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

Standard Evaluation Procedure

ENDOCRINE DISRUPTOR SCREENING PROGRAM
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Washington, DC 20460

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Table of Contents

I. INTRODUCTION

A. Use of the Standard Evaluation Procedure

This document was developed by EPA to provide guidance to EPA staff who will be reviewing the data submitted in response to Tier 1 Orders issued under the Endocrine Disruptor Screening Program (EDSP). This document provides general guidance and is not binding on either EPA or any outside parties. The use of language such as "will," "is," "may," "can" or "should" in this document does not connote any requirement for either EPA or any outside parties. As such, EPA may depart from the guidance where circumstances warrant and without prior notice.

This Standard Evaluation Procedure (SEP) provides guidance on how to review studies conducted using the OCSPP Guideline 890 890.1250 Estrogen Receptor Binding Assay Using Rat Uterine Cytosol that are submitted to support requirements imposed under the U.S. Environmental Protection Agency's Endocrine Disruptor Screening Program (EDSP). The product of the review will be a Data Evaluation Record (DER) that reflects how well the study conforms to the Guideline, and evaluates how well the study was performed, and provides the appropriate conclusions supported by the data. The DER will include, for example, a list of any significant deviations from the protocol as well as their potential impacts, a list of significant information missing from the study report, and any other information about the performance of the study that affects interpretation of the data within the context of the EDSP.

The DER should contain adequate information to provide the EPA with the ability to determine whether the study is scientifically acceptable. The objective of EDSP Tier 1 assays is to characterize the potential of a chemical to interact with the endocrine system.

The Guideline recommends the critical materials, methods, and analyses that lead to successful performance of the assay. If a particular material, method, or analysis is named in the Guideline, it is usually because other materials, methods, or analyses are either known to be inappropriate or at least have not been validated or that there is concern for their potential influence on results. The Agency has posted Corrections and Clarifications on Technical Aspects of the EDSP Tier 1 Assays (OCSPP Test Guideline Series 890) in the docket; the link to this document may be found by way of the EDSP web page (http://www.epa.gov/endo/). It is therefore important to note deviations from specific materials, methods, or analyses in the DER, and provide the reviewer's opinion on whether the deviation/deficiency has an impact on the performance and results of the study or the acceptability of the study.

II. ESTROGEN RECEPTOR BINDING ASSAY

A. Purpose of the Assay

The estrogen receptor binding assay identifies chemicals that have the potential to interact with the estrogen receptor (ER) *in vitro*. The goal of the Estrogen Receptor Binding Assay Using Rat Uterine Cytosol is to determine if chemicals can interact with estrogen receptors (ERs) prepared from rat uterine cytosol by displacing the endogenous hormone 17β-estradiol. As

conducted, this assay utilizes all cytosolic estrogen receptor subtypes that are expressed in this tissue including ERα and ERβ. The ER binding assay only has the ability to determine if a chemical can interact and displace the endogenous hormone; it provides no information on whether a chemical will act as an agonist or antagonist at the receptor(s) to either activate or inactivate an estrogen-dependent response. The hormone binding domain (HBD) of the estrogen receptor is highly conserved across species. Therefore substances that bind the ER from rats are presumed to be capable of binding the ER in humans.

B. Study Design

The ER binding assay measures the competitive inhibition of binding of radiolabeled estradiol to rat ER by a test chemical. The assay consists of two sets of experiments: a Saturation Binding Experiment; and a Competitive Binding Experiment. Each experiment (saturation and competitive binding) consists of three runs and each run contains three replicates at each concentration.

1. Saturation Binding Experiment

The purpose of the Saturation Binding Experiment is to demonstrate that the ER isolated from rat uterine cytosol is present in reasonable numbers and is functioning with appropriate affinity for the radio-labeled reference estrogen prior to routinely conducting ER competitive binding experiments. The guideline recommends that proper saturation binding of the reference ligand to the ER be demonstrated with each batch of rat uterine cytosol (the source of the ER for this assay) before using the cytosol to conduct competitive binding assays. The Saturation Binding Experiment measures binding at equilibrium of various concentrations of radioligand (*i.e.*, [^{3}H]-estradiol) both with and without the presence of 100-fold higher concentrations of unlabeled (inert) ligand. Radioactivity counts or disintegrations per minute (dpms) derived from increasing concentrations of [^{3}H]-estradiol binding in the absence of unlabeled ligand indicate the Total Binding (TB). In addition to binding to the ER, the radioligand may also bind non-specifically to other sites. Dpms derived from increasing concentrations of [^{3}H]-estradiol binding in the presence of 100-fold excess inert estradiol measures the Non-Specific Binding (NSB). The difference between the TB and NSB is the specific receptor binding.

