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**Estrogen Receptor Binding (Rat Uterine Cytosol)** 

**Final Report** 

**DATA REQUIREMENT(S):** OPPTS 890.1250 (2009)

AUTHOR(S):

**STUDY COMPLETION DATE:** January 27, 2012

TEST FACILITY:

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

LABORATORY PROJECT ID: Study Number: 9070-100107ERB Sponsor Contract No. HHSN273200900005C NIEHS Control No. N01-ES-00005

SPONSOR(S):	NIEHS
	530 Davis Drive, MD K2-12
	PO BOX 12233
	Durham, NC 27713

**STUDY MONITOR:** 

(ILS, Inc, Durham, NC)

# 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.



27 Jan 2012

Study Number: 9070-100107ERB

# 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.

Quality Assurance Auditor

4717 Campus Drive Kalamazoo, MI 49008

27 Jan 2012 Date

## **GENERAL INFORMATION**

#### Contributors

The following contributed to this report in the capacities indicated:

Name	Title
	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

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 17 $\beta$ -estradiol. The total binding tubes allowed for the identification of maximal binding of [<sup>3</sup>H]-17 $\beta$ -estradiol. Non-specific binding (NSB) was also assessed in replicates of 3 by determining the [<sup>3</sup>H]-17 $\beta$ -estradiol bound in the presence of 100-fold excess unlabeled 17 $\beta$ -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 [<sup>3</sup>H]-17 $\beta$ -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 17 $\beta$ -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 4°C, 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<sub>i</sub> 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<sub>50</sub> of -5.5 M while the LogIC<sub>50</sub> of 17 $\beta$ -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<sub>50</sub> of -5.5 M while the LogIC<sub>50</sub> of  $17\beta$ -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<sub>50</sub> of -5.6 M while the LogIC<sub>50</sub> of 17β-estradiol was -8.8 M.

The mean relative binding affinity, or RBA (calculated by dividing the LogIC<sub>50</sub> of the control/test material by the LogIC<sub>50</sub> of the positive control 17 $\beta$ -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.

Octyl salicylate, 2-ethylhexyl salicylate
(Octylsalate)
Sigma-Aldrich
118-60-5
Colorless liquid
DMSO
44698PJ
Not Provided
99.6%
$C_{15}H_{22}O_3$
250.33
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 <sub>24</sub> H <sub>27</sub> NO <sub>2</sub>
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  $17\beta$ -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  $[{}^{3}H]$ -17 $\beta$ -estradiol had a specific activity (SA) of 130.2 Ci/mmol on the certification date (06-May-2011). The SA<sub>adjusted</sub> 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.

 $17\beta$  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:

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 x 10 <sup>-2</sup> M	1 x 10 <sup>-3</sup> M
TC2	Use 100 µl of dilution TC1 (50 mM)	900 µl	1 ml	5 x 10 <sup>-3</sup> M	1 x 10 <sup>-4</sup> M
TC3	Use 100 µl of dilution TC2 (5 mM)	900 µl	1 ml	5 x 10 <sup>-4</sup> M	1 x 10 <sup>-5</sup> M
TC4	Use 100 µl of dilution TC3 (500 µM)	900 µl	1 ml	5 x 10 <sup>-5</sup> M	1 x 10 <sup>-6</sup> M
TC5	Use 100 µl of dilution TC4 (50 µM)	900 µl	1 ml	5 x 10 <sup>-6</sup> M	1 x 10 <sup>-7</sup> M
TC6	Use 100 µl of dilution TC5 (5 µM)	900 µl	1 ml	5 x 10 <sup>-7</sup> M	1 x 10 <sup>-8</sup> M
TC7	Use 100 µl of dilution TC6 (500 nM)	900 µl	1 ml	5 x 10 <sup>-8</sup> M	1 x 10 <sup>-9</sup> M
TC8	Use 100 µl of dilution TC7 (50 nM)	900 µl	1 ml	5 x 10 <sup>-9</sup> M	1 x 10 <sup>-10</sup> M

Example Dilution Procedure for oxybenzone, octylmethoxycinnamate, octylsalate and octocrylene.

\*Final concentration of test chemical in assay tube when 10  $\mu$ l of diluted concentration is used in a total volume of 500  $\mu$ l.

#### 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  $1 \times 10^{-10}$  to  $1 \times 10^{-3}$  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  $3.16 \times 10^{-9}$  to  $1 \times 10^{-4}$  M with DMSO kept at approximately 2%.

T1 #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted positive control	Positive Control Concentration	
Tube #				Diluted	Final in ER assay tube
P1	Use 400 µl of stock positive control (10 mM)	400 µ1	800 µl	5 x 10 <sup>-3</sup> M	1 x 10 <sup>-4</sup> M
P2	Use 150 µl of stock positive control (10 mM)	800 µ1	950 µl	1.58 x 10 <sup>-3</sup> M	3.16 x 10 <sup>-5</sup> M
P3	Use 100 µl of P2 (1.58 mM)	900 µl	1 ml	1.58 x 10 <sup>-4</sup> M	3.16 x 10 <sup>-6</sup> M
Intermed	Use 100 µl of P1 (5 mM)	900 µl	1 ml	5 x 10 <sup>-4</sup> M	Not used
P4	Use 100 µl of Intermed (500 µM)	900 µl	1 ml	5 x 10 <sup>-5</sup> M	1 x 10 <sup>-6</sup> M
P5	Use 100 µl of P3 (158 µM)	900 µl	1 ml	1.58 x 10 <sup>-5</sup> M	3.16 x 10 <sup>-7</sup> M
P6	Use 100 μl of P4 (50 μM)	900 µl	1 ml	5 x 10 <sup>-6</sup> M	1 x 10 <sup>-7</sup> M
P7	Use 100 µl of P5 (15.8 µM)	900 µl	1 ml	1.58 x 10 <sup>-6</sup> M	3.16 x 10 <sup>-8</sup> M
P8	Use 100 μl of P7 (1.58 μM)	900 µl	1 ml	1.58 x 10 <sup>-7</sup> M	3.16 x 10 <sup>-9</sup> M

**Example Dilution Procedure for 19-norethindrone** 

The positive contol, 17 $\beta$ -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 17 $\beta$ -estradiol ranged from 1 x 10<sup>-7</sup> to 1 x 10<sup>-11</sup> M as described below. Fresh 50  $\mu$ M 17 $\beta$ -estradiol stock was prepared and serial dilutions of the reference standard were performed in DMSO (final concentration of 2%).

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 <sup>-6</sup> M	1 x 10 <sup>-7</sup> M
S2	Use 100 µl of dilution NSB1 (5 µM)	900 µl	1 ml	5 x 10 <sup>-7</sup> M	1 x 10 <sup>-8</sup> M
<b>S</b> 3	Use 277 µl of dilution S2 (500 nM)	600 µl	877 µl	1.58 x 10 <sup>-7</sup> M	3.16 x 10 <sup>-9</sup> M
S4	Use 100 µl of dilution S2 (500 nM)	900 µl	1 ml	5 x 10 <sup>-8</sup> M	1 x 10 <sup>-9</sup> M
S5	Use 100 µl of dilution S3 (158 nM)	900 µl	1 ml	1.58 x 10 <sup>-8</sup> M	3.16 x 10 <sup>-10</sup> M
<b>S</b> 6	Use 100 µl of dilution S4 (50 nM)	900 µl	1 ml	5 x 10 <sup>-9</sup> M	1 x 10 <sup>-10</sup> M
<b>S</b> 7	Use 100 µl of dilution S6 (5 nM)	900 µl	1 ml	5 x 10 <sup>-10</sup> M	1 x 10 <sup>-11</sup> M

Example Dilution Procedure for 17β-estradiol

## 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  $4\pm2^{\circ}$ C. A 1 M Tris buffer was prepared and the pH was adjusted to 7.4. The buffer can be stored at  $4\pm2^{\circ}$ 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  $4\pm2^{\circ}$ C before adjusting to pH 7.4 and stored at  $4\pm2^{\circ}$ 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  $4\pm 2^{\circ}$ 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 ( $4\pm 2^{\circ}$ C). The slurry was mixed gently and allowed to settle for approximately 2 hours at  $4\pm 2^{\circ}$ C. After the second wash, the HAP slurry settled overnight (at least 8 to 10 hours at  $4\pm 2^{\circ}$ 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

		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	e (as serial dilutions)	100 pM to 1 mM	
Temperature		$4\pm 2^{\circ}C$	
Incubation time		16-20 hours	
Composition of assay buffer	Tris	10 mM (pH 7.4)	
	EDTA	1.5 mM	
Glycerol Protease Inhibitor		10% (v/v)	
		0.5% (v/v)	
	DTT	1 mM	

**Summary Table of Assay Conditions** 

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** [<sup>3</sup>H]-17β-estradiol Preparation

 $[^{3}H]$ -17 $\beta$ -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  $1.54 \times 10^{-4}$ /day). Time = days since the date on the stock bottle from the manufacturer. The  $[{}^{3}H]$ -17 $\beta$ -estradiol was diluted with TEDG + PI buffer so that each assay tube contained 1 nM final concentration of  $[{}^{3}H]$ -17 $\beta$ -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  $[{}^{3}H]$ -17 $\beta$ -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  $[{}^{3}H]$ -17 $\beta$ -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:

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

Master Mixture for Competitive Binding Assay

#### 3.5.4 Individual Tubes

For the assay tubes, 390  $\mu$ l of the master mixture above was added and kept on ice. For the total radioligand added (TRA) tubes, 50  $\mu$ l (1 nM [<sup>3</sup>H]-17 $\beta$ -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  $\mu$ l of chemical was added per tube. After all chemicals were added to the tubes, 100  $\mu$ l of cytosol was added to each tube for a final volume of 500  $\mu$ l. 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.

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

#### **Competitive Binding Assay Additions**

## **3.5.5** Separation of bound [<sup>3</sup>H]-17β-estradiol from free [<sup>3</sup>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  $\mu$ l 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 [<sup>3</sup>H]-17β-estradiol was immediately decanted and discarded. The HAP pellet contained the estrogen receptor bound [<sup>3</sup>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 $[^{3}H]$ -17 $\beta$ -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 (<sup>3</sup>H, <sup>14</sup>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 <sup>3</sup>H and <sup>14</sup>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  $17\beta$ -estradiol displaced [<sup>3</sup>H]- $17\beta$ -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  $[{}^{3}H]$ -17 $\beta$ -estradiol added per assay tube was no greater than 15%.

The parameter values (top, bottom, and slope) for  $17\beta$ -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  $\leq 10\%$ . 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)									
Demometer	I Init		Esti	adiol			19-Noretl	nindrone	
Parameter	Umt								

Deremator	Unit	Esti	adiol	19-Norethindrone		
Faranieter		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:2Equivocal:1Not Interactive:0

Chemical classification, based on the average of all the runs performed for a chemical:Interactive: $average \ge 1.5$ Equivocal: $0.5 \le average < 1.5$ 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  $1 = \sim 1.67$  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  $17\beta$ estradiol displaced [<sup>3</sup>H]- $17\beta$ -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  $17\beta$ -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  $17\beta$ -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 4°C, 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<sub>i</sub> 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<sub>50</sub> of -5.5 M while the LogIC<sub>50</sub> 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<sub>50</sub> of -5.5 M while the LogIC<sub>50</sub> 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<sub>50</sub> of -5.6 M while the LogIC<sub>50</sub> of 17β-estradiol was -8.8 M.

The mean relative binding affinity, or RBA (calculated by dividing the LogIC<sub>50</sub> of the control/test material by the LogIC<sub>50</sub> of the positive control 17 $\beta$ -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

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
	-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
Estradiol (NSB)	-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
	-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
10 Norothindrono	-6	72.1	0.4	0.2	0.5
19-Noreumarone	-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
	-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
Octultriathoxysilana	-6	94.0	3.1	1.8	3.3
Octyntienioxysilaile	-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 1Results 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
	-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
Ovubanzana	-6	95.1	3.1	1.8	3.2
Oxydelizolie	-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
	-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
Octyl-	-6	98.3	3.4	2.0	3.4
methoxycinnamate	-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
	-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
Octylsalate	-6	90.7	0.5	0.3	0.6
Octyfsalate	-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
	-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
Octocrylene	-6	101.7	1.1	0.7	1.1
Octoerytene	-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

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

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

TABLE 3	<b>Results of 1<sup>st</sup> Valid Binding Assay - Upper and Lower Parameters in</b>
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	

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
	-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
Estradiol (NSB)	-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
	-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
10 Norothindrone	-6	65.1	4.0	2.3	6.2
19-inoretimitatorie	-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
	-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
Ootultriothoyusilano	-6	92.3	4.3	2.5	4.6
Octynniemoxysnane	-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 4
 Results of 2<sup>nd</sup> Valid Binding Assay – Controls – August 01, 2011

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
	-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
Ovubanzana	-7	95.5	4.4	2.5	4.6
Oxydelizolie	-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
	-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
Octyl-	-7	89.4	5.9	3.4	6.6
methoxycinnamate	-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
	-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
Octvlsalate	-7	91.2	2.3	1.3	2.5
Octylsalate	-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
	-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
Octocrylene	-7	92.1	2.8	1.6	3.0
Octoerytene	-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 5Results of 2nd Valid Binding Assay – Test Articles – August 01, 2011

TABLE 6	<b>Results of 2<sup>nd</sup></b>	Valid Binding	Assay - Upper	and Lower Parameters
in Competit	ive Assay Bind	ing 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	

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
	-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
Estradiol (NSB)	-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 7Results 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
	-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
Owhensen	-7	98.3	3.1	1.8	3.2
Oxybenzone	-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
	-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
Octyl-	-7	98.8	0.7	0.4	0.8
methoxycinnamate	-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
	-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
Octylsalate	-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 8 Results of 3<sup>rd</sup> Valid Binding Assay – Test Articles – August 03, 2011

# TABLE 9Results of 3<sup>rd</sup> Valid Binding Assay - Upper and Lower Parametersin 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**

Study Number: 9070-100107ERB



FIGURE 1 Specific Binding for 1<sup>st</sup> 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 $\pm$ 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 2<sup>nd</sup> 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 $\pm$ Standard Deviation) from the third independent run of the assay (n=3).



**FIGURE 3** Specific Binding for 3<sup>rd</sup> 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 $\pm$ 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 $\pm$ Standard Deviation) from the third independent run of the assay (n=3).

## **APPENDICES SECTION**

## APPENDIX 1 Raw and Normalized Data 1<sup>st</sup> Valid Run – July 25, 2011

xperiment Date: est substance:	25-Jul-11 Oxybenzone		Study Number:	9070-10010	7ERB				Assays Cor	nducted by:					
/6/2011 17:42														-	
		10 uL of 50 nM E2- Therefore there are ug protein/assay tube -	80.0	DPM and 0. DPM/mole	5 x10-12 mol	es									
				Specific	Total									-	
	Tube	Sample Type	DPM (1mL)	Binding DPM (1mL) - NSB	Specific Binding (1.6mL)	Mean									
	1 2		17709 17865	-	138130.2 139347.0										
	3	Total Activity (Master Mix)	17769 17719	-	138598.2 138208.2	138465.6									
	5		17718 17732	-	138200.4 138309.6										
	7 8	Total Binding (Solvent Control)	22497 22817 21752	21933.3 22253.3 21199.3	32900.0 33380.0	32687.5								-	
			21/32	21100.3	31762.5										
DPM nL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL)	Total Specific Binding	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total	]	
513.0	10	Estradiol (NSB)	-7	- NSB -50.7	-76.0	-0.2	-0.6	0.3	0.0	0.7	0.4	3.2E+17	0.6		
739.0	11 12	E abaa di al	-7	175.3	263.0	0.8	0.5	0.9					0.5		
2328.0	13	Estradio	-8	1764.3	2646.5	7.6	-0.4	0.0	7.7	0.3	0.2	4.0	2.5	90	t•
5737.0	15	Estradiol	-8	5173.3	7760.0	23.7	0.6	0.2	24.9	1.1	0.6	4.3	6.2	2 70	F
6180.0	17		-8.5	5516.3	8424.5	25.3	2.1	4.6					6.6	60 50	Ē.
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	01000 40	È
11345.0	21	Estradiol	-9	10781.3	16172.0 25082.0	49.5	-0.3	0.1	75.4	1.8	1.0	2.3	12.3	- 30 - 20	Ē
16557.0 17119.0	23 24		-9.5 -9.5	15993.3 16555.3	23990.0 24833.0	73.4	-1.4	1.8					17.9 18.5	10	F
21014.0 19720.0	25 26	Estradiol	-10 -10	20450.3 19156.3	30675.5 28734.5	93.8 87.9	6.1 0.1	36.8	89.3	4.1	2.3	4.6	22.8		-11
19318.0 21033.0	27 28	Estradiol	-10	18754.3 20469.3	28131.5 30704.0	86.1 93.9	-1.7	2.9	92.8	1.3	0.7	1.4	20.9	1	
20859.0 20483.0	29 30		-11 -11	20295.3 19919.3	30443.0 29879.0	93.1 91.4	-0.9	0.9					22.6 22.2	1	
805.0 722.0	31	19-Norethindrone	4 4	241.3 158.3	362.0	1.1	-0.2	0.1	0.9	0.2	0.1	21.6	0.9		
750.0	33	19-Norethindrone	-4	186.3	279.5	0.9	-0.5	0.3	6.7	0.4	0.2	5.9	0.8	100	
2109.0	35		-4.5	1545.3	2318.0	7.1	1.0	1.0				1	2.3	90	Ľ∙
9984.0	30	19-Norethindrone	-5.5	9420.3	14130.5	43.2	-1.4	1.9	44.0	2.0	1.1	4.5	10.8	2 80 2 70	F
9825.0	38	40.1111	-5.5	9261.3	13892.0	46.2	-2.1	4.4		-			10.6	100 E0	Ē
16245.0	40	19-Norethindrone	-6	15681.3 15814.3	23522.0	72.0	1.0	1.0	72.1	0.4	0.2	0.5	17.6	00 50 40	F
16223.0 18403.0	42	19-Norethindrone	-6	15659.3 17839.3	23489.0 26759.0	71.9	0.9 -3.6	0.8	84.2	2.1	1.2	2.5	17.6	30	F
19313.0 19010.0	44 45		-6.5 -6.5	18749.3 18446.3	28124.0 27669.5	86.0 84.6	0.6	0.4					20.9	10	È
20117.0 20187.0	46 47	19-Norethindrone	-7 -7	19553.3 19623.3	29330.0 29435.0	89.7 90.0	-1.0 -0.7	1.1	90.7	1.4	0.8	1.5	21.8 21.9		8.6
20664.0 22360.0	48 49	19-Norethindrone	-7 -7.5	20100.3 21796.3	30150.5 32694.5	92.2 100.0	1.5	2.2	94.0	5.9	3.4	6.3	22.4 24.2	}	
20967.0 19786.0	50 51		-7.5 -7.5	20403.3 19222.3	30605.0 28833.5	93.6 88.2	1.2	1.4					22.7	}	
20235.0 21057.0	52 53	19-Norethindrone	-8.5 -8.5	19671.3 20493.3	29507.0 30740.0	90.3 94.0	-2.8	7.8	92.2	1.9	1.1	2.0	21.9 22.8	}	
20647.0	54	Octvitriethoxysilane	-8.5	20083.3	30125.0	92.2	-0.9	0.8	84.9	1.8	1.1	22	22.4	1	
19152.0	56	octymicatoxystane	-3	18588.3	27882.5	85.3	0.4	0.2		1.0		2.2	20.7	1	
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	120	F
21914.0	60	Ochultdathamailean	4	21350.3	32025.5	98.0	2.5	6.4	05.5				23.4	£ 100	⊦:
21281.0	62	Octymethoxysilane	-5	20905.3	31076.0	95.9	-0.8	0.6	35.6	0.5	0.3	0.5	23.3	2 so	Ļ⁼
21521.0	64	Octyltriethoxysilane	-6	20399.3	31436.0	96.2	0.3	0.1	94.0	3.1	1.8	3.3	23.3	en #2, 60	F
21370.0 20276.0	65 66		-6 -6	20806.3 19712.3	31209.5 29568.5	95.5 90.5	-0.4 -5.4	0.2					23.2 22.0	8 40	F
22349.0 21196.0	67 68	Octyltriethoxysilane	-7 -7	21785.3 20632.3	32678.0 30948.5	100.0 94.7	4.1	16.7	96.3	3.2	1.8	3.3	24.2 23.0	20	F
21110.0 21533.0	69 70	Octyltriethoxysilane	-7 -8	20546.3 20969.3	30819.5 31454.0	94.3 96.2	-1.6	2.6	94.7	1.7	1.0	1.8	22.9	0	Ļ
21291.0 20786.0	71		-8 -8	20727.3 20222.3	31091.0 30333.5	95.1 92.8	-0.8 -3.1	0.6 9.5					23.1 22.5	<u> </u>	-10
22344.0 22286.0	73	Octyltriethoxysilane	-9 -9	21780.3 21722.3	32670.5 32583.5	99.9 99.7	4.1	16.5 14.4	98.0	3.2	1.8	3.2	24.2 24.1	}	
21123.0 21688.0	75	Octyltriethoxysilane	-9 -10	20559.3 21124.3	30839.0 31686.5	94.3 96.9	-1.5	2.4	96.6	5.9	3.4	6.1	22.9	{	
22858.0 20303.0	77 78		-10 -10	22294.3 19739.3	33441.5 29609.0	102.3 90.6	6.4 -5.3	41.2 28.2					24.8 22.0	}	
12540 16677	79	Oxybenzone	-3	11976.3	17964.5 24170.0	55.0 73.9	-15.1 3.9	226.8 15.4	70.0	13.5	7.8	19.3	13.6		
18249	81	Oxybenzone	-3	17685.3	26528.0	81.2 84.7	11.1	124.0	83.2	1.6	0.9	2.0	19.8	110	г
18315	83		-4	17751.3	26627.0	81.5	-1.7	3.0					19.8	100	[
21755	85	Oxybenzone	-5	21191.3	31787.0	97.2	1.9	3.6	96.0	2.2	1.3	2.3	23.6	8 80	ŕ
20944 21768	86 87		-5	20380.3 21204.3	30570.5 31806.5	93.5 97.3	-1.8 2.0	3.3					22.7	5 70 60	Ē
21503 20523	88 89	Oxybenzone	-6 -6	20939.3 19959.3	31409.0 29939.0	96.1 91.6	0.8	0.6	95.1	3.1	1.8	3.2	23.3 22.2	10 50	E
21806	90	Oxybenzone	-6 -7	21242.3 20607.3	31863.5 30911.0	97.5 94.6	2.1	4.6	95.3	2.8	1.6	2.9	23.6	5 30	F
20813 22005	92 93		-7 -7	20249.3 21441.3	30374.0 32162.0	92.9 98.4	-2.4	5.8 9.3					22.5 23.8	20 × 20	Ē
20876	94 95	Oxybenzone	-8 -8	20312.3 20726.3	30468.5 31089.5	93.2 95.1	-2.1	4.5	95.8	3.0	1.8	3.2	22.6	0	-10
22170	96	Oxybenzone	-8	21606.3	32409.5	99.1 91.4	3.8	14.5	94.9	5.8	33	61	24.0	4	
20546	98	expeditions.	-9	19982.3	29973.5	91.7	-3.6	13.3		5.0	2.2	3.1	22.3	1	
21305	100	Oxybenzone	-10	20741.3	31112.0	95.2	-0.2	0.0	95.0	4.8	2.8	5.1	24.6	1	
20178	101		-10	19614.3	29421.5	90.0	-5.3	28.4		-			21.9	ł	

