEHS
Study Sponsor

6/29/11
Date

[redacted] LS
Study Monitor

6/29/11
Date

[redacted]
Study Director

29 June 2011
Date

1. Title of Study

Estrogen Receptor Binding (Rat Uterine Cytosol)

2. Purpose of Study

The objective of this protocol is to describe procedures for conduct of the Estrogen Receptor Binding assay using Rat Uterine Cytosol for the source of the receptor as a Tier 1 screen. This assay will be used to provide information on the ability of a substance to interact with the estrogen receptors (ERs) isolated from the rat uterus. This assay is not intended to be used to show that the interaction is, specifically, one-site competitive binding, or to characterize precisely the strength of the binding interaction. It therefore may not be appropriate to use in quantitative structure-activity relationship 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 interact with the estrogen hormonal system.

3. Compliance Statement

This study will be conducted in accordance with Good Laboratory Practice regulations as promulgated by the United States Environmental Protection Agency's (U.S. EPA) Good Laboratory Practice (GLP) Regulations (40 CFR Part 160) with the exception of section 160.113. Dose concentrations of test substance and control substances will not be verified using analytical methods. Also in accordance with the Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1250 all changes to the study protocol will be approved by the Sponsor by protocol amendment.

4. Quality Assurance

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

5. Regulatory Citations

OPPTS 890.1250: Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC)

6. Test Facility

CeeTox, Inc.
4717 Campus Drive
Kalamazoo, MI 49008

7. Test Substance

Note: A certificate of analysis 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. For positive, negative reference substances and radioligand, certificates of analysis will be obtained from the vendor and will be stored in the study data and appended to the study report. Test substance will be either returned to the Sponsor or destroyed following finalization of the study report.

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

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

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

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

7.3 Test Substance: Octyl Salicylate (Octylsalate)

CAS No.	118-60-5

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

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

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

8. Preparation of Test Substances

Each test substance will be dissolved in an appropriate vehicle (DMSO, ethanol, purified water) that solubilizes the test substance. Any vehicle used to dissolve test substances will be tested with the reference substance, if possible, for the run as well, unless the solvent is ineffective (i.e., reference and controls insoluble in that solvent). The maximum percent of ethanol allowed in assay tubes is 3% of the total volume. The maximum percent of DMSO allowed in assay tubes is 10% of the total volume. Dose concentrations of test and control substances will not be verified using analytical methods.

Test substance solubility will be evaluated by visual inspection for precipitation.

Serial Dilutions of Test Substances

Serial dilutions of test substances will be prepared in the appropriate vehicle, to yield the final concentrations indicated in Table 1, unless solubility limits the top concentration tested.

Table 1. Test Substance Dilution Procedure

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted test substance	Diluted test substance concentration	*Final test substance concentration in ER assay tube
TC1	Use 500 µl of stock test substance (100 mM)	500 µl	1 ml	5×10^{-2} M	1×10^{-3} M
TC2	Use 100 µl of dilution TC1 (50 mM)	900 µl	1 ml	5×10^{-3} M	1×10^{-4} M
TC3	Use 100 µl of dilution TC2 (5 mM)	900 µl	1 ml	5×10^{-4} M	1×10^{-5} M
TC4	Use 100 µl of dilution TC3 (500 µM)	900 µl	1 ml	5×10^{-5} M	1×10^{-6} M
TC5	Use 100 µl of dilution TC4 (50 µM)	900 µl	1 ml	5×10^{-6} M	1×10^{-7} M
TC6	Use 100 µl of dilution TC5 (5 µM)	900 µl	1 ml	5×10^{-7} M	1×10^{-8} M
TC7	Use 100 µl of dilution TC6 (500 nM)	900 µl	1 ml	5×10^{-8} M	1×10^{-9} M
TC8	Use 100 µl of dilution TC7 (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.

8.1 Positive and Negative Reference Substances

Octyltriethoxysilane is the negative reference substance. The concentration range for the negative reference is 1×10^{-10} to 1×10^{-3} M. A 100mM stock of octyltriethoxysilane will be prepared in solvent (2.765 mg/ml).

The weak positive reference will be norethynodrel. The final concentration range tested for the weak positive control will be from $1 \times 10^{-8.5}$ to 1×10^{-4} M. A 10 mM stock of norethynodrel will be prepared in solvent (2.984 mg/ml). Dilute serially as described in Table 2 below.

Table 2. Example Dilution Procedure for Norethynodrel

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 (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

The reference substance (17β-estradiol) will be included to ensure that the run has been properly performed and to allow an assessment of variability in the conduct of the assay across time. Final concentrations of unlabeled 17β-estradiol will range from 1×10^{-7} to 1×10^{-11} M as described below in Table 3. Serial dilutions of the reference substance will be in ethanol, DMSO or appropriate solvent, depending on the solvent used for the test substance. 50 µM 17β-estradiol stock will be prepared (0.136 mg/ml).