# APPENDIX 1 Raw and Normalized Data 1<sup>st</sup> Valid Run (continued) – July 25, 2011

Experiment Date:	25-Jul-11		Study Number:	9070-10010	7ERB				Assays Con	ducted by:						
Test substance:	Methoxycinn	amate														
12/6/2011 17:42																
		10 uL of 50 nM E2-	_	DPM and 0.	5 x10-12 mol	es										
		Therefore there are ug protein/assay tube =	80.0	DPM/mole												
	Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL)	Total Specific Binding	Mean										
	-		47700	- NSB	(1.6mL)											
	2		17709	-	138130.2											
	3	Total Activity (Master Mix)	17769	-	138598.2	138465.6										
	5		17718	-	138208.2											
	6		17732	-	138309.6											
	8	Total Binding (Solvent Control)	22497 22817	21933.3 22253.3	32900.0	32687.5										
	9	1	21752	21188.3	31782.5	<u> </u>						_				
DPM	Tube	Sample Type	Concentration	Specific Binding	Total Specific Binding	Specific Binding	Recidual	Squared	Mean Specific	Standard	SEM	% CV	% Ligand Bound vs.			
(Inte) nom ese			100[m]	- NSB	(1.6mL)	(%)		Redual	(%)	Deviation			Aotivity			
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	_		
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	_	80-	
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	£.	70-	
6080.0	17		-8.5	5516.3	8274.5	25.3	2.1	4.6					6.6	Ę.	60-	
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	8	50	
11152.0	20		-9	10588.3	15882.5	48.6	-1.1	1.3				1	12.1	ped	40-	
11345.0	21	Estradiol	-9	10781.3	16172.0	49.5	-0.3	0.1	75.4	1.0	1.0	2.3	12.3	5	30-	
16557.0	23	Estadio	-9.5	15993.3	23990.0	73.4	-1.4	1.8	7.5.4		1.0	2.3	17.9	Ne	10	
17119.0	24	Est-	-9.5	16555.3	24833.0	76.0	1.2	1.5	00.5			1.5	18.5		o	
21014.0	25	Estradiol	-10	20450.3	30675.5	93.8 87.9	6.1	36.8	89.3	4.1	2.3	4.6	22.8		-11	
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			
20483.0	30		-11	19919.3	29879.0	91.4	-2.7	7.0					22.0			
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			
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		100-	
2109.0	35		-4.5	1545.3	2318.0	7.1	1.0	1.0					2.3		90-0-	_
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	E	80-	
10634.0	38		-5.5	10070.3	15105.5	46.2	1.6	2.6					11.5	ę.	50	
16245.0	39	19-Norethindrone	-5.5	9261.3	23522.0	42.5	-2.1	4.4	72.1	0.4	0.2	0.5	10.6	8	50	
16378.0	41		-6	15814.3	23721.5	72.6	1.6	2.6					17.7	100	40-	
16223.0	42	19-Norethindrone	-6	15659.3	23489.0	71.9	0.9	0.8	94.7	2.1	12	26	17.6	s	30-	
19313.0	44	19-Norechindrone	-6.5	18749.3	28124.0	86.0	0.6	0.4	04.2	2.1	1.2	2.5	20.9	Nes	20-	
19010.0	45		-6.5	18446.3	27669.5	84.6	-0.8	0.6					20.6		10-	
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		-8.6	
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	!		
19786.0	51		-7.5	19222.3	28833.5	88.2	-4.2	17.8					21.4	1		
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	1		
21057.0 20647.0	53		-8.5	20493.3 20083.3	30740.0	94.0	-0.9	1.0					22.8			
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	1		
19152.0	56		-3	18588.3	27882.5	85.3	0.4	0.2					20.7			
20554.0	58	Octyltriethoxysilane	-3	19990.3	29985.5	91.7	-3.7	13.8	95.5	3.3	1.9	3.5	20.2		1	
21637.0	59		-4	21073.3	31610.0	96.7	1.3	1.6					23.4		120-	
21914.0	60	Octvitriethoxysilane	-4	21350.3	32025.5	98.0	2.5	6.4	95.6	0.5	0.3	0.5	23.7	E	100-2	
21281.0	62		-5	20717.3	31076.0	95.1	-0.8	0.6					23.1	ŝ	-00	-
21463.0	63	Caballadathaannallaaa	-5	20899.3	31349.0	95.9	0.0	0.0		24			23.3	5		
21321.0	65	octynnethoxysnane	-6	20357.3	31209.5	95.5	-0.4	0.2	54.0	3.1	1.8	3.3	23.3	edito	60-	
20276.0	66	Och Heat	-6	19712.3	29568.5	90.5	-5.4	29.5					22.0	ŝ	40-	
22349.0 21196.0	67	Octyltnethoxysilane	-7	21785.3 20632.3	32678.0	94.7	4.1	16.7	96.3	3.2	1.8	3.3	24.2	Nea	20-	
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		-10	
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	ł		
22286.0	74		-9	21722.3 20559.3	32583.5 30839.0	99.7	3.8	14.4				1	24.1			
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	1		
22858.0 20303.0	77		-10	22294.3	33441.5 29609.0	102.3	6.4	41.2	-			1	24.8			
19642	79	Methoxycinnamate	-3	19078.3	28617.5	87.5	2.9	8.6	84.0	3.1	1.8	3.7	21.3	1		
18451	80		-3	17887.3	26831.0	82.1	-2.5	6.4					20.0			
20648	81	Methoxycinnamate	-3	20084.3	30126.5	92.2	4.5	4.9	89.2	5.0	2.9	5.6	20.1		110	
18746	83		-4	18182.3	27273.5	83.4	-4.3	18.3					20.3		100	•
20601	84	Methoxycinnamate	-4	20037.3	30056.0	91.9	4.2	17.9	90.7	7.0	4.1	7.8	22.3	ê	80	
20766	86		-5	20202.3	30303.5	92.7	-0.1	0.0				1 ····	22.5	Ĵ,	70-	
18619	87	Li athanna i	-5	18055.3	27083.0	82.9	-9.9	98.5	00.5				20.2	-	60-	
21546 21578	88	Metnoxycinnamate	-6	20982.3	31473.5 31521.5	96.3	0.1	0.0	98.3	3.4	2.0	3.4	23.3		50-	
22840	90		-6	22276.3	33414.5	102.2	6.1	36.9					24.7	ŝ	40-	
21260	91	Methoxycinnamate	-7	20696.3	31044.5	95.0	-2.3	5.4	97.8	2.4	1.4	2.5	23.0	Weat	20	
22142	93		-7	21578.3	32367.5	99.0	1.7	3.0					24.0		10	
22206	94	Methoxycinnamate	-8	21642.3	32463.5	99.3	1.7	3.0	97.5	1.6	0.9	1.7	24.1		-10	
21697	95		-8	20956.3	31/00.0	96.2	-0.6	2.0					23.5	_		
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 21549	98		-9	21534.3 20985.3	32301.5 31478.0	98.8 96.3	1.2	1.4				1	23.9 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	i		
21855	101		-10	21291.3	31937.0	97.7	0.0	0.0					23.7			

# APPENDIX 1 Raw and Normalized Data 1<sup>st</sup> Valid Run (continued) – July 25, 2011

Experiment Date:	25-Jul-11		Study Number:	9070-10010	7ERB				Assays Co	nducted by:			-	
Test substance: 12/6/2011 17:42	Octylsalicyla	te												
		10 uL of 50 nM E2-	_	DPM and 0.	5 x10-12 mol	es								
		ug protein/assay tube =	80.0	DPM/mole										
				Specific	Total		i i							
	Tube	Sample Type	DPM (1mL)	DPM (1mL)	Binding	Mean								
	1	-	17709	- NSB	(1.6ML) 138130.2									
	2	Total Activity (Master Mix)	17865	-	139347.0 138598.2	138465.6								
	4 5		17719	-	138208.2									
	6 7		17732 22497	21933.3	138309.6 32900.0									
	8	Total Binding (Solvent Control)	22817 21752	22253.3 21188.3	33380.0 31782.5	32687.5								
DPM	Tube	Sample Type	Concentration	Specific Binding	Total Specific	Specific	Paridual	Squared	Mean Speolfio	Standard		54 CV	% Ligand Bound vs.	
(1mL) from LSC	1000	sample Type	log[M]	DPM (1mL) - NSB	(1.6mL)	(%)	Roaddar	Residual	Binding (%)	Deviation	0 EM		Totai Activity	
513.0 439.0	10	Estradiol (NSB)	-7 -7	-50.7	-76.0	-0.2	-0.6	0.3	0.0	0.7	0.4	3.2E+17	0.6	
739.0	12	Estradiol	-7	175.3	263.0	0.8	0.5	0.2	7.7	0.3	0.2	4.0	0.8	
2216.0	14		-8	1652.3	2478.5	7.6	-0.4	0.2					2.4	90-
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	2 70-
6080.0	17 18		-8.5 -8.5	5516.3 5616.3	8274.5 8424.5	25.3 25.8	2.1 2.6	4.6 6.7					6.6 6.7	8 60 - 8 50 -
10397.0 11152.0	19 20	Estradiol	-9 -9	9833.3 10588.3	14750.0 15882.5	45.1 48.6	-4.6	21.2	47.7	2.3	1.3	4.8	11.3 12.1	ag 40 -
11345.0 17285.0	21	Estradiol	-9	10781.3	16172.0 25082.0	49.5 76.7	-0.3	0.1	75.4	1.8	1.0	2.3	12.3	9 30 - 5 20 -
16557.0	23		-9.5	15993.3	23990.0 24833.0	73.4	-1.4	1.8					17.9	≥ 10-
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	• <u>+</u>
19720.0	26		-10	19156.3	28/34.5 28131.5	87.9	-1.7	2.9					21.4	
21033.0 20859.0	28 29	Estradiol	-11	20469.3 20295.3	30704.0 30443.0	93.9 93.1	-0.1	0.0	92.8	1.3	0.7	1.4	22.8 22.6	
20483.0 805.0	30	19-Norethindrone	-11	19919.3 241.3	29879.0 362.0	91.4	-2.7	7.0	0.9	0.2	0.1	21.6	22.2	
722.0	32		-4	158.3	237.5	0.7	-0.6	0.4					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	100-
1937.0	36		-4.5	1373.3	2060.0	6.3	0.2	0.1					2.3	- 00
10634.0	37	19-Noretnindrone	-5.5	10070.3	14130.5	43.2	-1.4	2.6	44.0	2.0	1.1	4.5	10.8	2 70 -
9825.0 16245.0	39	19-Norethindrone	-5.5	9261.3	13892.0 23522.0	42.5	-2.1	4.4	72.1	0.4	0.2	0.5	10.6	2 50 -
16378.0 16223.0	41 42		-6 -6	15814.3 15659.3	23721.5 23489.0	72.6	1.6	2.6					17.7	8 40 - 9 30 -
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	20-
19010.0	45	19-Norathindmaa	-6.5	18446.3	27669.5	84.6	-0.8	0.6	90.7	14		1.6	20.6	10-
20187.0	47	15 Woreeningrone	-7	19623.3	29435.0	90.0	-0.7	0.5	50.7		0.0	1.3	21.9	-8.6
20664.0	48	19-Norethindrone	-7 -7.5	20100.3	30150.5	92.2	7.6	2.2	94.0	5.9	3.4	6.3	22.4	
20967.0 19786.0	50		-7.5 -7.5	20403.3 19222.3	30605.0 28833.5	93.6 88.2	-4.2	1.4					22.7 21.4	
20235.0 21057.0	52 53	19-Norethindrone	-8.5 -8.5	19671.3 20493.3	29507.0 30740.0	90.3 94.0	-2.8	7.8	92.2	1.9	1.1	2.0	21.9 22.8	
20647.0	54	Octvitrietboxysilane	-8.5	20083.3	30125.0	92.2	-0.9	0.8	84.9	1.8	11	22	22.4	
19152.0	56	octymic moxy and re	-3	18588.3	27882.5	85.3	0.4	0.2					20.7	
20554.0	58	Octyltriethoxysilane	-3	19990.3	29985.5	91.7	-2.0	4.1	95.5	3.3	1.9	3.5	20.2	120
21637.0 21914.0	59 60		4	21073.3 21350.3	31610.0 32025.5	96.7 98.0	1.3	1.6					23.4 23.7	£ 100-
21469.0 21281.0	61	Octyltriethoxysilane	-5	20905.3 20717.3	31358.0 31076.0	95.9	0.1	0.0	95.6	0.5	0.3	0.5	23.3 23.1	e
21463.0 21521.0	63	Octyltriethoxysilane	-5	20899.3 20957.3	31349.0 31436.0	95.9 96.2	0.0	0.0	94.0	3.1	1.8	3.3	23.3 23.3	a 60
21370.0	65		-6	20806.3	31209.5	95.5	-0.4	0.2					23.2	bed bed
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	40 -
21196.0	69		-7	20632.3	30948.5	94.7	-1.2	2.6					23.0	20
21533.0 21291.0	70 71	Octyltriethoxysilane	-8 -8	20969.3 20727.3	31454.0 31091.0	96.2 95.1	0.3	0.1	94.7	1.7	1.0	1.8	23.3 23.1	-10
20786.0 22344.0	72	Octyltriethoxysilane	-8 -9	20222.3 21780.3	30333.5 32670.5	92.8	-3.1	9.5	98.0	3.2	1.8	3.2	22.5 24.2	
22286.0 21123.0	74		-9 -9	21722.3 20559.3	32583.5 30839.0	99.7 94.3	3.8	14.4					24.1 22.9	
21688.0 22858.0	76	Octyltriethoxysilane	-10	21124.3	31686.5 33441.5	96.9 102.3	1.0	1.1	96.6	5.9	3.4	6.1	23.5	
20303.0	78	Ostulaalisulata	-10	19739.3	29609.0	90.6	-5.3	28.2	60.3	4.5		4.0	22.0	
13643	80	octynancynite	-3	13079.3	19619.0	60.0	-0.3	0.1		1.4	9.7	1.9	14.8	
13993	81	Octylsalicylate	-3	13429.3	20144.0	61.6 70.5	1.3	1.7 20.8	74.9	3.8	2.2	5.1	15.2	110
17337 17389	83 84		4	16773.3	25160.0 25238.0	77.0	1.9	3.7					18.8 18.8	90-
19745 19933	85	Octylsalicylate	-5 -5	19181.3 19369.3	28772.0 29054.0	88.0 88.9	-0.2	0.0	88.8	0.8	0.4	0.9	21.4 21.6	~ 80 - -
20080	87	Octylsalicylate	-5	19516.3	29274.5	89.6	1.3	1.8	90.7	0.5	03	0.6	21.8	E 60-
20253	89		-6	19689.3	29534.0	90.4	-1.9	3.6		3.9	3.5	0.0	21.9	50 - 40 -
20273	90	Octylsalicylate	-6	20072.3	29564.0	90.4	-1.8	0.8	93.2	1.8	1.0	1.9	22.0	5 30- 37 ac
21318 20638	92		-7 -7	20754.3 20074.3	31131.5 30111.5	95.2 92.1	2.2	4.9					23.1 22.4	10-
20924 21691	94 95	Octylsalicylate	-8 -8	20360.3	30540.5 31691.0	93.4 97.0	0.3	0.1	96.2	2.5	1.4	2.6	22.7 23.5	-10
21982	96	Octylealicylate	-8	21418.3	32127.5	98.3	5.1	26.4	97.4	0.4	0.7	0.4	23.8	
20758	98	octynaancynate	-9	20194.3	30291.5	92.7	-0.5	0.3	22.4	<i></i>	9.2	0.4	22.5	
20615	99	Octylsalicylate	-9 -10	20051.3	30077.0	92.0	-1.2	1.3	91.8	0.2	0.1	0.2	22.3	
20609 20549	101		-10	20045.3	30068.0 29978.0	92.0	-1.2	1.4	1	1		1	22.3	

# APPENDIX 1 Raw and Normalized Data 1<sup>st</sup> Valid Run (continued) – July 25, 2011

Experiment Date:	25-Jul-11		Study Number:	9070-10010	TERB				Assays Con	ducted by:						
Test substance: 12/6/2011 17:42	Octocrylene												Γ			
12/6/2011 17:42																
		Therefore there are	—	DPM and 0. DPM/mole	5 x10-12 mol	es										
		ug protein/assay tube =	80.0													
				Specific	Total									1		
	Tube	Sample Type	DPM (1mL)	Binding DPM (1mL)	Specific Binding	Mean										
	1		17709	- N8B	(1.6mL)											
	2		17865	-	139347.0											
	3	Total Activity (Master Mix)	17769	-	138598.2	138465.6										
	5		17718 17732	-	138200.4 138309.6											
	7		22497	21933.3	32900.0											
	8	Total Binding (Solvent Control)	22817 21752	22253.3 21188.3	33380.0 31782.5	32687.5										
				Specific	Total	Specific			Mean				% Ligand	1		
DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Binding DPM (1mL)	Binding	Binding	Residual	Recidual	Specific Binding	Standard Deviation	SEM	% CV	Bound vs. Total	L		
513.0	10	Estradiol (NSB)	-7	- NSB -50.7	(1.6mL) -76.0	-0.2	-0.6	0.3	(%) 0.0	0.7	0.4	3.2E+17	Activity 0.6	•		
439.0	11		-7	-124.7	-187.0	-0.6	-0.9	0.9					0.5	ᄂ		
739.0 2328.0	12	Estradiol	-7 -8	175.3	263.0	0.8	0.5	0.2	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		80-00	
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	8	70-	
6080.0 6180.0	17		-8.5 -8.5	5516.3 5616.3	8274.5 8424.5	25.3 25.8	2.1	4.6					6.6	Bind	60-	
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	8	40-	
11345.0	20		-9	10781.3	16172.0	49.5	-0.3	0.1					12.3	S.	30-	
17285.0	22	Estradiol	-9.5 -9.5	16721.3	25082.0 23990.0	76.7	2.0	3.9	75.4	1.8	1.0	2.3	18.7	2	20-	
17119.0	24	Caterdial	-9.5	16555.3	24833.0	76.0	1.2	1.5	80.3		2.2		18.5	1		
19720.0	25	Estradio	-10	19156.3	28734.5	87.9	0.1	0.0	69.3	4.1	2.3	4.6	22.8		-11	
19318.0	27	Estradial	-10	18754.3	28131.5	86.1 93.9	-1.7	2.9	97.8	13	07	14	20.9	1		
20859.0	29	L'androi	-11	20295.3	30443.0	93.1	-0.9	0.9	52.0		0.7		22.6	1		
20483.0	30	19-Norethindrone	-11	241.3	29879.0	91.4	-2.7	7.0	0.9	0.2	0.1	21.6	0.9	1		
722.0	32		4	158.3	237.5	0.7	-0.6	0.4					0.8	ᄂ		
2032.0	33	19-Norethindrone	-4.5	1468.3	2202.5	6.7	0.5	0.3	6.7	0.4	0.2	5.9	2.2		100-	
2109.0 1937.0	35		-4.5	1545.3	2318.0 2060.0	7.1 6.3	1.0	1.0					2.3	~	90-0-	
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	8	70	
9825.0	39		-5.5	9261.3	13892.0	40.2	-2.1	4.4					10.6	2	60-	
16245.0 16378.0	40	19-Norethindrone	-6	15681.3	23522.0	72.0	1.0	1.0	72.1	0.4	0.2	0.5	17.6	8	50 40	
16223.0	42		-6	15659.3	23489.0	71.9	0.9	0.8					17.6	8	30-	
18403.0 19313.0	43	19-Norethindrone	-6.5	17839.3 18749.3	26759.0 28124.0	81.9	-3.6	0.4	84.2	2.1	1.2	2.5	19.9	Nea	20-	
19010.0	45	19-Norethindrone	-6.5	18446.3	27669.5	84.6	-0.8	0.6	90.7	14	0.8	15	20.6	{	٥.	
20187.0	47		-7	19623.3	29435.0	90.0	-0.7	0.5	20.1		0.0		21.9		-8.6	
22360.0	48	19-Norethindrone	-7	20100.3	30150.5	92.2	7.6	57.7	94.0	5.9	3.4	6.3	22.4	1		
20967.0	50		-7.5	20403.3	30605.0	93.6	1.2	1.4				-	22.7	Ł		
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	1		
21057.0 20647.0	53		-8.5	20493.3 20083.3	30740.0 30125.0	94.0	-0.9	1.0					22.8	1		
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	1		
18614.0	57		-3	18050.3	27075.5	82.8	-2.0	4.1					20.7			
20554.0 21637.0	58	Octyltriethoxysilane	4	19990.3	29985.5 31610.0	91.7	-3.7	13.8	95.5	3.3	1.9	3.5	22.3		120	
21914.0	60	Octultriathownslippe	-4	21350.3	32025.5	98.0	2.5	6.4	95.5	0.5	0.3		23.7	3	100-2	•
21281.0	62	octynnethoxysnane	-5	20305.3	31076.0	95.1	-0.8	0.6	55.6	0.5	0.3	3.5	23.3	ŝ	-00	
21463.0	63	Octvitriethoxysilane	-5	20899.3	31349.0	95.9	0.0	0.0	94.0	31	1.8	33	23.3	5		
21370.0	65		-6	20806.3	31209.5	95.5	-0.4	0.2				1	23.2	100	60-	
20276.0 22349.0	66	Octyltriethoxysilane	-6 -7	21785.3	32678.0	90.5	-5.4	29.5	96.3	3.2	1.8	3.3	22.0	5	40-	
21196.0	68		-7	20632.3	30948.5	94.7	-1.2	1.5					23.0	2	20	
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	1	اه	
21291.0 20786.0	71		-8 -8	20727.3	31091.0	95.1	-0.8	0.6 9.5					23.1 22.5	<u> </u>	-10	
22344.0	73	Octyltriethoxysilane	-9	21780.3	32670.5	99.9 99.7	4.1	16.5	98.0	3.2	1.8	3.2	24.2	{		
21123.0	75		-9	20559.3	30839.0	94.3	-1.5	2.4					22.9	1		
21688.0 22858.0	76	Octyltriethoxysilane	-10	21124.3 22294.3	31686.5 33441.5	96.9 102.3	1.0	1.1 41.2	96.6	5.9	3.4	6.1	23.5 24.8	{		
20303.0	78	Otherstere	-10	19739.3	29609.0	90.6	-5.3	28.2	00.5				22.0	1		
20289	80	Octochylene	-3	19309.3	29588.0	90.5	1.9	3.6	90.7	4.4	1.3	2.5	21.5			
20845	81	Octocrylene	-3	20281.3	30422.0	93.1 100.3	4.5	19.9	102.4	2.0	1.1	1.9	22.6	1	110	-
22945	83		-4	22381.3	33572.0	102.7	14.1	198.7				1	24.9		100	-
23263	84	Octocrylene	-5	22699.3	34049.0 33786.5	104.2	15.6	242.0	103.9	1.2	0.7	1.2	25.2	8	80	
23018 23511	86 87		-5	22454.3 22947.3	33681.5 34421.0	103.0	14.4	208.3 278.7					24.9 25.5	E.	70-	
22848	88	Octocrylene	-6	22284.3	33426.5	102.3	13.7	186.4	101.7	1.1	0.7	1.1	24.8	2	50	
22449 22903	89 90		-6 -6	21885.3 22339.3	32828.0 33509.0	100.4	11.8 13.9	139.7 193.3					24.3 24.8	ŝ	40-	
21786	91	Octocrylene	-7	21222.3	31833.5	97.4	8.8	77.1	130.9	56.8	32.8	43.3	23.6	Mean	20	
43379	93		-7	42815.3	64223.0	196.5	10.4	11635.3					47.0	1	10	
23018 22833	94 95	Octocrylene	-8 -8	22454.3 22269.3	33681.5 33404.0	103.0	14.4 13.6	208.3	103.4	1.4	0.8	1.4	24.9 24.7		-10	
23435	96	Octocorlege	-8	22871.3	34307.0	105.0	16.3	267.2	97.0	47	27	19	25.4	1		
21105	98	Octochylene	-9	20533.3	30812.0	94.3	5.7	32.0	57.0	·•./	2.1	4.9	22.9	1		
22891 20768	99 100	Octocrylene	-9	22327.3 20204.3	33491.0 30306.5	102.5 92.7	13.8	191.8	94.1	2.0	1.2	2.1	24.8	1		
20856	101		-10	20292.3	30438.5 31509.5	93.1 96.4	4.5	20.3					22.6	1		

Experiment Date:	1-Aug-11		Study Number:	9070-10010	7ERB				Assays Cor	ducted by:			-	
Test substance:	Oxybenzone													1
1/2//2012 16:13														
		10 uL of 50 nM E2= Therefore there are	<b>—</b>	DPM and 0. DPM/mole	5 x10-12 mol	es								
		ug protein/assay tube -	80.0											
				Specific	Total									
	Tube	Sample Type	DPM (1mL)	DPM (1mL)	Binding	Mean								
	1		17533	- NSB	(1.6mL) 136757.4									
	2		17478 17405	-	136328.4 135759.0	135000 5								
	4	Total Activity (Master Mix)	17532	-	136749.6 137319.0	136505.5								
	6		17762		138543.6									1
	8	Total Binding (Solvent Control)	15894	15521.7	23282.5	24693.0								
			1/122	16/43./	25124.5									
				Specific	Total	Specific			Mean				% Ligand	1
DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Binding DPM (1mL)	Specific Binding	Binding	Residual	Recidual	Specific Binding	Standard Deviation	SEM	% CV	Bound vs. Total	
414.0	10	Estradiol (NSB)	-7	- N8B 41.7	(1.6mL) 62.5	0.3	0.1	0.0	(%)	0.2	0.1	2.0E+17	Activity 0.5	
356.0 347.0	11		-7	-16.3	-24.5	-0.1	-0.2	0.0					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	an L .
1626.0 1570.0	14		-8	1253.7	1880.5 1796.5	7.6	-1.1	1.3					1.8	2 80-
4889.0 4289.0	16	Estradiol	-8.5	4516.7 3916.7	6775.0 5875.0	27.4	4.1	16.7	26.2	2.1	1.2	7.9	5.4	2 70 -
4870.0	18	Estradiol	-8.5	4497.7	6746.5	27.3	4.0	15.7	44.4	0.0	0.4	17	5.3	50 50
7813.0	20	Canada	-9	7440.7	11161.0	45.2	-2.0	3.9		0.0	0.4		8.6	10 - 40 -
7563.0 12394.0	21 22	Estradiol	-9 -9.5	7190.7 12021.7	10786.0 18032.5	43.7	-3.5	7.6	70.8	1.9	1.1	2.7	8.3	5 20-
11797.0 11911.0	23		-9.5 -9.5	11424.7 11538.7	17137.0 17308.0	69.4 70.1	-0.9	0.8					12.9	10-
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	-11
13855.0	26		-10	13482.7	20224.0	81.9	-1.8	3.4					15.2	
16046.0 14839.0	28	Estradiol	-11	15673.7	23510.5 21700.0	95.2	3.8	14.1	90.2	4.3	2.5	4.8	17.6	
14779.0	30	19-Norethindrone	-11	14406.7	21610.0	87.5	-3.9	15.5	0.8	0.1	0.1	12.5	16.2	
514.0	32		-4	141.7	212.5	0.9	1.2	1.5					0.6	
492.0 1226.0	33	19-Norethindrone	-4 -4.5	119.7 853.7	179.5	0.7 5.2	-2.7	1.2	6.3	1.0	0.6	16.1	0.5	100-0
1434.0 1554.0	35		-4.5 -4.5	1061.7 1181.7	1592.5	6.4 7.2	-1.5	2.2					1.6	90-
6886.0 7807.0	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	2 70 -
7863.0	39		-5.5	7490.7	11236.0	45.5	2.3	5.4					8.6	8 60-
10332.0	40	19-Noretningrone	-6	10971.7	16457.5	66.6	-3.6	6.3	65.1	4.0	2.3	6.2	11.3	10 - 40 -
11584.0 13652.0	42	19-Norethindrone	-6 -6.5	11211.7 13279.7	16817.5 19919.5	68.1 80.7	4.0	15.8	79.2	3.3	1.9	4.2	12.7	<sup>60</sup> 30 -
12780.0	44		-6.5	12407.7	18611.5	75.4	-4.2	18.0					14.0	10-
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 14283.0	47		-7 -7	15125.7 13910.7	22688.5 20866.0	91.9 84.5	3.3	11.2					17.0	-0.0
15099.0 15142.0	49	19-Norethindrone	-7.5	14726.7	22090.0 22154.5	89.5 89.7	-3.5	12.4	90.1	0.8	0.5	0.9	16.5	
15355.0	51	19-Norethindrone	-7.5	14982.7	22474.0	91.0	-2.0	3.9	99.4	17	10	17	16.8	{
16530.0	53	13 Horeannaiche	-8.5	16157.7	24236.5	98.2	2.2	5.0			1.0		18.1	
7933.0	55	Octyltriethoxysilane	-8.5	7560.7	11341.0	45.9	4.3	0.0	45.8	0.2	0.1	0.5	8.7	1
7938.0 7874.0	56		-3	7565.7	11348.5 11252.5	46.0	0.0	0.0					8.7	
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	120-
15381.0	60		-4	15008.7	22513.0	91.2	2.7	7.4					16.9	£ 100-
16553.0	61	Octylthethoxysilane	-5	16180.7	24065.5	97.5	6.5	42.0	98.4	1.1	0.6	1.1	18.0	₹ a0
16763.0 15302.0	63	Octyltriethoxysilane	-5	16390.7	24586.0 22394.5	99.6 90.7	7.8	60.1 1.3	92.3	4.3	2.5	4.6	18.4	a
15039.0	65		-6	14666.7	22000.0	89.1	-2.7	7.4					16.5	8
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	5 40 -
14825.0	69		-7	146/3./ 14452.7	21679.0	90.4 87.8	-1.5	16.2					16.7	20-
15139.0 14731.0	70	Octyltriethoxysilane	-8 -8	14766.7 14358.7	22150.0 21538.0	89.7 87.2	-2.1	4.5	87.3	2.3	1.3	2.6	16.6	-10
14382.0 16156.0	72	Octyltriethoxysilane	-8	14009.7	21014.5	85.1 95.9	-6.7 4.1	45.0	93.0	3.0	1.7	3.2	15.8	1
15710.0	74		-9	15337.7	23006.5	93.2	1.4	1.8					17.2	
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	1
15433.0	77		-10 -10	14683.7	22591.0 22025.5	91.5 89.2	-0.3	0.1 6.8					16.9	1
12787 13091	79	Oxybenzone	4 4	12414.7 12718.7	18622.0 19078.0	75.4	-1.6 0.2	2.6	76.4	0.9	0.5	1.2	14.0	
12994	81	Ownhamman	-4	12621.7	18932.5	76.7	-0.4	0.1		2.0			14.2	110
15695	83	Oxybenzone	-5	15322.7	22984.0	93.1	2.3	5.3	53.4	2.0	1.2	2.2	17.2	100
16091 15372	84 85	Oxybenzone	-5	15718.7 14999.7	23578.0 22499.5	95.5	4.7	22.1	91.2	0.5	0.3	0.5	17.6	80 - 80
15475 15325	86 87		-6 -6	15102.7 14952.7	22654.0 22429.0	91.7 90.8	-3.4	11.5					17.0 16.8	5 70 - E 60 -
15618	88	Oxybenzone	-7	15245.7	22868.5	92.6	-3.9	15.0	95.5	4.4	2.5	4.6	17.1	
16919	90		-7	16546.7	24820.0	100.5	4.0	16.2					18.5	∂ 40 -
16930 15279	91 92	Oxybenzone	-8 -8	16557.7 14906.7	24836.5 22360.0	100.6	3.7	13.5 40.4	95.9	5.1	2.9	5.3	18.5	20-
16276 16948	93	Oxybenzone	-8	15903.7	23855.5 24863.5	96.6 100.7	-0.3 3.7	0.1	101.2	3.2	1.8	3.1	17.8	
17582	95		-9	17209.7	25814.5 24255 5	104.5	7.5	56.3					19.3	-11
15838	97	Oxybenzone	-10	15465.7	23198.5	93.9	-3.1	9.8	97.1	3.0	1.7	3.0	17.4	1
16449 16799	98		-10 -10	16076.7 16426.7	24115.0 24640.0	97.7 99.8	0.6	0.3					18.0	
16092 16022	100	Oxybenzone	-11	15719.7 15649.7	23579.5 23474.5	95.5 95.1	-1.6	2.6	96.6	2.3	1.3	2.4	17.6	
16712	102		-11	16339.7	24509.5	99.3	2.2	4.7		1			18.3	1