Table 3. 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	5×10^{-6} M	1×10^{-7} M
S2	Use 100 µl of dilution NSB1 (5 µM)	900 µl	1 ml	5×10^{-7} M	1×10^{-8} M
S3	Use 277 µl of dilution S2 (500 nM)	600 µl	877 µl	1.58×10^{-7} M	3.16×10^{-9} M
S4	Use 100 µl of dilution S2 (500 nM)	900 µl	1 ml	5×10^{-8} M	1×10^{-9} M
S5	Use 100 µl of dilution S3 (158 nM)	900 µl	1 ml	1.58×10^{-8} M	3.16×10^{-10} M
S6	Use 100 µl of dilution S4 (50 nM)	900 µl	1 ml	5×10^{-9} M	1×10^{-10} M
S7	Use 100 µl of dilution S6 (5 nM)	900 µl	1 ml	5×10^{-10} M	1×10^{-11} M

8.2 Stock Solution Preparation

200mM EDTA Stock Solution:

For example, add 7.444g disodium EDTA to 100 ml purified H_2O. Solution will be stored at approximately 4°C.

1M Tris Buffer:

For example, add 147.24g Tris-HCl and 8.0g Tris base to 800 ml purified H_2O. Bring the final volume to 1 Liter. Adjust pH to approximately 7.4 and store 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, add the following in this order:

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

Note: Cool to approximately 4°C before adjusting to pH ~7.4, and then bring volume to 100 ml with purified H^2O and store at approximately 4°C 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]:

Prepare daily as needed.

For example to make 100 ml, add the following in this order:

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

Note: Discard any unused 1X buffer

For example to prepare 60% hydroxyapatite (HAP) slurry (prepare one day before use):

- Mix HAP gently to resuspend and add approximately 25 ml of slurry to a 100 ml graduated cylinder for washing.
- Add TEDG + PI buffer to a final volume of 100 ml, cap container, mix by inversion and refrigerate for at least 2 hours.
- Aspirate or decant the supernatant and resuspend the HAP in fresh TEDG + PI buffer (approximately 4°C) to 100 ml. Mix gently. Allow the HAP to settle for approximately 2 hours at approximately 4°C and repeat the wash step.

- After the last wash, the HAP slurry will be left to settle overnight (at least 8 to 10 hours at approximately 4°C).
- On the next day (the day of use), note the volume of HAP on the graduated cylinder. Aspirate or decant the supernatant and resuspend the HAP 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.

Uterine Homogenate Collection and Saturation Binding

Cytosol prepared from Rat uteri was collected, processed, and validated per EPA guideline and CeeTox SOP for use on this study. Related data will be maintained separate from this study and available upon request.

Standardization of Receptor Concentration for Competition Binding

Before performing a binding assay, the receptor concentration of cytosol prepared from rat uteri will be adjusted to minimize the likelihood of ligand depletion. Ligand depletion occurs when a high percentage of the [^{3}H]-17β-estradiol is bound to ER causing the concentration of the unbound (free) [^{3}H]-17β-estradiol to differ significantly from the concentration of [^{3}H]-17β-estradiol that was originally added to the assay tube.

Competition Binding

For the competitive binding assay, 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.

9. Competitive Radioligand Binding Assay

Table 4. 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		~°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

Prepare Assay Buffer. 2X TEG stock solutions will be prepared (as described previously) and can be stored at approximately 4°C for up to 3 months. On the day of assay, working assay buffer will be prepared and DTT and PI will be added immediately before use in assay (TEDG+PI).

Check test substance solubility.

[^{3}H]-17β-estradiol will be prepared on the day of assay. The specific activity (SA) 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.

Standardization of Receptor Concentration and Assay Volume.

Assay Preparations. Use 12 x 75 mm siliconized tubes for the assay. Prepare a master mixture of radioligand and buffer to be used for the assay. 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. Trace tubes are 50 µl TEDG Buffer + PI with diluted [3H]-17β-estradiol. For example the following table describes the preparation of a master mixture for 155 tubes.

Table 5. 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, 390 µl of the master mixture above will be added to each assay tube and keep on ice. For the trace tubes, 50 µl will be added directly to 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 6 and added to the assay tubes. 10 µl of substance will be added per tube. After all substances have been added to the tubes, 100 µl of cytosol will be added to each tube for a final volume of 500 µl according to Table 6 below. Assay tubes will be vortexed after additions and incubated at approximately 4°C for 16 to 20 hours on a rotator.