## APPENDIX 1 Raw and Normalized Data 2<sup>nd</sup> Valid Run (continued) – August 01, 2011

Experiment Date:	1-Aug-11		Study Number:	9070-10010	7ERB				Assays Con	ducted by:		_			
Test substance:	Methoxycinn	namate													
1/27/2012 16:14															
		10 uL of 50 nM E2=		DPM and 0.	5 x 10-12 mol	es									
		Therefore there are	80.0	DPM/mole											
		ag protein assay tabe -	00.0												
				Specific	Total										
	Tube	Sample Type	DPM (1mL)	DPM (1mL)	Binding	Mean									
				- NSB	(1.6mL)										
	1 2	-	17533		136757.4										
	3	Total Activity (Master Mix)	17405	-	135759.0	136909.5									
	4		17532		136749.6										
	6		17762	-	138543.6										
	7	Total Disting (Datast Control)	17487	17114.7	25672.0	24502.0									
	9	Total Binding (Solvent Control)	15894	16749.7	25124.5	24693.0									
	-			Specific	Total				Mean				%Ligand	1	
DPM	Tube	Remote Type	Concentration	Binding	Specific	Specific	Decidual	Squared	Specific	Standard		14 CV	Bound vs.	I	
(1mL) from LSC			log[M]	DPM (1mL)	Binding	(%)	Roundan	Recidual	Binding	Deviation	U.L.	~~~	Total	I	
414.0	10	Estradiol (NSB)	-7	41.7	(1.6mL) 62.5	0.3	0.1	0.0	0.0	0.2	0.1	2.0E+17	0.5	1	
356.0	11		-7	-16.3	-24.5	-0.1	-0.2	0.0					0.4		
347.0	12	Estradial	-7	-25.3	-38.0	-0.2	-0.3	0.1			0.3	6.2	0.4		
1626.0	14	Estradio	-8	1253.7	1880.5	7.6	-1.1	1.3	1.1	0.5	0.5	0.5	1.8		90
1570.0	15		-8	1197.7	1796.5	7.3	-1.5	2.2					1.7	æ	80-
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	8	70-
4870.0	18		-8.5	4497.7	6746.5	27.3	4.0	15.7					5.3	2	60-
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	÷.	40
7813.0	20		-9	7440.7	10786.0	45.2	-2.0	3.9				1	8.6	8	30
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	5	20
11797.0	23		-9.5	11424.7	17137.0	69.4	-0.9	0.8					12.9	2	10
11911.0	24	Estradiol	-9.5	11538.7	22435.0	70.1	-0.2	50.7	85.5	4.7	2.7	5.5	13.0		°E
14149.0	26		-10	13776.7	20665.0	83.7	0.0	0.0					15.5		-11
13855.0	27	Esteration	-10	13482.7	20224.0	81.9	-1.8	3.4	90.2	47	2.5		15.2	1	
16046.0	28	Estradio	-11	15673.7	23510.5	95.2	-3.6	14.1	90.2	4.3	2.5	4.8	17.6	{	
14779.0	30		-11	14406.7	21610.0	87.5	-3.9	15.5					16.2	í	
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		
492.0	32		-4	141.7	179.5	0.9	1.2	1.5				-	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		100-0
1434.0	35	-	-4.5	1061.7	1592.5	6.4	-1.5	2.2					1.6		90-
6886.0	36	19-Norethindrone	-5.5	6513.7	9770.5	39.6	-3.6	13.0	43.4	3.3	1.9	7.7	7.5	£	80-
7807.0	38		-5.5	7434.7	11152.0	45.2	2.0	3.9					8.6	ŝ.	70-
7863.0	39		-5.5	7490.7	11236.0	45.5	2.3	5.4		10			8.6	8	50
10332.0	40	19-Norethindrone	-6	10971.7	16457.5	66.6	-3.6	6.3	65.1	4.0	2.3	6.2	11.3	5	40-
11584.0	42		-6	11211.7	16817.5	68.1	4.0	15.8					12.7	8	30-
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	Nean	20-
13776.0	44		-6.5	13403.7	20105.5	81.4	1.8	3.3					15.1		10-
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		-0.0
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	1	
15142.0	50		-7.5	14769.7	22154.5	89.7	-3.3	10.6					16.6	1	
15355.0	51	19-Norethindrone	-7.5	14982.7	22474.0	91.0	-2.0	3.9	99.4	17	1.0	17	16.8	ł	
16530.0	53	13 Horethindrone	-8.5	16157.7	24236.5	98.2	2.2	5.0	50.4		1.4		18.1	1	
16866.0	54		-8.5	16493.7	24740.5	100.2	4.3	18.2					18.5	1	
7933.0	55	Octyltnethoxysllane	-3	7560.7	11341.0	45.9	0.0	0.0	45.8	0.2	0.1	0.5	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		120
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	e.	100
16553.0	62		-5	16180.7	24271.0	98.3	6.5	42.0					18.1	÷.	80-
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	-	60
15039.0	65		-6	14666.7	22000.0	89.1	-2.7	7.4					16.5	8	~" <b>F</b>
16361.0	66	Octvitriethoxysilane	-6	15988.7	23983.0	97.1	-0.1	28.2	89.9	2.0	11	22	17.9	S S	40
15246.0	68	Cong an entroxy single?	-7	14873.7	22310.5	90.4	-1.5	2.1					16.7	2	20
14825.0	69	Contractor in	-7	14452.7	21679.0	87.8	-4.0	16.2					16.2		- I .
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		-10
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	{	
15/10.0	75		-9	15337.7 14793.7	23006.5	93.2	-1.9	3.8					17.2	1	
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	1	
15433.0	77		-10	15060.7	22591.0	91.5	-0.3	0.1				1	16.9	{	
16017	79	Methoxycinnamate	-4	15644.7	23467.0	95.0	-0.3	0.1	93.4	1.4	0.8	1.5	17.5	1	
15679	80		-4	15306.7	22960.0	93.0	-2.4	5.6					17.2	<u> </u>	
15565	81	Methoxycinnamate	-4	15192.7	22789.0	92.3	-3.1	9.3	97.3	0.4	0.2	0.4	17.1		שיי
16305	83		-5	15932.7	23899.0	96.8	1.4	2.1					17.9		100-
16444	84	Mathewart	-5	16071.7	24107.5	97.6	2.3	5.2	01.5				18.0	ŝ	a0 8
16025	85	Methoxycinnamate	-6	15652.7	23479.0	95.1	-0.1	0.7	94.2	0.8	0.5	0.9	17.6	S.	70-
15759	87		-6	15386.7	23080.0	93.5	-0.8	0.6					17.3	Bind	60-
15530	88	Methoxycinnamate	-7	15157.7	22736.5	92.1	1.9	3.6	89.4	5.9	3.4	6.6	17.0	€	50-
15767	89		-7	15394.7	23092.0	93.5	3.3	11.1				1	17.3	8	40-
15221	91	Methoxycinnamate	-8	14848.7	22273.0	90.2	0.0	0.0	91.0	1.4	0.8	1.5	16.7	5	30-
15234	92		-8	14861.7	22292.5	90.3	0.1	0.0					16.7	-	10
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		-11
15467	96	Methoxycinnamate	-9	15094.7	22642.0	91.7	1.5	2.3	90.9	04	0.2	0.5	16.9	{	
15353	98	inclusivy chinamate	-10	14980.7	22471.0	91.0	0.8	0.7			5.2	3.5	16.8	1	
15383	99	Mathews	-10	15010.7	22516.0	91.2	1.0	1.0	00.1				16.9	{	
15270	100	Methoxycinnamate	-11	14897.7	22346.5	90.5	-2.8	8.0	90.1	2.5	1.5	2.8	16.7	{	
15581	102		-11	15208.7	22813.0	97.4	22	49	1	1		1	17.1	1	

## APPENDIX 1 Raw and Normalized Data 2<sup>nd</sup> Valid Run (continued) – August 01, 2011

Experiment Date:	1-Aug-11		Study Number:	9070-10010	7ERB				Assays Cor	ducted by:				
Test substance:	OctylSalicyla	ite												
1/2//2012 16:15														
		10 uL of 50 nM E2= Therefore there are	_	DPM and 0. DPM/mole	5 x10-12 mol	es								
		ug protein/assay tube -	80.0											
				Specific	Total									
	Tube	Sample Type	DPM (1mL)	Binding DPM (1mL)	Specific	Mean								
				- NSB	(1.6mL)									
	1	-	17533 17478	-	136757.4 136328.4									
	3	Total Activity (Master Mix)	17405	-	135759.0	136909.5								
	4		17532 17605	-	136749.6 137319.0									
	6		17762		138543.6									
	8	Total Binding (Solvent Control)	15894	15521.7	23282.5	24693.0								
	9	4	17122	16749.7	25124.5									
DPM		Complex Trees	Concentration	Specific Binding	Specific	Specific	Desident	Squared	Mean Speolflo	Standard			% Ligand Bound vs.	
(1mL) from LSC	TUDe	sample Type	log[M]	DPM (1mL)	Binding	(%)	Recidual	Recidual	Binding	Deviation	SEM	% CV	Total	
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	1
1728.0	12	Estradiol	-7	1355.7	2033.5	-0.2	-0.3	0.1	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	90-
4889.0	15	Estradiol	-8	4516.7	6775.0	27.4	4.1	16.7	26.2	2.1	1.2	7.9	5.4	2 70
4289.0	17		-8.5	3916.7	5875.0	23.8	0.4	0.2					4.7	E 60-
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	2 50
7813.0	20		-9	7440.7	11161.0	45.2	-2.0	3.9					8.6	20 40 - 30 -
12394.0	21	Estradiol	-9	12021.7	18032.5	43.7	-3.5	7.6	70.8	1.9	1.1	2.7	8.3	50-
11797.0	23		-9.5	11424.7	17137.0	69.4	-0.9	0.8					12.9	≥ 10-
11911.0 15329.0	24	Estradiol	-9.5 -10	11538.7	17308.0 22435.0	70.1	-0.2	0.0	85.5	4.7	2.7	5.5	13.0	•
14149.0	26		-10	13776.7	20665.0	83.7	0.0	0.0					15.5	-11
13855.0	27	Estradiol	-10	13482.7	20224.0	81.9	-1.8	3.4	90.2	43	2.5	4.8	15.2	
14839.0	29		-11	14466.7	21700.0	87.9	-3.6	12.8					16.3	l
14779.0	30	19-Norethindmos	-11	14406.7	21610.0	87.5	-3.9	15.5	0.0	0.1	0.1	17.6	16.2	
514.0	32	ra waretningrone	4	141.7	212.5	0.9	1.2	1.5	3.0	3.1	3.1	12.5	0.6	i
492.0	33	19-Northindone	-4	119.7	179.5	0.7	1.1	1.2	6.3	10	0.5	15.4	0.5	4001 -
1434.0	35	istworetningrone	-4.5	1061.7	1592.5	6.4	-1.5	2.2	0.3	1.0	0.6	(6.1	1.6	
1554.0	36	10 blog this down	-4.5	1181.7	1772.5	7.2	-0.7	0.6			10		1.7	e0 -
6886.0	37	19-Norethindrone	-5.5	6513.7	9770.5	39.6	-3.6	3.9	43.4	3.3	1.9	7.7	7.5	g 70-
7863.0	39		-5.5	7490.7	11236.0	45.5	2.3	5.4					8.6	E 60-
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	20 50 20 40 -
11584.0	42		-6	11211.7	16817.5	68.1	4.0	15.8					12.7	S 30-
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	20-
13776.0	45		-6.5	13403.7	20105.5	81.4	1.8	3.3					15.1	10-
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	-8.6
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	
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	
16866.0	54		-8.5	16493.7	24236.5	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	
7874.0	57		-3	7501.7	11252.5	45.6	-0.4	0.0					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	120
14889.0	60		-4	14516.7	21775.0	91.2	-0.3	7.4					16.3	2 100
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	ē ""[
16553.0 16763.0	62		-5	16180.7	24271.0 24586.0	98.3	6.5	42.0					18.1	-0s
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	£ 60-
16361.0	65		-6	14666.7	22000.0	89.1 97.1	-2.7	28.2					16.5	8 40
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	Le la
15246.0 14825.0	68		-7	14873.7	22310.5	90.4	-1.5	2.1					16.7	20
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	وليب
14731.0 14382.0	71		-8	14358.7	21538.0	87.2	-4.6	21.1					16.1	-10
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	1
15710.0	74		-9	15337.7	23006.5	93.2	-1.9	1.8					17.2	i i
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	l
15433.0	77		-10	15060.7	22591.0	91.5	-0.3	0.1					16.9	
11592	79	OctylSalicylate	-4	11219.7	16829.5	68.2	-0.6	0.4	68.7	0.5	0.3	0.8	12.7	ł
11746	80		-4	11373.7	17060.5	69.1	0.3	0.1					12.9	1
13671	81	OctylSalicylate	-5	13298.7	19948.0	80.8	-2.4	5.8	83.2	2.2	1.3	2.7	12.9	110
14148	83		-5	13775.7	20663.5	83.7	0.5	0.2					15.5	100-
14397	84	OctylSalicylate	-5	14024.7	21037.0	85.5	-3.6	4.0	88.8	2.9	1.6	3.2	15.8	8 80-
15246	86		-6	14873.7	22310.5	90.4	1.2	1.5					16.7	ž 70-
15284	8/	OctylSalicylate	-6	14911.7	22367.5	93.8	3.9	14.8	91.2	2.3	1.3	2.5	16.7	······································
15259	89		-7	14886.7	22330.0	90.4	0.5	0.2					16.7	
15091	90	OctviSalicviste	-7	14718.7	22078.0	89.4	-0.5	0.3	91.2	15	0.8	1.6	16.5	<b>5</b> 30-
15148	92	Cityrouncyldie	-8	14775.7	22163.5	89.8	-0.3	0.1	21.3				16.6	ž 20-
15619	93	Ochul@allaulata	-8	15246.7	22870.0	92.6	2.6	6.8	00.0	6.0	25	67	17.1	10-
14659	94	octyrsancylate	-9	14086.7	21130.0	85.6	-4.5	19.9	89.6	6.0	3.5	6.7	15.8	-11
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	
15919	99		-10	15546.7	23320.0	94.4	4.4	19.5	-		_		17.4	
14822	100	OctylSalicylate	-11	14449.7	21674.5 22058.5	87.8 89.3	-2.3	5.1 0.5	89.0	1.0	0.6	1.2	16.2	1
15149	102	1	-11	14775 7	22165.0	89.8	-0.3	0.1	1	1	1	1	16.6	1

## APPENDIX 1 Raw and Normalized Data 2<sup>nd</sup> Valid Run (continued) – August 01, 2011

Experiment Date:	1-Aug-11 Octocoviene		Study Number:	9070-10010	7ERB				Assays Cor	ducted by:					
1/27/2012 16:16	octocificite														
		10 uL of 50 nM E2-		DPM and 0.	5 x10-12 mol	es									
		ug protein/assay tube =	80.0	DPM/mole											
				Specific	Total										
	Tube	Sample Type	DPM (1mL)	DPM (1mL)	Binding	Mean									
	1	-	17533	- NSB	(1.6ML) 136757.4										
	2	Total Activity (Master Mix)	17478 17405	-	136328.4 135759.0	136909.5									
	4		17532 17605	-	136749.6 137319.0										
	6		17762 17487	17114.7	138543.6 25672.0										
	8	Total Binding (Solvent Control)	15894 17122	15521.7 16749.7	23282.5 25124.5	24693.0									
DPM (1ml.) from L SC	Tube	Sample Type	Concentration	Specific Binding	Total Specific Binding	Specific Binding	Residual	Squared	Mean Speolfio Binding	Standard	SEM	% CV	% Ligand Bound vs.		
414.0	10	Estradiol (NSB)	-7	- N8B	(1.6mL)	(%)	0.1		(%)	0.7	0.1	2.05+17	Activity		
356.0	11	Estration (NOB)	-7	-16.3	-24.5	-0.1	-0.2	0.0	0.0	0.2	0.1	2.02+17	0.4		
1728.0	12	Estradiol	-8	1355.7	2033.5	8.2	-0.5	0.3	7.7	0.5	0.3	6.3	1.9	100	
1626.0 1570.0	14		-8 -8	1253.7	1880.5 1796.5	7.6	-1.1	1.3					1.8	÷ - ي	
4889.0 4289.0	16	Estradiol	-8.5	4516.7 3916.7	6775.0 5875.0	27.4 23.8	4.1	16.7	26.2	2.1	1.2	7.9	5.4	- <sup>80</sup>	
4870.0 7687.0	18	Estradiol	-8.5	4497.7	6746.5 10972.0	27.3	4.0	15.7	44.4	0.8	0.4	1.7	5.3 8.4	a 60-	
7813.0	20		-9 -9	7440.7	11161.0	45.2	-2.0	3.9					8.6	<b>8</b> 40 -	
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	20	
11911.0	23	<b>F</b> -4	-9.5	11538.7	17308.0	70.1	-0.9	0.0					13.0	<b>.</b>	
15329.0	25	Estradiol	-10 -10	14956.7 13776.7	22435.0 20665.0	90.9 83.7	7.1	50.7	85.5	4.7	2.7	5.5	16.8 15.5	-11	1
13855.0 16046.0	27 28	Estradiol	-10	13482.7	20224.0 23510.5	81.9 95.2	-1.8 3.8	3.4	90.2	4.3	2.5	4.8	15.2		-
14839.0 14779.0	29 30		-11 -11	14466.7 14406.7	21700.0 21610.0	87.9 87.5	-3.6 -3.9	12.8 15.5					16.3 16.2		
526.0 514.0	31	19-Norethindrone	4	153.7	230.5	0.9	1.3	1.7	0.8	0.1	0.1	12.5	0.6		
492.0	33	10 Norathing man	-4	119.7	179.5	0.7	1.1	1.2	63	10	0.6	45.4	0.5	г	
1434.0	35	15 Norechingtone	-4.5	1061.7	1592.5	6.4	-1.5	2.2	0.5	1.0	0.0	10.1	1.6	100 - 📒	
6886.0	36	19-Norethindrone	-4.5	6513.7	9770.5	39.6	-0.7	13.0	43.4	3.3	1.9	7.7	7.5	eo - 08	
7807.0 7863.0	38 39		-5.5 -5.5	7434.7 7490.7	11152.0 11236.0	45.2 45.5	2.0	3.9 5.4					8.6 8.6	8 60-	
10332.0 11344.0	40	19-Norethindrone	-6 -6	9959.7 10971.7	14939.5 16457.5	60.5 66.6	-3.6	13.2	65.1	4.0	2.3	6.2	11.3	2 40 -	
11584.0 13652.0	42	19-Norethindrone	-6 -6.5	11211.7 13279.7	16817.5 19919.5	68.1 80.7	4.0	15.8	79.2	3.3	1.9	4.2	12.7	5	
12780.0 13776.0	44		-6.5 -6.5	12407.7	18611.5 20105.5	75.4	-4.2 1.8	18.0 3.3					14.0	2 **	
15139.0 15498.0	46	19-Norethindrone	-7	14766.7	22150.0	89.7	1.2	1.3	88.7	3.8	2.2	4.3	16.6	°Ea.6	
14283.0	48	10 blorathing man	-7	13910.7	20866.0	84.5	-4.0	16.3					15.6		
15142.0	50	ishvoretnindrone	-7.5	14769.7	22154.5	89.7	-3.3	10.6	50.1	0.8	0.5	0.9	16.6		
16305.0	51	19-Norethindrone	-7.5	14982.7	23899.0	91.0	0.9	0.7	98.4	1.7	1.0	1.7	16.8		
16530.0 16866.0	53 54		-8.5 -8.5	16157.7 16493.7	24236.5 24740.5	98.2 100.2	2.2 4.3	5.0 18.2					18.1 18.5		
7933.0 7938.0	55	Octyltriethoxysilane	-3	7560.7	11341.0 11348.5	45.9	0.0	0.0	45.8	0.2	0.1	0.5	8.7 8.7		
7874.0 14573.0	57	Octyltriethoxysilane	-3	7501.7	11252.5 21301.0	45.6 86.3	-0.4	0.2 4.8	88.5	2.5	1.4	2.8	8.6	יייר	
14889.0 15381.0	59		4	14516.7	21775.0	88.2	-0.3	0.1					16.3	100-	-
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	€ 80- 2° 70	
16763.0	63	Ostulidation	-5	16390.7	24586.0	99.6	7.8	60.1		47	25		18.4	60-	
15302.0	64	Octyltriethoxysilane	-6	14929.7 14666.7	22394.5 22000.0	90.7 89.1	-1.1	1.3 7.4	92.3	4.3	2.5	4.6	16.8 16.5	10 50 40	
16361.0 15466.0	66	Octyltriethoxysilane	-6 -7	15988.7 15093.7	23983.0 22640.5	97.1 91.7	5.3 -0.1	28.2	89.9	2.0	1.1	2.2	17.9	S 30-	
15246.0 14825.0	68 69		-7 -7	14873.7 14452.7	22310.5 21679.0	90.4 87.8	-1.5	2.1					16.7 16.2	20- 10-	
15139.0 14731.0	70	Octyltriethoxysilane	-8 -8	14766.7 14358.7	22150.0 21538.0	89.7 87.2	-2.1	4.5	87.3	2.3	1.3	2.6	16.6 16.1	-10	<u> </u>
14382.0	72	Octvitriethovvsilane	-8	14009.7	21014.5	85.1	-6.7	45.0	93.0	3.0	17	3.2	15.8		
15710.0	74	erery meeting y and re	-9	15337.7	23006.5	93.2	1.4	1.8		2.0			17.2		
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 15056.0	77		-10 -10	15060.7 14683.7	22591.0 22025.5	91.5 89.2	-0.3 -2.6	0.1 6.8					16.9 16.5		
13433 14039	79	Octocrylene	4 4	13060.7 13666.7	19591.0 20500.0	79.3 83.0	-2.3	5.2	81.6	2.0	1.2	2.4	14.7		
13954 15096	81 82	Octocrylene	-4 -5	13581.7 14723.7	20372.5 22085.5	82.5 89.4	0.9	0.8	90.3	2.4	1.4	2.7	15.3	110	
14922 15680	83 84		-5 -5	14549.7 15307.7	21824.5 22961.5	88.4 93.0	-1.9	3.7					16.3	90 - B	
15193	85	Octocrylene	-6	14820.7	22231.0	90.0 93.8	-2.2	4.8	92.4	2.1	1.2	2.2	16.6	2 80 - 2 70 -	
15750	87	Octocoviene	-6	15377.7	23066.5	93.4	1.2	1.4	97.1	2.0	15	20	17.3	- 60 -	
15042	89	Octocrytene	-7	14669.7	22004.5	89.1	-3.5	12.3	94.1	2.8	1.6	3.0	16.5	등 50 용 40 -	
15637 15521	90	Octocrylene	-7 -8	15264.7 15148.7	22897.0 22723.0	92.7 92.0	0.1	0.0	93.0	1.3	0.7	1.4	17.1	S 30-	
15922 15598	92 93		-8 -8	15549.7 15225.7	23324.5 22838.5	94.5 92.5	1.7	3.1					17.4	- 20-	
15501 15055	94 95	Octocrylene	-9 -9	15128.7 14682.7	22693.0 22024.0	91.9 89.2	-0.8 -3.5	0.7	91.8	2.5	1.5	2.7	17.0	<u>الــــــــــــــــــــــــــــــــــــ</u>	1
15882 16654	96 97	Octocrylene	-9 -10	15509.7	23264.5 24422.5	94.2 98.9	1.5	2.2 38.1	93.8	4.8	2.7	5.1	17.4		
15108	98		-10	14735.7	22103.5	89.5	-3.2	10.4					16.6		
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	104/8./	23218.0	94.0	1.3	4.7	1	1		1	17.4		

<b>APPENDIX 1</b>	Raw and Normalized Data 3 <sup>r</sup>	<sup>'d</sup> Valid Run – August 03, 2011
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Experiment Date: Test substance:	3-Aug-11 Oxybenzone		Study Number:	9070-10010	7ERB				Assays Cor	ducted by:				
1/27/2012 16:16														-
		10 uL of 50 nM E2=	—	DPM and 0.	5 x10-12 mol	es								
		ug protein/assay tube =	80.0	Cr minior										
	Tube	Sample Type	DPM (1mL)	Speelfie Binding DPM (1mL) - NSB	Total Specific Binding (1.5mL)	Mean								
	1	-	17691		137989.8									
	3	Total Activity (Master Mix)	17342	-	135267.6	137285.2								
	5		17914	-	139729.2									
	6 7		17588	15438.0	137186.4 23157.0									
	8	Total Binding (Solvent Control)	18098 14953	17745.0 14600.0	26617.5 21900.0	23891.5								
DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Speelfie Binding DPM (1mL) - NSB	Totai Specific Binding (1.6mL)	Speolflo Binding (%)	Residual	Squared Recidual	Mean Speoifio Binding (%)	Standard Deviation	8EM	% CV	% Ligand Bound vs. Total Aotivity	
330.0 369.0	10	Estradiol (NSB)	-7 -7	-23.0	-34.5 24.0	-0.1	1.6	2.4	0.0	0.1	0.1	#DIV/0!	0.4	
360.0	12	Estradiol	-7 -8	7.0	10.5 1882.5	0.0	1.7	3.0 33.4	9.3	1.3	0.8	14.3	0.4	r r
1869.0	14		-8	1516.0	2274.0	9.5	-4.1	17.1					2.0	100
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	8 80 -
5002.0	18		-8.5	4649.0	6973.5	29.2	-3.1	9.6					5.5	8 60 -
7930.0 10344.0	19 20	Estradiol	-9	7577.0	11365.5 14986.5	47.6	-8.1	66.4 49.1	56.9	8.1	4.7	14.3	8.7	20 40 -
9954.0 11512.0	21 22	Estradiol	-9 -9.5	9601.0 11159.0	14401.5 16738.5	60.3 70.1	4.6	20.8	73.1	2.9	1.7	4.0	10.9	5 20
12049.0	23		-9.5	11696.0	17544.0	73.4	-1.8	3.3					13.2	
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	
14447.0	26		-10	14094.0	21141.0	88.5	1.8	3.4					15.8	
15295.0 15296.0	28 29	Estradiol	-11	14942.0 14943.0	22413.0 22414.5	93.8 93.8	-0.4	0.2	95.5	2.9	1.7	3.0	16.7 16.7	
16096.0 506.0	30	19-Norethindrone	-11	15743.0	23614.5 229.5	98.8	4.6	21.4	1.6	1.0	0.6	62.1	17.6	+
801.0 536.0	32		-4	448.0	672.0 274.5	2.8	1.1	1.1					0.9	
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	100-
1503.0	36		-4.5	1150.0	1725.0	7.2	0.5	0.2				2.0	1.6	æ
7226.0	37	19-Norethindrone	-5.5	6873.0	10309.5	41.0	1.2	1.4	41.7	1.3	0.7	3.0	7.5	£ -
6871.0 11340.0	39	19-Norethindrone	-5.5	6518.0 10987.0	9777.0	40.9	-1.0	0.2	68.8	0.7	0.4	1.1	7.5	2 60- 2
11182.0 11416.0	41 42		-6 -6	10829.0	16243.5 16594.5	68.0 69.5	-0.6	0.4					12.2	88 40 -
14118.0 13932.0	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	8 20 -
14721.0	45		-6.5	14368.0	21552.0	90.2	3.3	11.1			20		16.1	L
15865.0	40	15-Norethindrone	-7	15512.0	23268.0	97.4	2.0	45.0	34.2	3.1	2.5	3.4	17.3	-8.6
15783.0 16511.0	48	19-Norethindrone	-7 -7.5	15430.0	23145.0 24237.0	96.9	1.5 2.8	2.3 8.1	99.1	2.0	1.2	2.1	17.2	1
15991.0 15909.0	50		-7.5	15638.0 15556.0	23457.0 23334.0	98.2 97.7	-0.4	0.2					17.5	
16054.0 16389.0	52	19-Norethindrone	-8.5	15701.0	23551.5 24054.0	98.6 100.7	-1.5	2.4	100.3	1.6	0.9	1.6	17.5	
16552.0	54	Octvitrietboxysilane	-8.5	16199.0	24298.5	101.7	1.6	2.5	48.2	26	15	54	18.1	1
7634.0	56	Colymetricky Smalle	-3	7281.0	10921.5	45.7	-2.5	6.1					8.3	
15334.0	57	Octyltriethoxysllane	-3	14981.0	22471.5	94.1	3.2	10.2	90.9	2.8	1.6	3.1	16.8	110
14505.0 14637.0	59 60		-4	14152.0 14284.0	21228.0 21426.0	88.9 89.7	-2.0	4.0					15.8	90 8
16252.0 16864.0	61 62	Octyltriethoxysliane	-5	15899.0 16511.0	23848.5 24766.5	99.8 103.7	0.9	0.9	101.4	2.0	1.2	2.0	17.8	80- E 70-
16382.0 16894.0	63 64	Octvitriethoxysilane	-5 -6	16029.0	24043.5 24811.5	100.6	1.7	3.0 24.6	105.1	1.7	1.0	1.6	17.9	eo -
17408.0	65		-6	17055.0	25582.5	107.1	8.2	67.0					19.0	50 80 40 -
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	5 30 - 20 -
16274.0	69		-7	15921.0	23881.5	100.0	1.1	1.1					17.8	10-
15932.0 16050.0	70	Octyltriethoxysllane	-8 -8	15579.0	23368.5 23545.5	97.8	-1.1 -0.3	1.2 0.1	98.1	0.4	0.2	0.4	17.4	-10
15932.0 14920.0	72	Octyltriethoxysilane	-8 -9	15579.0	23368.5 21850.5	97.8 91.5	-1.1	1.2	93.9	2.1	1.2	2.2	17.4	
15460.0 15524.0	74		-9 -9	15107.0	22660.5 22756.5	94.8 95.2	-4.0 -3.6	16.4 13.3					16.9	
15033.0	76	Octyltriethoxysilane	-10 -10	14680.0	22020.0	92.2 92.6	-6.7	45.3 39.8	93.3	1.5	0.9	1.7	16.4	
15490.0	78	Oxybenzone	-10	15137.0	22705.5	95.0	-3.9	14.9	77.3	0.7	0.4	0.9	16.9	ł
12550	80		-4	12197.0	18295.5	76.6	-0.7	0.5				3.5	13.7	l
12643	81	Oxybenzone	-4	12290.0	23490.0	98.3	-0.1	0.0	97.7	0.9	0.5	1.0	13.8	110
15736 15976	83 84		-5	15383.0 15623.0	23074.5 23434.5	96.6 98.1	-1.1	1.1					17.2	90-
15613 16183	85 86	Oxybenzone	-6 -6	15260.0 15830.0	22890.0 23745.0	95.8 99.4	-2.7	7.4	98.1	2.0	1.2	2.0	17.1	80 - 5 70 -
16150 15445	87 88	Oxybenzone	-6 -7	15797.0	23695.5 22638.0	99.2 94.8	0.7	0.4	98.3	3.1	1.8	3.2	17.6	E 60-
16413	89		-7	16060.0	24090.0	100.8	2.3	5.2					17.9	50 80 40
16152	91	Oxybenzone	-/	15799.0	23698.5	99.2	0.5	0.3	100.2	1.6	0.9	1.6	17.6	5 30- 2 20-
16597 16195	92 93		-8 -8	16244.0 15842.0	24366.0 23763.0	102.0	3.4 0.9	11.8 0.8			-		18.1 17.7	10-
15865 16063	94	Oxybenzone	-9 -9	15512.0	23268.0 23565.0	97.4 98.6	-1.2	1.3	97.6	1.0	0.6	1.0	17.3	-11
15748 16166	96 97	Oxybenzone	-9 -10	15395.0	23092.5 23719.5	96.7 99.3	-1.9	3.6	99.7	2.9	1.6	2.9	17.2	1
15807	98		-10	15454.0	23181.0	97.0	-1.5	2.3					17.3	
15941	100	Oxybenzone	-11	15588.0	23382.0	97.9	-0.7	0.5	97.5	1.0	0.6	1.1	17.4	1
16002	102		-11	15649.0	23005.5	98.3	-2.3	0.1					17.1	1

## APPENDIX 1 Raw and Normalized Data 3<sup>rd</sup> Valid Run (continued) – August 03, 2011

Experiment Date:	3-Aug-11		Study Number:	9070-10010	7ERB				Assays Cor	ducted by:				
Test substance: 1/27/2012 16:17	Methoxycinn	lamate												
		10 uL of 50 nM E2-		DPM and 0.	5 x10-12 mol	es								
		Therefore there are ug protein/assay tube =	80.0	DPM/mole										
	Tube	Sample Type	DPM (1mL)	Specific Binding DPM (1mL)	Total Specific Binding	Mean								
	1 2 3		17691 17594 17342	- NSB	(1.6mL) 137989.8 137233.2 135267.6									
	4 5 6	Total Activity (Master Mix)	17475 17914 17588		136305.0 139729.2 137186.4	137285.2								
	7 8 9	Total Binding (Solvent Control)	15791 18098 14953	15438.0 17745.0 14600.0	23157.0 26617.5 21900.0	23891.5								
				Specific	Total				Mean				% Ligand	
DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Binding DPM (1mL) - NSB	Specific Binding (1.6mL)	Specific Binding (%)	Residual	Squared Recidual	Specific Binding (%)	Standard Deviation	SEM	% CV	Bound vs. Total Activity	
330.0 369.0 360.0	10 11	Estradiol (NSB)	-7 -7	-23.0 16.0	-34.5 24.0	-0.1	1.6 1.8	2.4 3.2 3.0	0.0	0.1	0.1	#DIV/0!	0.4	
1608.0 1869.0	13	Estradiol	-8	1255.0	1882.5	7.9	-5.8	33.4	9.3	1.3	0.8	14.3	1.8	100
2029.0	15	Estradiol	-8	1676.0 5814.0	2514.0 8721.0	10.5	-3.1 4.2	9.8 17.8	35.3	5.7	3.3	16.0	2.2	80-
6777.0 5002.0	17		-8.5 -8.5	6424.0 4649.0	9636.0 6973.5	40.3 29.2	8.1 -3.1	64.8 9.6					7.4	8 60 -
7930.0 10344.0	19 20	Estradiol	-9 -9	7577.0	11365.5 14986.5	47.6 62.7	-8.1 7.0	66.4 49.1	56.9	8.1	4.7	14.3	8.7 11.3	50
9954.0 11512.0	21	Estradiol	-9 -9.5	9601.0 11159.0	14401.5 16738.5	60.3 70.1	4.6	20.8	73.1	2.9	1.7	4.0	10.9	97 30 - 5 20 -
12049.0	23		-9.5	11696.0	17544.0	73.4	-1.8	3.3					13.2	2 10-
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	<u>وليا</u>
14447.0	20	Estradi-*	-10	14094.0	21141.0	88.5	1.8	3.4	05.7	36	4.7		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	
16096.0	30 31	19-Norethindrone	-11	15743.0	23614.5 229.5	98.8	4.6 -0.8	21.4	1.6	1.0	0.6	62.1	17.6 0.6	
801.0 536.0	32		4 4	448.0 183.0	672.0 274.5	2.8	1.1	1.1					0.9	
1336.0 1536.0	34	19-Norethindrone	-4.5 -4.5	983.0 1183.0	1474.5	6.2	-0.6	0.3	6.9	0.7	0.4	9.7	1.5	100-
1503.0	36	19-Norethindrone	-4.5	1150.0	1725.0	7.2	0.5	0.2	41.7	1.3	0.7	3.0	1.6	- 00 - 08
7226.0	38		-5.5	6873.0 6518.0	10309.5	43.2	1.2	1.4				5.5	7.9	2° 70 -
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	50
11182.0	41 42		-6	10829.0	16243.5	69.5	-0.6	0.4					12.2	8 40 - 2 30 -
14118.0 13932.0	43 44	19-Norethindrone	-6.5 -6.5	13765.0	20647.5 20368.5	86.4 85.3	-0.4	0.2	87.3	2.6	1.5	3.0	15.4 15.2	20- 10-
14721.0	45	19-Norethindrone	-6.5	14368.0	21552.0 21102.0	90.2 88.3	3.3	11.1 49.6	94.2	5.1	2.9	5.4	16.1 15.8	يا
15865.0 15783.0	47 48		-7 -7	15512.0 15430.0	23268.0 23145.0	97.4 96.9	2.0	4.1					17.3 17.2	-8.6
16511.0 15991.0	49	19-Norethindrone	-7.5 -7.5	16158.0 15638.0	24237.0 23457.0	101.4 98.2	2.8	8.1	99.1	2.0	1.2	2.1	18.0 17.5	
15909.0	51	19-Norethindrone	-7.5	15556.0	23334.0	97.7	-0.9	0.9	100.3	1.6	0.9	1.6	17.4	
16389.0	53		-8.5	16036.0	24054.0	100.7	0.6	0.3	100.5		0.0		17.9	
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	55		-3	7281.0 7635.0	10921.5	45.7	-2.5	0.1					8.3	
15334.0 14505.0	58	Octyltriethoxysilane	4	14981.0 14152.0	22471.5 21228.0	94.1 88.9	3.2	10.2	90.9	2.8	1.6	3.1	16.8 15.8	120-
14637.0 16252.0	60	Octyltriethoxysilane	-4 -5	14284.0	21426.0 23848.5	89.7 99.8	-1.2	1.4	101.4	2.0	1.2	2.0	16.0 17.8	£ 100-
16864.0 16382.0	62 63		-5 -5	16511.0	24766.5 24043.5	103.7	4.8	22.7					18.4 17.9	80-
16894.0 17408.0	64 65	Octyltriethoxysilane	-6 -6	16541.0 17055.0	24811.5 25582.5	103.9	5.0 8.2	24.6 67.0	105.1	1.7	1.0	1.6	18.5	eo -
16983.0	66 67	Octvitriethoxysilane	-6 -7	16630.0	24945.0	104.4	5.5	30.4	101.7	3.0	1.8	3.0	18.6	<del>9</del> 40 -
17111.0	68		-7	16758.0	25137.0	105.2	6.3	39.9					18.7	ž 20-
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	-10
15932.0	71		-8	15697.0	23545.5	97.8	-0.3	1.2					17.5	
14920.0 15460.0	73	Octyltriethoxysilane	-9	14567.0 15107.0	21850.5 22660.5	91.5 94.8	-7.4	55.3 16.4	93.9	2.1	1.2	2.2	16.3 16.9	
15524.0 15033.0	75	Octyltriethoxysilane	-9 -10	15171.0	22756.5 22020.0	95.2 92.2	-3.6 -6.7	13.3 45.3	93.3	1.5	0.9	1.7	17.0 16.4	
15100.0 15490.0	77 78		-10 -10	14747.0 15137.0	22120.5 22705.5	92.6 95.0	-6.3 -3.9	39.8 14.9					16.5 16.9	
14534 15366	79 80	Methoxycinnamate	4 4	14181.0 15013.0	21271.5 22519.5	89.0 94.3	-4.0	16.3 1.4	93.1	3.6	2.1	3.9	15.9 16.8	
15632 16534	81 82	Methoxycinnamate	-4	15279.0 16181.0	22918.5 24271.5	95.9 101.6	2.9	8.1 8.5	101.2	0.5	0.3	0.5	17.1 18.1	110
16381	83 84		-5	16028.0	24042.0	100.6	2.0	3.8					17.9	100- <b>8</b>
17070	85	Methoxycinnamate	-6	16717.0	25075.5	105.0	6.3	39.5	101.9	2.7	1.6	2.7	18.7	8 80 - 8 70
16449	87	Mathewaring	-6	16096.0	24144.0	101.1	2.4	5.7	00.0				18.0	60-
16216	88	Methoxycinnamate	-7	15863.0	23/94.5	99.6 98.7	0.9	0.9	98.8	U.7	0.4	0.8	17.7	50 50
15981 15558	90	Methoxycinnamate	-7 -8	15628.0	23442.0 22807.5	98.1 95.5	-0.6	0.3	93.7	2.5	1.4	2.7	17.5	g 30 -
14823 15444	92 93		-8 -8	14470.0 15091.0	21705.0 22636.5	90.8 94.7	-7.8 -3.9	61.2 15.4					16.2 16.9	10-
15823 15858	94 95	Methoxycinnamate	-9 -9	15470.0 15505.0	23205.0 23257.5	97.1 97.3	-1.5	2.4	96.8	0.8	0.5	0.8	17.3 17.3	-11
15622	96 97	Methoxycinnamate	-9 -10	15269.0 15348.0	22903.5 23022.0	95.9 96.4	-2.8	7.9	97.7	2.0	1.1	2.0	17.1	
15785	98		-10	15432.0	23148.0 23887 F	96.9	-1.8	3.2					17.2	
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	1	1		1	17.5	