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, 250 µl of ice cold HAP slurry (60% in TEDG + PI) will be added to each assay tube. The tubes will be vortexed for ~10 seconds at approximately 5 minute intervals for a total of ~15 minutes with tube remaining in the ice-water bath between vortexing. Following the vortexing step, 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 ~10 minutes at 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 mls 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 ~10 seconds at 5-minute intervals. The assay tubes will be centrifuged at room temperature for ~10 minutes at 1000 x g. A 1 ml aliquot will be pipetted, taking care to avoid the centrifuged pellet, into a 20 ml scintillation vial containing 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.

Table 6. Competitive Binding Assay Additions.

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

10. Solubility/Precipitation Assay

The limit of test substance solubility will be determined by visual observation. Substance solubility will be determined in solvent. It may be necessary to warm the stock solution of the test substance for 10 to 15 minutes before making the dilutions. In addition, the solutions will be watched closely when added to the experiment tube (as the test substance may precipitate upon addition to the assay tube mixtures). If solubility issues occur, appropriate documentation will be provided.

11. Data Interpretation Criteria

Competitive Binding Analyses

Definitions

Total [^{3}H]-17β-estradiol Added.

Radioactivity in DPMs added to each assay tube. The total radioligand added is approximated by the mean of the DPMs in the tubes that contain only radiolabeled ligand and buffer.

Total Binding

Radioactivity in DPMs bound eluted from the centrifuge pellet in the solvent control tubes (tubes that contain radioligand and receptor but no competitor).

Non-specific Binding (NSB)

Radioactivity in DPMs bound eluted from the centrifuge pellet in the tubes that contain 100-fold excess of unlabeled over labeled 17β-estradiol. NSB is the mean of all the NSB tubes included in a run.

Specific Binding

Total binding (in the presence of a given concentration of competitor) minus NSB, expressed as a percentage of total binding (in the absence of a competitor). Specific binding is plotted on the Y-axis of the competitive binding graph, against log-Molar concentration of competitor added on the X-axis.

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 nonlinear curve fitting software (GraphPad PRISM). The relative binding affinity (RBA) is calculated by comparing the log (IC_{50}) of 17β-estradiol with that of the test substance. APPENDIX C – HOW TO ESTIMATE IC50

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 (norethynodrel) are within the tolerance bounds provided in Table 7.

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 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 Norethynodrel)

Parameter	Unit	Estradiol		Norethynodrel	
		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

12. 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.

13. Test System

As per the guideline (OPPTS 890.1250) uteri from Sprague-Dawley female rats (85 to 100 days of age at time of kill) ovariectomized seven to 10 days prior to being humanely killed will be used to prepare the cytosol.

14. Study Reports

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

15. Alterations of the Study Design

Alterations of this protocol may be made as the study progresses. No changes in the protocol will be made without the specific written request or consent of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, CeeTox will honor such a change. However, written authorization will be obtained thereafter. All protocol

amendments and justifications will be documented, signed and dated by the Study Director, Study Monitor and Sponsor and added to the report. A copy of the protocol and all amendments will be issued to the Sponsor as well as CeeTox and placed into the study binder.

16. Data Retention and Archiving

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

Study Records to be maintained:

All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and chemicals used.

Protocol and any Amendments

List of any Protocol Deviations

CeeTox

Protocol Amendment

Study Number: 9070-100107ERB

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

Reason for Amendment to Protocol: A typo was observed in the text above Table 2. The information in the table is correct.

Change:
Section 8.1 paragraph 2 sentence 2 stated:

"The final concentration range tested for the weak positive control will be from 1 X $10^{-8.5}$ to 1 x 10^{-4} M."

Section 8.1 paragraph 2 sentence 2 will now state:

"The final concentration range tested for the weak positive control will be from 3.16 X 10^{-9} to 1 x 10^{-4} M."

Signature

CeeTox, Inc.

[redacted]
Study Director (Project Manager)

21 Sept 2011
Date

CeeTox

Protocol Amendment

Study Number: 9070-100107ERB

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

Reason for Amendment to Protocol: The Table of Contents had typographical errors.

Change:

The Table of Contents will now read:

Signature

CeeTox, Inc.

[redacted]

Study Director (Project Manager)

26 Sept 2011
Date

CeeTox

Protocol Amendment

Study Number: 9070-100107ERB

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

Reason for Amendment to Protocol: First choice weak positive control, norethynodrel, is not available.

Change: As per EPA Guideline OPPTS 890.1250, pgs 9 and 13, norethindrone may be substituted when norethynodrel is unavailable.

Signature

CeeTox, Inc.

[redacted]

Study Director (Project Manager)

18 Oct 2011
Date

CeeTox

Protocol Amendment

Study Number: 9070-100107ERB

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

Reason for Amendment to Protocol: Client requested amendment

Change:
Section Data Retention and Archiving will now state:

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

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

Signature

CeeTox, Inc.

[redacted]
Study Monitor

12-6-11
Date

[redacted]
Study Director (Project Manager)

06 Dec 11
Date