## APPENDIX 1 Raw and Normalized Data 3<sup>rd</sup> Valid Run (continued) – August 03, 2011

Experiment Date:	3-Aug-11		Study Number:	9070-10010	7ERB				Assays Cor	ducted by:					
Test substance: 1/27/2012 16:17	OctylSalicylat	te													
		10 uL of 50 nM E2-	_	DPM and 0.	5 x10-12 mol	es									
		Therefore there are ug protein/assay tube -	80.0	DPM/mole											
				Specific	Total										
	Tube	Sample Type	DPM (1mL)	Binding DPM (1mL)	Specific Binding	Mean									
	1		17691	- NSB	(1.6mL) 137989.8										
	2		17594 17342	-	137233.2 135267.6										
	4	Total Activity (Master Mix)	17475	-	136305.0 139729.2	137285.2									
	6		17588		137186.4									1	
	8	Total Binding (Solvent Control)	18098	17745.0	26617.5	23891.5									
			14335	14000.0	21300.0										
				Specific	Total	Specific			Mean				% Ligand	i –	
(1mL) from LSC	Tube	Sample Type	log[M]	DPM (1mL)	Binding	Binding (%)	Residual	Recidual	Binding	Deviation	SEM	% CV	Total		
330.0	10	Estradiol (NSB)	-7	- NSB -23.0	(1.6mL) -34.5	-0.1	1.6	2.4	(%)	0.1	0.1	#DIV/0!	O.4		
369.0 360.0	11		-7 -7	16.0 7.0	24.0	0.1	1.8	3.2					0.4	-	
1608.0 1869.0	13	Estradiol	-8 -8	1255.0	1882.5	7.9	-5.8	33.4	9.3	1.3	0.8	14.3	1.8		100
2029.0	15	Estradiol	-8	1676.0	2514.0 8721.0	10.5	-3.1	9.8 17.8	35.3	5.7	3.3	16.0	2.2	E	80-
6777.0	17		-8.5	6424.0	9636.0	40.3	8.1	64.8					7.4	and the second s	70 - 60 -
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	8	50
10344.0 9954.0	20		-9 -9	9991.0 9601.0	14986.5 14401.5	62.7	7.0	49.1 20.8					11.3	ŝ	30-
11512.0 12049.0	22 23	Estradiol	-9.5 -9.5	11159.0	16738.5 17544.0	70.1	-5.2	26.8 3.3	73.1	2.9	1.7	4.0	12.6	Near	20-
12434.0 13520.0	24	Estradiol	-9.5 -10	12081.0	18121.5	75.8 82.7	0.6	0.4	85.9	3.0	1.7	3.5	13.6 14.8		°-
14159.0	26		-10	13806.0	20709.0	86.7	0.0	0.0					15.5		-11
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	1	
15296.0 16096.0	29 30		-11	14943.0 15743.0	22414.5 23614.5	93.8 98.8	-0.4 4.6	0.2 21.4					16.7 17.6		
506.0 801.0	31	19-Norethindrone	4	153.0 448.0	229.5	1.0	-0.8	0.6	1.6	1.0	0.6	62.1	0.6		
536.0 1336.0	33 34	19-Norethindrone	-4	183.0	274.5	1.1	-0.6	0.4	6.9	0.7	0.4	9.7	0.6		
1536.0	35		-4.5	1183.0	1774.5	7.4	0.7	0.4					1.7		90 -
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	8	80 - 70 -
7226.0 6871.0	38 39		-5.5	6873.0 6518.0	10309.5 9777.0	43.2	-1.0	1.4					7.9	Bhd	60-
11340.0 11182.0	40	19-Norethindrone	-6 -6	10987.0	16480.5 16243.5	69.0 68.0	0.4	0.2	68.8	0.7	0.4	1.1	12.4	bedlic	50 40 -
11416.0 14118.0	42	19-Norethindrone	-6 -6.5	11063.0	16594.5 20647.5	69.5 86.4	0.9	0.8	87.3	2.6	1.5	3.0	12.5	ST ST	30 -
13932.0	44		-6.5	13579.0	20368.5	85.3	-1.6	2.6					15.2	Ň	10-
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		-8.6
15783.0	47		-7	15430.0	23268.0	96.9	1.5	2.3					17.2		-
16511.0 15991.0	49	19-Norethindrone	-7.5	16158.0	24237.0 23457.0	101.4 98.2	2.8	8.1 0.2	99.1	2.0	1.2	2.1	18.0 17.5		
15909.0 16054.0	51	19-Norethindrone	-7.5	15556.0	23334.0 23551.5	97.7	-0.9	0.9	100.3	1.6	0.9	1.6	17.4		
16389.0 16552.0	53 54		-8.5 -8.5	16036.0	24054.0 24298.5	100.7	0.6	0.3					17.9 18.1		
8461.0 7634.0	55	Octyltriethoxysilane	-3	8108.0	12162.0	50.9 45.7	2.7	7.4	48.2	2.6	1.5	5.4	9.2		
7988.0	57	Ontolitication	-3	7635.0	11452.5	47.9	-0.2	0.1					8.7		
14505.0	59	Octylthethoxysilane	4	14981.0	21228.0	88.9	-2.0	4.0	90.9	2.8	1.6	3.1	15.8		120-
14637.0 16252.0	60	Octyltriethoxysilane	-4 -5	14284.0	21426.0 23848.5	89.7 99.8	-1.2	0.9	101.4	2.0	1.2	2.0	16.0 17.8	E	100
16864.0 16382.0	62 63		-5 -5	16511.0	24766.5 24043.5	103.7	4.8	22.7					18.4 17.9	Bhđ	e0 -
16894.0 17408.0	64 65	Octyltriethoxysilane	-6 -6	16541.0 17055.0	24811.5 25582.5	103.9	5.0 8.2	24.6 67.0	105.1	1.7	1.0	1.6	18.5 19.0	selle	60-
16983.0 16273.0	66 67	Octvitriethoxysilane	-6 -7	16630.0	24945.0 23880.0	104.4	5.5	30.4	101.7	3.0	1.8	3.0	18.6	S S	40-
17111.0	68		-7	16758.0	25137.0	105.2	6.3	39.9				2.0	18.7	Ne	20-
15932.0	70	Octyltriethoxysilane	-8	15579.0	23081.5	97.8	-1.1	1.1	98.1	0.4	0.2	0.4	17.8		
16050.0 15932.0	71 72		-8 -8	15697.0	23545.5 23368.5	98.6 97.8	-0.3	0.1					17.5	-	0
14920.0 15460.0	73	Octyltriethoxysilane	-9 -9	14567.0 15107.0	21850.5 22660.5	91.5 94.8	-7.4	55.3 16.4	93.9	2.1	1.2	2.2	16.3 16.9		
15524.0	75	Octyltriethoxysilane	-9 -10	15171.0	22756.5	95.2 92.2	-3.6	13.3 45.3	93.3	1.5	0.9	1.7	17.0		
15100.0	77		-10	14747.0	22120.5	92.6	-6.3	39.8					16.5		
11386	79	OctylSallcylate	-4	11033.0	16549.5	69.3	-0.4	0.2	69.7	2.9	1.7	4.2	12.4	1	
11028	80		4	11604.0	17406.0	67.0 72.9	-2.7	7.3 9.8				_	12.0		119-
14262 14622	82 83	OctylSallcylate	-5	13909.0 14269.0	20863.5 21403.5	87.3 89.6	-2.6	6.6 0.1	89.9	2.7	1.6	3.0	15.6 16.0		100-
15131 15302	84	OctylSallcylate	-5	14778.0 14949.0	22167.0 22423.5	92.8 93.9	2.9	8.3	95.5	1.6	0.9	1.7	16.5 16.7	8	80-08
15810 15580	86 87		-6 -6	15457.0 15227.0	23185.5 22840.5	97.0 95.6	1.5	2.2					17.3 17.0	Shiding	70-
15214	88	OctylSallcylate	-7	14861.0	22291.5	93.3	-2.4	5.6	97.0	3.2	1.9	3.3	16.6	8	50-
16192	90	Octub Too too	-7	15839.0	23758.5	99.4	3.8	14.3					17.7	S C	40 - 30 -
16511 15643	91 92	Octyrsallcylate	-8 -8	15290.0	24237.0 22935.0	96.0	5.8 0.3	33.4 0.1	96.8	4.3	2.5	4.5	18.0 17.1	Nea	20
15148 14600	93	OctylSalicylate	-8	14795.0	22192.5 21370.5	92.9 89.4	-2.8	7.7 38.7	92.9	3.0	1.7	3.2	16.6 16.0		10-
15385 15444	95 96		-9 -9	15032.0	22548.0 22636.5	94.4 94.7	-1.3	1.7					16.8 16.9		-11
15364	97	OctylSallcylate	-10	15011.0	22516.5	94.2 97.1	-1.4	2.0	96.1	1.6	0.9	1.7	16.8 17.3		
15817	99	OctviSalloviate	-10	15464.0	23196.0	97.1	1.4	2.0	95.5	49	2.0	E 4	17.3		
16376	101	Octyrodiicyldre	-11	16023.0	24034.5	100.6	4.9	24.3	35.0		2.0	3.1	17.9	1	
15556	102			15202.0	<ul> <li>77804 E</li> </ul>		-07						170	-	

## APPENDIX 1 Raw and Normalized Data 3<sup>rd</sup> Valid Run (continued) – August 03, 2011

Experiment Date:	3-Aug-11		Study Number:	9070-10010	7ERB				Assays Cor	ducted by:				
Test substance: 1/27/2012 16:17	Octocrylene												_	
		10 ul. of 50 pM E2=		DPM and 0	5 x10-12 mol	-								
		Therefore there are		DPM/mole										
		ug protenvassay tube -	00.0	0	Takat									
	Tube	Sample Type	DPM (1mL)	Binding	Specific	Mean								
				DPM (1mL) - NSB	(1.6mL)									
	1 2	-	17691 17594	-	137989.8 137233.2									
	3	Total Activity (Master Mix)	17342 17475	-	135267.6 136305.0	137285.2								
	5		17914	-	139729.2									
	7	Total Binding (Solvent Control)	15791	15438.0	23157.0	77001.6								
	9	Total Binding (Solvent Control)	14953	14600.0	21900.0	23651.5								
DPM	Tube	Rampia Type	Concentration	Specific Binding	Total Specific	Specific	Residual	Squared	Mean Speolfio	Standard	0 EM	* CV	% Ligand Bound vs.	
(1mL) from LSC	1000	sample Type	log[M]	DPM (1mL) - NSB	Binding (1.6mL)	(%)	Roaddai	Recidual	Binding (%)	Deviation	GEM	2000	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	
360.0	12	Entry dist	-7	7.0	10.5	0.0	1.7	3.0					0.4	405
1869.0	13	Estradioi	-8	1255.0	2274.0	9.5	-5.8	17.1	9.3	1.3	0.8	14.3	2.0	90-
2029.0 6167.0	15	Estradiol	-8 -8.5	1676.0 5814.0	2514.0 8721.0	10.5	-3.1	9.8 17.8	35.3	5.7	3.3	16.0	2.2	€ 80 - 70 -
6777.0 5002.0	17		-8.5 -8.5	6424.0 4649.0	9636.0 6973.5	40.3 29.2	8.1	64.8 9.6					7.4	20
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	₩ 50 ₩ 40-
9954.0	20		-9	9601.0	14401.5	60.3	4.6	20.8				-	10.9	å <del>}</del> 30−
11512.0 12049.0	22 23	Estradiol	-9.5 -9.5	11159.0	16738.5 17544.0	70.1	-5.2	26.8	73.1	2.9	1.7	4.0	12.6	20- 210-
12434.0 13520.0	24	Estradiol	-9.5 -10	12081.0	18121.5	75.8 82.7	0.6	0.4	85.9	3.0	1.7	3.5	13.6 14.8	•–
14159.0	26		-10	13806.0	20709.0	86.7	0.0	0.0					15.5	-11
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 16096.0	29 30		-11	14943.0 15743.0	22414.5 23614.5	93.8 98.8	-0.4 4.6	0.2					16.7 17.6	
506.0 801.0	31	19-Norethindrone	4 4	153.0 448.0	229.5 672.0	1.0	-0.8	0.6	1.6	1.0	0.6	62.1	0.6	
536.0	33	10-blorathlodmos	-4	183.0	274.5	1.1	-0.6	0.4	6.0	0.7		0.7	0.6	
1536.0	35	19-Norethindrone	-4.5	1183.0	1774.5	7.4	0.7	0.3	0.3	0.7	0.4	3.7	1.5	100
1503.0 6890.0	36	19-Norethindrone	-4.5	1150.0 6537.0	1725.0 9805.5	7.2	-0.9	0.2	41.7	1.3	0.7	3.0	1.6	e0 -
7226.0 6871.0	38 39		-5.5 -5.5	6873.0 6518.0	10309.5 9777.0	43.2 40.9	1.2	1.4					7.9	20 70 - 20 60 -
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	11063.0	16243.5	69.5	0.9	0.4					12.2	8 40 - 30 -
14118.0 13932.0	43	19-Norethindrone	-6.5 -6.5	13765.0	20647.5 20368.5	86.4	-0.4	0.2	87.3	2.6	1.5	3.0	15.4	ag 20 -
14721.0 14421.0	45	19-Norethindrone	-6.5	14368.0	21552.0	90.2	3.3	11.1	94.2	5.1	2.9	5.4	16.1	•
15865.0	47		-7	15512.0	23268.0	97.4	2.0	4.1					17.3	-8.6
16511.0	48	19-Norethindrone	-7.5	16158.0	24237.0	101.4	2.8	8.1	99.1	2.0	1.2	2.1	17.2	
15991.0 15909.0	50		-7.5 -7.5	15638.0	23457.0 23334.0	98.2	-0.4	0.2					17.5	
16054.0 16389.0	52	19-Norethindrone	-8.5 -8.5	15701.0	23551.5 24054.0	98.6 100.7	-1.5	2.4	100.3	1.6	0.9	1.6	17.5	
16552.0	54	Ontolitications	-8.5	16199.0	24298.5	101.7	1.6	2.5	40.2	26			18.1	
7634.0	56	Octylinethoxysilane	-3	7281.0	10921.5	45.7	-2.5	6.1	40.2	2.0	1.5	3.4	8.3	
7988.0 15334.0	57	Octyltriethoxysilane	-3	7635.0	11452.5 22471.5	47.9 94.1	-0.2	0.1	90.9	2.8	1.6	3.1	8.7	4.00
14505.0 14637.0	59		-4	14152.0	21228.0	88.9 89.7	-2.0	4.0					15.8	20-
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	2 100 - 1
16382.0	62		-5	16029.0	24766.5	103.7	4.8	3.0					18.4	28 80-
16894.0 17408.0	64 65	Octyltriethoxysilane	-6 -6	16541.0	24811.5 25582.5	103.9	5.0	24.6 67.0	105.1	1.7	1.0	1.6	18.5 19.0	90
16983.0 16273.0	66	Octyltriethoxysilane	-6 -7	16630.0	24945.0 23880.0	104.4	5.5	30.4	101.7	3.0	1.8	3.0	18.6 17.8	5 40-
17111.0	68		-7	16758.0	25137.0	105.2	6.3	39.9					18.7	20 -
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	<u>ملب م</u>
16050.0 15932.0	71 72		-8 -8	15697.0	23545.5 23368.5	98.6 97.8	-0.3	0.1					17.5	-10
14920.0 15460.0	73 74	Octyltriethoxysilane	-9 -9	14567.0 15107.0	21850.5 22660.5	91.5 94.8	-7.4	55.3 16.4	93.9	2.1	1.2	2.2	16.3 16.9	
15524.0	75	Octvitriethovycillane	-9	15171.0	22756.5	95.2	-3.6	13.3	93.3	1.6	0.9	17	17.0	
15100.0	77	octymethoxysilane	-10	14747.0	22120.5	92.6	-6.3	39.8			0.9	1.7	16.5	
14543	78	Octocrylene	-10	14190.0	21285.0	89.1	-3.9	33.2	83.3	8.1	4.7	9.7	15.9	
12146 14187	80		4 4	11793.0 13834.0	17689.5 20751.0	74.0 86.9	-9.3 3.5	86.2					13.3 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	100
15960	84		-5	15607.0	23410.5	98.0	3.5	12.5					17.4	£ 90-
16172 15777	85	Octocrylene	-6 -6	15819.0	23728.5 23136.0	99.3 96.8	-1.5	2.3	101.1	5.3	3.1	5.3	17.7	€ 70-
17404	87	Octocrylene	-6	17051.0	25576.5 24268.5	107.1	6.2	38.7	101.2	0.9	0.5	0.9	19.0 18.1	8 60-
16296	89		-7	15943.0	23914.5	100.1	-0.7	0.6					17.8	900 40 -
16340	90	Octocrylene	-8	15987.0	24325.5	100.4	-0.5	0.9	102.9	2.2	1.3	2.1	17.9	5 30 -
16998 16878	92		-8 -8	16645.0	24967.5 24787.5	104.5	3.7	13.4 8.5					18.6 18.4	10
16807 16933	94 95	Octocrylene	-9 -9	16454.0	24681.0 24870.0	103.3	2.5	6.1 10.6	103.9	0.6	0.3	0.6	18.4	-11
16989	96	Octororiene	-9	16636.0	24954.0	104.4	3.6	13.0	100.4	24	1.0	24	18.6	
15921	98	Octocrytene	-10	15568.0	23352.0	97.7	-3.1	9.6	100.4	2.1	1.0	3.1	17.4	
16902 15680	99	Octocrylene	-10	16549.0	24823.5 22990.5	103.9 96.2	3.1	9.4 21.3	95.5	0.9	0.5	0.9	18.5	
15416	101		-11	15063.0	22594.5	94.6	-6.3	39.3					16.8	

### **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**

- $\Box$  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).
- $\Box$  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 -80°C.

### **Calibration Curve**



R	Raw	Data Plate	e Map										
pq		1	2	3	4	5	6	7	8	9	10	11	12
Ort	Α	3x	3x	3x		2mg/mL	2mg/mL	2mg/mL					
Ŋ	В	5x	5x	5x		1mg/mL	1mg/mL	1mg/mL					
Im	С	10x	10x	10x		0.5mg/mL	0.5mg/mL	0.5mg/mL					
ber	D	20x	20x	20x		0.25mg/mL	0.25mg/mL	0.25mg/mL					
.: 9	Е	40x	40x	40x		0.125mg/mL	0.125mg/mL	0.125mg/mL					
07	F	80x	80x	80x		0.06mg/mL	0.06mg/mL	0.06mg/mL					
<u>-</u>	G	backgrnd	backgrnd	backgrnd		backgrnd	backgrnd	backgrnd					
00	Н	backgrnd	backgrnd	backgrnd		backgrnd	backgrnd	backgrnd					
10		cytosol	cytosol	cytosol		BSA standards	BSA standards	BSA standards					
JT(		samples	samples	samples	blank	(mg/mL)	(mg/mL)	(mg/mL)					
١R													
В	Rau	v Data											

## **Raw Data** Plate Seq#: 8306

$au Seq \pi. 0500$												
Comment:	A	cquired: Tue	sday, Septem	ber 28, 2010	2:38 PM Ter	mperature Min	n/Max: 0.0/0.0	)°C				
Absorbance-A		File Repo										
	1	2	3	4	5	6	7	8	9	10	11	12
А	0.954	0.939	0.941	0.039	1.244	1.223	1.193	0.039	0.04	0.040	0.041	0.042
В	0.661	0.682	0.667	0.041	0.849	0.895	0.841	0.041	0.042	0.040	0.041	0.042
С	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
Е	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
Н	0.388	0.389	0.389	0.051	0.394	0.389	0.388	0.041	0.043	0.042	0.044	0.046



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 890.1<del>150</del> based upon the reference control results shown in this study report.

270472011



Senior Scientist/Endocrine Group Leader CeeTox, Inc.

27007 2011 Date



Director of Project Management CeeTox, Inc.

27 OCF 2011 Date

Study Number: 9070-100107ERB

## APPENDIX 3 Deviation Forms

	~ =			Form #:	SOP-1003-F-1.0
la	vitre models to predict toxicity	Deviation & Invest	ligation		
Stu	udy Number (if applicable):	9 <del>146V-100337ST</del>	R O		
510					
Do au	ate of Reporting:22 Jul 201 udit	1	Reporting Associate:	_QA Director_	in process
Do	ate of Occurrence:20 and 2	21# Jul2011	Associate Involved: _	n/a	
De	escription of Deviation:				
The July rem and tem del rar mis nou du	e temperatures for refridgerate y 21, 2011. The impact of the atterials for study number 9146 amined for signs of freeze/that mained in range for the July 20 and other studies due to the miss imperatures were examined for thermined from the min/max re- inge for the 24 hour time period ssed reading time period. The one were identified. The # 6 min ring the July 20 <sup>th</sup> and July 21 <sup>th</sup>	ors 1, 2, 3, 7, 9 an is deviation for this 5V-100337STER. T w and no sign was 0 <sup>th</sup> and July 21 <sup>st</sup> . It ed temperature rec refridgerators 1,2, adings that these re d before the first mi e contents of the free inus 80 freezer log time period. No e	ad freezers 4,5 6, 8 w study is specific to Fr he contents of the #8 found. Thus it can be was determined that to ording of freezer #8 c 3,7,9 and freezers 4, efridgerators and freezers issed reading and the ezers were examined precorder was examinant xcursions were identif	rere not recorde reezer # 8 that minus 80 freeze e expected that here was no in on these two do 5, additionally zers were withi 24 hour period for signs of fre red for tempero fied.	ed on July 20 and contained ter were t the temperature npact on this study ays. The min/max . It was n the determined d after the second eze/thaw and ature excursions
Ту	pe of Deviation (determined b	y Study Director/Pr	incipa <b>l</b> Investigator):		
	Facility Deviation from SO	P-4007			
Su	ummary of Deviation Investigat	ion by SD/PI/Test	Facility Management/	Designee:	
Th fre	ne records of the temperatures eezers were examined for sign	of the listed refrig s of freeze/thaw.	perators and freezers	were examine	d. All contents of
Ad	ction Taken and Determination	of Impact on Study	v Data and/or Facility	Compliance:	
Th ter 20	ne result of the above listed i mperature excursions that cou D <sup>th</sup> and July 21 <sup>er</sup> time period	nvestigation conclu Id have been a re	ided there was no G sult of the missed ten	iLP study impa nperature mon	ct due to possible itoring for the July
Sig	gnature:SD/PI/Test	Facility Manageme	Date:/	8-AUL -:	2011
© This was t identified all studi studi	the study number that , but as a facility de es ongoing during th dard Operating Procedure	the deviation wation, it approves the deviation of the days.	on was plies to 26 Aug 201	CO	Poge 1 of 1

<u>П. Т.</u>		Form #:	SOP-1003-F-
In vitro models to predict toxicity	& Investigation		
Study Number (if applicable): 9070 -100	DIOTERB		
Date of Reporting: _26 Aug 201	Reporting Associat	e: _	
25 Jul Zoll Ol Aug Zoll Date of Occurrence: 03 Aug Zoll	Associate Involved	:	
Description of Deviation:			
Dxybenzone (2. hydroxy - 4. m	thosy benzone) lot	# in protocol	Was 20090
however lot supplied by Spo	nsor was 201008	OI. No Co	of A for lot
20100501 and Cof A for loi	20080801 says it	expired or	4 Aug 2010.
Signature (Keporting Associate):	Date:	26 Au	2011
Type of Deviation (determined by Study Dire	ector/Principal Investigator	):	
SOP Deviation	Deviation 🔲 GLP Devi	ation 🔲 N	lo Deviation
Summary of Deviation Investigation by SD/	PI/Test Facility Managemei	nt/Designee:	
Incorrect lot and Cof,	4 provided		
	1		
Action Taken and Determination of Impact of	on Study Data and/or Faci	lity Compliance:	
Ask Sponsor to supply	proper Cof A		
	_	21. 1	2
Signature: SD/91/Tèst Factility/Ma	nagement	che Aug	<u> </u>
indard Operating Procedure			Page 1 of

Internet by predict tasky         Study Number (II applicable): <u>9070-100107ER8</u> Date of Reporting: <u>04-jan-12</u> Reporting Associate:         Date of Cocurrence: <u>and 18-Oct-11</u> Associate Involved:         Description of Deviation:       Sponsor was not asked to sign amendments according to the protocol.         Signature	Ceelox	Deviation &	Investigation	Form #:	SOP-1003-F
Study Number (II applicable):       90/0-10010/ERB         Date of Reporting:       04-Jan-12       Reporting Associate:         Date of Occurrence:       and 18-Oct-11       Associate Involved:         Description of Deviation:       Sponsor was not asked to sign amendments according to the protocol.         Signature	In vitro models to predict toxicity				
Date of Reporting:       04-Jan-12       Reporting Associate:         Date of Cccurrence:       21-Sep-11, 26-Sep-11       Associate Involved:         Description of Deviation:       Sponsor was not asked to sign amendments according to the protocol.         Signature	Study Number (if app	licable): 9070-10	DO TO 7 ERB	2	
Date of Occurrence:       21-Sep-11, 26-Sep-11 and 18-Oct-11       Associate Involved:         Description of Deviation:       Sponsor was not asked to sign amendments according to the protocol.         Signature	Date of Reporting: _	04-Jan-12	_ Reporting Associate: _		
Description of Deviation:          Sponsor was not asked to sign amendments according to the protocol.         Signature	Date of Occurrence:	21-Sep-11, 26-Sep-1 and 18-Oct-11	Associate Involved:		
Sponsor was not asked to sign amendments according to the protocol.         Signature	Description of Deviati	on:			
Signature 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: Date: Date: 04-Jan-12	Sponsor was not aske	d to sign amendment	s according to the proto	col.	
Signature       Date:       04-Jan-12         Image: Type of Deviation (determined by Study Director/Principal Investigator):       SOP Deviation       No Deviation         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:					
Signature       Date:       04-Jan-12         (Reporting/Associate)       Type of Deviation (determined by Study Director/Principal Investigator):       No Deviation         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:	·				
Signature	Constan		Deter	04.14	an 10
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:		(Constanting of the start of th	Dale:	04-30	un-12
Type of Deviation (determined by Study Director/Principal Investigator):       No Deviation         SOP Deviation       Protocol 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:       Oate:       04-Jan-12	(	(Reporting/Associate)			
SOP Deviation       Protocol Deviation       Image: 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:       Date:       04-Jan-12	Type of Deviation (de	termined by Study Dir	ector/Principal Investigat	or):	
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:  Sponsor Sponsor Facility Management	SOP Deviation	Protocol Devia	tion GLP Deviation	n 🗆	No Deviation
Sponsor was notified of pending Amendments but were not asked to sign the amendments as <pre>stated in the protocol.</pre> 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 <pre>study.</pre> Signature: Date: 04-Jan-12	Summary of Deviation	n Investigation by SD/F	PI/Test Facility Manageme	ent/Designe	e:
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:	Sponsor was notified of	of pending Amendme	ents but were not asked t	o sign the ar	mendments as
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: Date: 04-Jan-12	stated in the protocol	l.			
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:       Date:       04-Jan-12         /SD/PI/Test Facility Management					
None. Sponsor signature and date will be required for all future amendments, if any, for this study. Signature: Date: 04-Jan-12 CSD/PI/Test Factlety Management					
study. Signature: Date: 04-Jan-12 /SD/PI/Test Factlyty Management	Action Taken and Det	termination of Impact	on Study Data and/or Fo	acility Comp	liance:
Signature: Date:04-Jan-12 /SD/PI/Test Factlyfy Management	Action Taken and Del	termination of Impact	on Study Data and/or Fo	acility Comp	liance: any, for this
Signature: Date: 04-Jan-12	Action Taken and Del None. Sponsor signat	termination of Impact lure and date will be r	on Study Data and/or Fo	acility Comp andments, if	liance: any, for this
Signature: Date: 04-Jan-12	Action Taken and Del None. Sponsor signat study.	termination of Impact lure and date will be r	on Study Data and/or Fo	acility Comp andments, if	liance: any, for this
/SD/PI/Test Facility Management	Action Taken and Det None. Sponsor signat study.	termination of Impact	on Study Data and/or Fo	acility Comp	liance: any, for this
	Action Taken and Dev None. Sponsor signat study.	termination of Impact	on Study Data and/or Fo equired for all future ame Date:	acility Comp endments, if	liance: any, for this an-12
	Action Taken and De <u>None.</u> Sponsor signat study. Signature: 	termination of Impact lure and date will be n	on Study Data and/or Fo equired for all future ame Date:	acility Comp endments, if 04-Ji	liance: any, for this an-12

ConTour			F	orm #:	SOP-1003-F
In vitro models to predict to	idly Devic	non & investig	jalion		
Study Number (if a	pplicable):	9070-100107EF	2B		
Date of Reporting:	04-Jan-	12 Rep	orting Associate: _		
Date of Occurrence	25-Jul-11, 01- e: <u>and 03-Aug-</u>	Aug-11 11 Asso	ciate Involved:		
Description of Devi	ation:				
Wrong purity was u	used for methoxyc	cinnamate. Us	ed 98% instead of 99	9.8%.	
Signature			Date:	04-Jc	n-12
l	(Reporting Ass	locíate)			
Type of Deviation (	determined by St	udy Director/F	rincipal Investigator	):	
	on ØProtoco	Deviation	GLP Deviation	/·	lo Deviation
Summary of Devia	ion Investigation	by SD/PI/Test F	acility Managemen	t/Designee	<del>.</del>
Wrong purity was i	ised for methowy	cionamate			
Thong bony tray o		A mamaro.			
Action Taken and I	Determination of	Impact on Stu	dy Data and/or Fac	ility Compl	iance:
None. After dilutio	ns, the difference	is negligible.			
Signature:			Date:	04-Jo	in-12
SC	/PI/Test Fachity N	fanagement			

Deviation & Investigation
Study Number (if applicable): ERB002 Batch
Date of Reporting:08-Nov-11 Reporting Associate:
25-Jul-11, 01-Aug-11         Date of Occurrence:         and 03-Aug-11         Associate Involved:
Description of Deviation:
Protocol states that PI (protease inhibitor) will be used while the OPPTS guideline states that PMSF will be
used specifically.
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.
SOP Deviation       Protocol 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.
SOP Deviation       OProtocol Deviation       OProtocol Deviation       OProtocol 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:

Form #:         SOP-1003-F-1           Deviation & Investigation         Deviation
Study Number (if applicable): ERB002 Batch
Date of Reporting: 08-Nov-11 Reporting Associate:
25-Jul-11, 01-Aug-11         Date of Occurrence:       and 03-Aug-11         Associate Involved:
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.
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: Date: 08-Nov-11 / SD/PI/Test/Fecility Management



## CERTIFICATE OF ANALYSIS Product 29116

Specifications

Appearance Infrared spectrometry Seperat, techn, GC Acid value Specific abs, A (1%/1cm) Specific

CLEAR COLOURLESS TO YELLOW LIQUID AUTHENTIC 975.5 % <1 mg ROH19 9880 (d1 307 to 308 mm in methanol) (2505°C) 1007 to 1012 1.5401 to 1.5470 (d2°C, 589 mm) 0.05 to 0.1 % BHT

#### General Product Data

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



### Lot Specific Data for Lot No.: A0293319

Appearance
nfrared spectrometry
Separat. techn. GC
Acid value
Specific abs. A (1%/1cm)
Specific gravity
Refractive index
Stabilizer

CLEAR COLOURLESS LIQUID CLEAR COLOURLESS LIQUID AUTHENTC 99.8 % 0.1 mg KOH/g 856 (at 307 to 308 nm in methanol) (25/25\*C) 1.0396 1.5453 (30° C, 589 nm) 0.09 % BHT



ssued: 10-08-10 Quality Assurance Manager

Acros Organics

GeelWest Zone 2, Janssen Pitarmacoutication 3e, 8-2440 Geel, Belgium Tel +32 14/57.52, 11 - Fax +32 14/59.34.34 internet: http://www.acros.com I Regent Lone, Fair Lown, NJ 07410,USA Fax 201-796-1329



MRI-NTEVTesk 1492

A-1

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### **Certificate of Analysis** LORCH.

#### Product Name

Product Number Product Brand CAS Number Molecular Formula Molecular Weight

2-Ethylhexyl salicγlate, ≥99% VV514500 ALDRICH 118-60-5 (H0)0<sub>6</sub>H<sub>4</sub>00<sub>2</sub>CH<sub>2</sub>CH(0<sub>2</sub>H<sub>6</sub>)(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub> 250.33

≤3.0 ppm

≤1.0 ppm

<1.0 ppm

≤10.0 ppm

TEST Appearance (Color) Appearance (Form) Refractive index at 20 ° C Infrared spectrum Purity (GC) Color Test Arsenic (A s) Cadmium (Co) Mercury (Hg) Lead (Pb) Specification Date: Date of QC Release: Print Date:

SPECIFICATION Colorless Liquid 1.500 - 1.504 Conforms to Structure ≥99.0 % ≤100 APHA

#### LOT 44696PJ RESULTS

Calorless Liquid 1.502 Conforms 99.6 % 10 APHA < 1.0 ppm < 1.0 ppm < 1.0 ppm < 1.0 ppm DEC 2008 DEC 2008 DEC 19 2009

Supervisor Quality Centrol Milwaukes, Wisconsin USA

Page 1 of 1

## Certificate of Analysis

SPECIFICATION

Conforms to Structure

Viscous Liquid

Yellow

≥96.5 %

#### Product Name

Product Number Product Brand CAS Number Nolecular Formula Nolecular Weight 2-Ethy hexyl 2-cyano-3,3-diphenyla: ylate, 97% 415920 ALDRICH 6197-30-4  $(C_6H_2)_2C=C(CN(CO_2CH_2CH(C_2H_2)(CH_2)_3CH_3)$ 361,48

TEST

Appearance (Color) Appearance (Form) Infrared spectrum Purity (G C) Specification Date: Date of QC Release: Print Date:



LOT 01697MJ RESULTS

Yellow Viscous Liquid Conforms 99.2 % OCT 2008 OCT 2008 OCT 22 2008

### APPENDIX 5 Protocol and Protocol Amendments



TO BE COMPLETED BY THE STUDY SPONSOR: Study Sponsor: NIEHS/NTP Thief Toxicology Branch) Address: P.O. Box 12233 Research Triangle Park, NC Phone: Study Monitor: E-mail: Sponsor Protocol/Project No.: Test Substance Name(s): Octyl Salicylate, 2-Ethylhexyl p-methoxydinnamate, 2 Ethylhexyl 2-Cyano-3,3-Diphenylacrylate, 2-Hydroxy-4-Methoxybenzophenone NIEHS/NTP Investigator Telephone No.: Facsimile No.: E-mail: Contract Office Technical Representative (Contract No. HHSN273200900005C; NIEHS Control No. N01-ES-00005) Study Monitor [ILS, Inc, Durham, NC) Telephone No.: Facsimile No.: E-mail:		TEST PROTOCOL			
Study Sponsor:       NIEHS/NTP       Thief Toxicology Branch)         Address:       P.O. Box 12233       Research Triangle Park, NC       Phone:         Study Monitor:       E-mail:       Sponsor Protocol/Project No.:         Test Substance Name(s):       Octyl Salicylate, 2-Ethylhexyl p-methoxydinnamate, 2         Ethylhexyl 2-Cyano-3,3-Diphenylacrylate, 2-Hydroxy-4-Methoxybenzophenone         NIEHS/NTP Investigator         Telephone No.:       Facsimile No.:         E-mail:       Contract Office Technical Representative         (ILS, Inc, Durham, NC)       Telephone No.:         Facsimile No.:       Facsimile No.:         E-mail:       ILS, Inc, Durham, NC)         Telephone No.:       Facsimile No.:         Facsimile No.:       Facsimile No.:         E-mail:       ILS, Inc, Durham, NC)	TO BE COMPLETED	TO BE COMPLETED BY THE STUDY SPONSOR:			
Address:       P.O. Box 12233         Research Triangle Park, NC       Phone:         Study Monitor:       E-mail:         Sponsor Protocol/Project No.:       E-mail:         Sponsor Protocol/Project No.:       E-mail:         Study Monitor:       E-mail:         Study Monitor:       E-mail:         Sponsor Protocol/Project No.:       E-mail:         Test Substance Name(s):       Octyl Salicylate, 2-Ethylhexyl p-methoxycinnamate, 2         Ethylhexyl 2-Cyano-3,3-Diphenylacrylate, 2-Hydroxy-4-Methoxybenzophenone       NIEHS/NTP Investigator         Telephone No.:       E-mail:       Email:         Contract Office Technical Representative       (ILS, Inc, Durham, NC)       N01-ES-00005)         Study Monitor       (ILS, Inc, Durham, NC)       Telephone No.:       E-mail:         Facsimile No.:       E-mail:       E-mail:       E-mail:	Study Sponsor:	NIEHS/NTP	chief Toxicology Branch)		
Research Triangle Park, NC       Phone:         Study Monitor:       E-mail:         Sponsor Protocol/Project No.:       E-mail:         Test Substance Name(s): Octyl Salicylate, 2-Ethylhexyl p-methoxycinnamate, 2         Ethylhexyl 2-Cyano-3,3-Diphenylacrylate, 2-Hydroxy-4-Methoxybenzophenone         NIEHS/NTP Investigator         Telephone No.:         Facsimile No.:         E-mail:         Contract Office Technical Representative         (Contract No. HHSN273200900005C; NIEHS Control No. N01-ES-00005)         Study Monitor         (ILS, Inc, Durham, NC)         Telephone No.:         Facsimile No.:         E-mail:         (ILS, Inc, Durham, NC)         Telephone No.:         Facsimile No.:         E-mail:	Address:	P.O. Box 12233			
E-mail:         E-mail:         Sponsor Protocol/Project No.:         Test Substance Name(s): Octyl Salicylate, 2-Ethylhexyl p-methoxydinnamate, 2         INIEHS/NTP Investigator         NIEHS/NTP Investigator         Telephone No.:         Facsimile No.:         E-mail:         Contract Office Technical Representative         (ILS, Inc, Durham, NC)         Telephone No.:         Facsimile No.:         E-mail:         (ILS, Inc, Durham, NC)         Telephone No.:         Facsimile No.:         E-mail:		Research Triangle Park, NC	Phone:		
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         Telephone No.:         Facsimile No.:         E-mail:         Contract Office Technical Representative         (Contract No. HHSN273200900005C; NIEHS Control No. N01-ES-00005)         Study Monitor         (ILS, Inc, Durham, NC)         Telephone No.:         Facsimile No.:         E-mail:         (ILS, Inc, Durham, NC)         Telephone No.:         Facsimile No.:         E-mail:	Study Monitor:		E-mail:		
Test Substance Name(s): Octyl Salicylate, 2-Ethylhexyl p-methoxycinnamate, 2         Ethylhexyl 2-Cyano-3,3-Diphenylacrylate, 2-Hydroxy-4-Methoxybenzophenone         NIEHS/NTP Investigator         Telephone No.:         Facsimile No.:         E-mail:         Contract Office Technical Representative         (Contract No. HHSN273200900005C; NIEHS Control No. N01-ES-00005)         Study Monitor         (ILS, Inc, Durham, NC)         Telephone No.:         Facsimile No.:         E-mail:	ponsor Protocol/	/Project No.:			
Ithylhexyl 2-Cyano-3,3-Diphenylacrylate, 2-Hydroxy-4-Methoxybenzophenone         NIEHS/NTP Investigator         Telephone No.:         Facsimile No.:         E-mail:         Contract Office Technical Representative         (Contract No. HHSN273200900005C; NIEHS Control No. N01-ES-00005)         Study Monitor         [ILS, Inc, Durham, NC)         Telephone No.:         Facsimile No.:         E-mail:	est Substance	Name(s): Octyl Salicylate,	2-Ethylhexyl p-methoxycinnamate, 2		
E-moil:					
	Study Monitor Telephone No.: Facsimile No.:	r (ILS, Inc, Durh	am, NC)		
	Study Monito Telephone No.: Facsimile No.: E-mail:	r (ILS, Inc, Durh	am, NC)		
	Study Monito Telephone No.: Facsimile No.: E-mail:	r (ILS, Inc, Durh	am, NC)		
	Study Monito Telephone No.: Facsimile No.: E-mail:	r (ILS, Inc, Durh	am, NC)		
	Study Monito Telephone No.: Facsimile No.: E-mail:	r (ILS, Inc, Durh	am, NC)		
	Study Monito Telephone No.: Facsimile No.: E-mail:	r (ILS, Inc, Durh	am, NC)		
	Study Monito Telephone No.: Facsimile No.: E-mail:	r (ILS, Inc, Durh	am, NC)		
	Study Monito Telephone No.: Facsimile No.: E-mail:	r (ILS, Inc, Durh	am, NC)		
	Study Monito Telephone No.: Facsimile No.: E-mail:	r (ILS, Inc, Durh	am, NC)		

### Ceeton PROTOCOL - ESTROGEN RECEPTOR BINDING ASSAY USING RUC

Study Number:9070-100107ERB

### Table of Contents

Signatures
1. Title of Study
2. Purpose of Study
3. Compliance Statement
4. Quality Assurance
5. Regulatory Citations
6. Test Facility
7. Test Substance
7.1 Test Substance: 2-Hydroxy-4-Methoxybenzophenone (Oxybenzone)
7.2 Test Substance: 2-Ethylhexyl p-methoxycinnamate (Octylmethoxycinnamate)
7.3 Test Substance: Octyl Salicylate (Octylsalate)
7.4 Test Substance: 2-Ethylhexyl 2-Cyano-3,3-Diphenylacrylate (Octocrylene)
8. Preparation of Test Substances
8.1 Positive and Negative Reference Substances
8.2 Stock Solution Preparation
Uterine Homogenate Collection and Saturation Binding
9. Competitive Radioligand Binding Assay
10. Solubility/Precipitation Assay
11. Data Interpretation Criteria
Saturation Binding Analyses Error! Bookmark not defined.
Definitions: Error! Bookmark not defined.
General Considerations
Additional Considerations
Definitions
Page 3 of 15

CeeTo	Ham PROTOCOL - ESTROGEN RECEPTOR BINDING ASSAY USING RUC
	Competitive Binding Performance Criteria
12.	Classification Criteria
13.	Test System
14.	Study Reports
15.	Alterations of the Study Design
16.	Data Retention and Archiving


# 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

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Na app be will sub	<b>te:</b> A certificate of analyspended to the study report. Overified by the Sponsor. Follower be obtained from the vendestance will be either returned. <i>Test Substance: 2-Hydro</i> CAS No. Source:	sis will be provided by the Sponsor and will be stored in the study data an Confirmation of the identity of the test substance, characterization and stability wi r positive, negative reference substances and radioligand, certificates of analysi or and will be stored in the study data and appended to the study report. Test to the Sponsor or destroyed following finalization of the study report. oxy-4-Methoxybenzophenone (Oxybenzone) 131-57-7
7.1	Test Substance: 2-Hydro CAS No. Source:	oxy-4-Methoxybenzophenone (Oxybenzone) 131-57-7
	CAS No. Source:	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-Ethylł	nexyl 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 S	alicylate (Octylsalate)
	CAS No.	118-60-5

Cee <b>To</b>	Kan PROTOCOL – ESTROGEN	RECEPTOR BINDING ASSAY USING RUC Study Number: 9070-100107ERB				
	Source:	Sigma-Aldrich				
	Lot/Batch No.:	44698PJ				
	ILS Repository No.:	11-30				
	Formula:	C <sub>15</sub> H <sub>22</sub> O <sub>3</sub>				
	Description:	Colorless liquid				
	Storage	Room Temperature				
7.4	Test Substance: 2-Ethyl	hexyl 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				
3.	<b>Preparation of Test Substances</b> Each test substance will be dissolved in an appropriate vehicle (DMSO, ethanol, purific water) that solubilizes the test substance. Any vehicle used to dissolve test substances we be tested with the reference substance, if possible, for the run as well, unless the solvent ineffective (i.e., reference and controls insoluble in that solvent). The maximum percent ethanol allowed in assay tubes is 3% of the total volume. The maximum percent of DMS allowed in assay tubes is 10% of the total volume. Dose concentrations of test and controls allowed in assay tubes is 10% of the total volume.					
	substances will not be v Test substance solubility	erified using analytical methods. will be evaluated by visual inspection for precipitation.				
	Serial Dilutions of Test S					

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#### CeeTone PROTOCOL - ESTROGEN RECEPTOR BINDING ASSAY USING RUC

Study Number:9070-100107ERB

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.

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 x 10 <sup>2</sup> M	1 x 10 <sup>3</sup> M
TC2	Use 100 µl of dilution TC1 (50 mM)	900 µl	1 ml	5 x 10 <sup>3</sup> M	1 x 104 M
TC3	Use 100 µl of dilution TC2 (5 mM)	900 µl	1 ml	5 × 104 M	1 x 10 <sup>5</sup> M
TC4	Use 100 µl of dilution TC3 (500 µM)	900 µl	1 ml	5 x 10 <sup>5</sup> M	1 x 10° M
TC5	Use 100 µl of dilution TC4 (50 µM)	900 µl	1 ml	5 x 10° M	1 x 10 <sup>7</sup> M
TC6	Use 100 µl of dilution TC5 (5 µM)	900 µl	1 ml	5 x 10 <sup>7</sup> M	1 x 10 <sup>8</sup> M
TC7	Use 100 µl of dilution TC6 (500 nM)	900 µl	1 ml	5 x 10 <sup>8</sup> M	1 x 10 <sup>9</sup> M
TC8	Use 100 µl of dilution TC7 (50 nM)	900 µl	1 ml	5 x 10° M	1 × 10 <sup>-10</sup> M

#### Table 1. Test Substance Dilution Procedure

\*Final concentration of test substance in assay tube when 10  $\mu$ l of diluted concentration is used in a total volume of 500  $\mu$ l.

#### 8.1 Positive and Negative Reference Substances

Octyltriethoxysilane is the negative reference substance. The concentration range for the negative reference is  $1 \times 10^{10}$  to  $1 \times 10^{3}$  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  $1 \times 10^4$  M. A 10 mM stock of norethynodrel will be prepared in solvent (2.984 mg/ml). Dilute serially as described in Table 2 below.

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Study Number: 9070-100107ERB

Tube #	Volume of stock to add for diluted	Volume of solvent to add	Total volume of diluted positive	Positive Control Concentration		
	concentration	Sec. 1	control	Diluted	Final in ER assay tube	
P1	Use 400 µl of stock positive control (10 mM)	400 µl	800 <i>µ</i> l	5 x 10 <sup>3</sup> M	1 x 10 <sup>4</sup> M	
P2	Use 150 µl of stock positive control (10 mM)	اµ 008	950 <i>µ</i> l	1.58 x 10 <sup>3</sup> M	3.16 x 10 <sup>5</sup> M	
P3	Use 100 µl of P2 (1.58 mM)	900 µl	1 ml	1.58 x 104 M	3.16 x 10 <sup>6</sup> M	
Intermed	Use 100 µl of P1 (5 mM)	900 µl	1 ml	5 x 104 M	Not used	
P4	Use 100 µl of Intermed (500 µM)	900 µl	1 ml	5 x 10 <sup>5</sup> M	1 × 10° M	
P5	Use 100 µl of P3 (158 µM)	900 µl	1 ml	1.58 x 10 <sup>5</sup> M	3.16 x 10 <sup>-7</sup> M	
P6	Use 100 µl of P4 (50 µM)	اµ 900	1 ml	5 x 10° M	1 × 10 <sup>-7</sup> M	
P7	Use 100 µl of P5 (15.8 µM)	اµ 900	1 ml	1.58 x 10° M	3.16 x 10 <sup>8</sup> M	
P8	Use 100 μl of P7 (1.58 μM)	900 µl	1 ml	1.58 x 107 M	3.16 x 10° M	

# Table 2. Example Dilution Procedure for Norethynodrel

The reference substance (17 $\beta$ -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 17 $\beta$ -estradiol will range from 1 x 10<sup>7</sup> to 1 x 10<sup>11</sup> 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  $\mu$ M 17 $\beta$ -estradiol stock will be prepared (0.136 mg/ml).

# Table 3. Example of Dilution Procedure for Reference Standard 17<sub>β</sub>-estradiol

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to odd	Total volume of 17β- estradial	Diluted 17β-estradiol concentration	Final 17β-estradio concentration in Ef assay tube
NSB1	Use 100 μl of stock 17β-estradiol (50 μM)	900 µl	1 ml	5 x 10° M	1 × 10 <sup>-7</sup> M
S2	Use 100 µl of dilution NSB1 (5 µM)	900 µl	1 ml	5 x 10 <sup>7</sup> M	1 x 10 <sup>8</sup> M
\$3	Use 277 µl of dilution S2 (500 nM)	ыц 006	اµ 877	1.58 x 10 <sup>7</sup> M	3.16 x 10 <sup>9</sup> M
S4	Use 100 µl of dilution S2 (500 nM)	900 µl	1 ml	5 x 10 <sup>8</sup> M	1 x 10° M
S5	Use 100 µl of dilution S3 (158 nM)	900 µl	1 ml	1.58 x 10 <sup>8</sup> M	3.16 x 10 <sup>10</sup> M
S6	Use 100 µl of dilution S4 (50 nM)	900 µl	1 ml	5 x 10° M	1 x 10 <sup>10</sup> M
S7	Use 100 µl of dilution S6 (5 nM)	900 µl	1 ml	5 × 10 <sup>-10</sup> M	1 x 10 <sup>11</sup> M

CEION	PROTOCOL – ESTROGEN RECEPTOR BINDING ASSAY USING RUC Study Number: 9070-100107ERB
	8.2 Stock Solution Preparation
	200mM EDTA Stock Solution:
	For example, add 7.444g disodium EDTA to 100 ml purified $\rm H_2O.$ 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.
<u>Pr</u> • •	eparation 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 <sub>2</sub> O 2.0 ml 1M Tris Buffer 20 ml glycerol 1.5 ml 200 mM EDTA
	<b>Note:</b> Cool to approximately 4°C before adjusting to pH $\sim$ 7.4, and then bring volume to 100 ml with purified H <sup>2</sup> O and store at approximately 4°C up to 3 months.
Pro ac 7.	eparation of Working Assay Buffer (10 mM Tris, 1.5 mM EDTA (Ethylenediaminetetraacetic id), 1 mM DTT(Dithiothreitol), 0.5% Protease Inhibitor (with PMSF)(v/v), 10% glycerol, pH 4) [TEDG + PI]:
Pre	epare 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 <sub>2</sub> O.
	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 + Pl buffer to a final volume of 100 ml, cap container, mix by inversion and refrigerate for at least 2 hours.

#### CeeToner PROTOCOL - ESTROGEN RECEPTOR BINDING ASSAY USING RUC

- 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]-17 $\beta$ -estradiol is bound to ER causing the concentration of the unbound (free) [ ${}^{3}$ H]-17 $\beta$ -estradiol to differ significantly from the concentration of [ ${}^{3}$ H]-17 $\beta$ -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		l 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).

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#### CONTINUE PROTOCOL - ESTROGEN RECEPTOR BINDING ASSAY USING RUC

Check test substance solubility.

 $[^{3}H]$ -17 $\beta$ -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<sub>adiusted</sub>/SA = Fraction Isotope Remaining (FIR)

Where:

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

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

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

The  $[^{3}H]$ -17 $\beta$ -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]-17 $\beta$ -estradiol to add to the dilution (for example having a final concentration of 1 nM in 500  $\mu$ l 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<sup>6</sup> nmole/mmole \* 1000 ml/L = (Y/X) \* 10<sup>6</sup> nM

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

The 50 nM [ $^{3}$ H]-17 $\beta$ -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  $\mu$ l TEDG Buffer + Pl with diluted [3H]-17 $\beta$ -estradiol. For example the following table describes the preparation of a master mixture for 155 tubes.

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Substance	Target Volume/Tube (µl)		# of Tubes		Total Volume	Master Mix Volumes (ml)	
	Assay Tubes	Trace Tubes	Assay Tubes	Trace Tubes	Assay Tubes	Trace Tubes	
TEDG Buffer + Pl	380	48.72	155	6	58.9	0.292	59.192
Diluted [ <sup>3</sup> 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  $\mu$ l of the master mixture above will be added to each assay tube and keep on ice. For the trace tubes, 50  $\mu$ l 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  $\mu$ l of substance will be added per tube. After all substances have been added to the tubes, 100  $\mu$ l of cytosol will be added to each tube for a final volume of 500  $\mu$ l 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 [3H]-17B-estradiol from free [3H]-17B-estradiol

ER assay tubes will be removed from the rotator and placed in an ice-water bath. Using a repeating pipette, 250  $\mu$ l 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  $[{}^{3}H]-17\beta$ -estradiol will be discarded. The HAP pellet will contain the estrogen receptor bound  $[{}^{3}H]-17\beta$ -estradiol. Two ml of icecold 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 [3H]-17B-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

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CONTINUE PROTOCOL - ESTROGEN RECEPTOR BINDING ASSAY USING RUC

Study Number:9070-100107ERB

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 + [ <sup>3</sup> 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 [<sup>3</sup>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  $17\beta$ -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.

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Estimating the IC<sub>50</sub>

An ER competitive binding assay measures the binding of a single concentration of [<sup>3</sup>H]-17β-estradiol in the presence of increasing concentrations of a test substance. The competitive binding curve is plotted as specific [<sup>3</sup>H]-17β-estradiol binding (as a percent of total binding) versus the concentration (log<sub>10</sub> units) of the competitor. The concentration of the test substance that inhibits 50% of the maximum specific [<sup>3</sup>H]-17β-estradiol binding is the IC<sub>50</sub> value. Estimates of IC<sub>50</sub> values are determined using nonlinear curve fitting software (GraphPad PRISM). The relative binding affinity (RBA) is calculated by comparing the log (IC<sub>50</sub>) 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  $17\beta$ -estradiol displace [<sup>3</sup>H]- $17\beta$ -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  $[^{3}H]$ -17 $\beta$ -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.

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CeeTox PROTOCOL - ESTROGEN RECEPTOR BINDING ASSAY USING RUC

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Table 7. S	Suggested Upper a	nd Lower Limits	for Parameters in	Competitive	<b>Binding Assay</b>	Curves
for the Sta	andards (Radioiner	t Estradiol and N	lorethynodrel)			

Parameter	Unit	Estradiol		Norethynodrel	
		Lower Limit	Upper Limit	Lower Limit	Upper Limit
Log <sub>e</sub> (S <sub>yx</sub> )	-	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

# 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  $10^6\,\,\text{M}.$ 

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

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# 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

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#### CeeTon M PROTOCOL - ESTROGEN RECEPTOR BINDING ASSAY USING RUC

Study Number:9070-100107ERB

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

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Protocol Amendment

Study Number: 9070-100107ERB

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

<u>Reason for Amendment to Protocol:</u> 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  $\times$  10<sup>-8.5</sup> to 1  $\times$  10<sup>-4</sup> 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.

Study Director (Project Manager)

<u>21 Sept 2011</u> Date

CeeTox Study # 9070-100107ERB

21-Sep-11

Study Number: 9070-100107ERB



#### 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.

The Table of Contents will now read:Signatures51.Title of Study62.Purpose of Study63.Compliance Statement64.Quality Assurance65.Regulatory Citations66.Test Facility67.Test Substance67.1Test Substance: 2-Hydroxy-4-Methoxybenzophenone (Oxybenzone)77.2Test Substance: 2-Ethylhexyl p-methoxycinnamate (Octylmethoxycinnamate)77.3Test Substance: 2-Ethylhexyl p-methoxycinnamate (Octocrylene) 88.Preparation of Test Substances88.1Positive and Negative Reference Substances98.2Stock Solution Preparation11Uterine Homogenate Collection and Saturation Binding129.Competitive Radioligand Binding Assay1511.Data Interpretation Criteria1512.Classification Criteria1612.Classification Criteria1613.Test System1814.Study Reports1815.Alterations of the Study Design1816.Data Retention and Archiving19	Cha	nge:					
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1.   Title of Study   6     2.   Purpose of Study   6     3.   Compliance Statement   6     4.   Quality Assurance   6     5.   Regulatory Citations   6     6.   Test Facility   6     7.   Test Substance   6     7.   Test Substance: 2-Hydroxy-4-Methoxybenzophenone (Oxybenzone)7   7     7.2   Test Substance: 2-Ethylhexyl p-methoxycinnamate (Octylmethoxycinnamate)7   7     7.3   Test Substance: Octyl Salicylate (Octylsalate)   7     7.4   Test Substance: 2-Ethylhexyl 2-Cyano-3,3-Diphenylacrylate (Octocrylene) 8   8     8.   Preparation of Test Substances   8     8.1   Positive and Negative Reference Substances   9     8.2   Stock Solution Preparation   11     Uterine Homogenate Collection and Saturation Binding   12     9.   Competitive Radioligand Binding Assay   15     11.   Data Interpretation Criteria   15     0.   Solubility/Precipitation Assay   15     11.   Data Interpretation Criteria   16     12.   Classification Criteria	Signo	atures	5				
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16. Data Retention and Archiving 19	15.	Alterations of the Study Design	18				
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#### Signature

CeeTox, Inc.



26 Sept 2011 Date

CeeTox Study # 9070-100107ERB

26-Sep-11



**Protocol Amendment** 

Study Number: 9070-100107ERB

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

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

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

# Signature

CeeTox, Inc.



18 OCT 2011 Date

CeeTox Study # 9070-100107ERB

18-Oct-11

Study Number: 9070-100107ERB

# Cee**Tox**≥

#### **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

615 Davis Drive, Suite 300 Durham, NC 27713

**Signature** 

CeeTox, Inc.



<u>|2-6-//</u> Date



Db Dec 11 Date

CeeTox Study # 9070-100107ERB

6-Dec-11

Study Number: 9070-100107ERB