The saturation binding experiment characterizes the relationship between specific receptor binding and the ligand concentration to determine the number of specific binding sites (B_{max}) and the radioligand affinity (the equilibrium dissociation constant, K_d). (See OCSPP Guideline 890.1250 for details).

2. Competitive Binding Experiment

The Competitive Binding Experiment measures the ability of increasing concentrations of test chemical to displace a single concentration of radioligand from the ER. Details of the assay are provided in the Test Guideline. The experiment is generally conducted as follows: Using a constant volume of cytosol, a single concentration of radioligand is combined with increasing concentrations of test compound. This mixture is incubated overnight (16-20 h) to allow ER binding of the components to come to equilibrium. A hydroxyapatite (HAP) slurry is then added

to each assay tube, mixed and then tubes are centrifuged to pellet the HAP. Any ligand bound ER will be retained in the HAP pellet. Unbound (free) ligand is discarded in the supernatant.

Ligand-bound ER is released from the HAP by the addition of an ethanol solution. The ER-bound radioligand can then be quantified as dpms using a scintillation counter. If the test compound competes for binding to the receptor, then the dpms should decrease as the concentration of test compound increases.

The binding is quantified by the concentration of competitor which inhibits 50% of the binding of the radioligand (IC_{50}) to the receptor and by the Relative Binding Affinity (RBA, % binding affinity relative to estradiol). The extent of displacement of the radiolabeled estradiol is used to characterize the test chemical as interacting, not interacting, or generating an equivocal response.

In addition to the chemical to be tested, each experimental run also includes a weak positive control (e.g., norethylnodrel), negative control (e.g., octyltriethoxysilane) and solvent control to demonstrate proper assay performance. A run will also typically contain replicates of radioligand solution only, to represent the total radioactivity added to each tube in the experiment and replicates with 100-fold molar excess radioligand to determine NSB.

3. Evaluation of estrogen receptor preparations

The source of the ER for this assay is the Rat Uterine Cytosol (obtained from Sprague-Dawley rats). The Saturation Binding Experiment may be used to determine the optimal protein concentration for binding, and is recommended to ensure that the ER is present in reasonable concentrations and is functioning with appropriate affinity for 17β-estradiol. The Guideline recommends that the saturation binding experiment be performed for each batch of cytosol prepared as long as the cytosol is properly stored (see Test Guideline for details). The Agency recommends that three adequate saturation binding runs (each containing three replicates per concentration) be performed initially to characterize a cytosol batch.

III. EVALUATION OF STUDY CONDUCT

This section provides a summary description of the information that would generally be expected to be obtained from a study that had been conducted following the recommendations in the Test Guideline. As described in this section, the DER reviewer is responsible for summarizing how the study was conducted, the extent to which that is consistent with the Guideline, and how, if at all, that affected the validity of the study. This information will factor into the Agency's interpretations of the data contained in the study report. Specific points that are important for the DER to address are highlighted in the individual sections below, as appropriate.

The summary in this section is offered as a general outline to aid in preparation of the DER. The purpose of this section is not to serve as substitute for the Test Guideline, nor to provide any guidance on how the study should be conducted. Rather, this summary is intended to provide context and examples illustrating to the reviewer what the DER would be expected to contain.

A. Test Compound

It is recommended that the Estrogen Receptor Binding Assay be performed with the technical (purest) form of the chemical intended for commercial use. The DER should record the specifications of the test material including the following information: CAS number, molecular formula, molecular weight, source, lot number, purity, storage conditions, and the identity of any contaminants present at concentrations ≥1%.

B. Radioligand (Hexatritiated 17β-estradiol)

The Guideline recommends the following information be reported for the radioligand used in the study: name of supplier, catalog number, batch number, CAS numbers, number and locations of radiolabels, specific activity on date of production, date of production, date of use and the results of any purity determinations. The DER should record any justification given in the lab report for use of any other radioligand than hexatritiated 17β-estradiol as certain other radioisotopes have the potential to interfere sterically with binding. It is recommended that the specific activity for hexatritiated 17β-estradiol at date of production not be significantly less than 110 Ci/mmol, as this may result in reduced radioactivity counts and thus reduce the sensitivity of the assay.

C. Reference Standard Ligand (Unlabeled 17β-estradiol)

It is recommended that information on the supplier, purity, catalog number, batch number and CAS number of the unlabeled 17β-estradiol be included in the DER.

D. Controls (Weak Positive, Negative and Solvent Controls)

The Guideline recommends the following information be reported for the controls used in the study: name of supplier, purity, and CAS number of the weak positive control, negative control, and the solvent used in the competitive binding assays. The recommended weak positive is norethynodrel and the recommended negative control is octyltriethoxysilane. The DER should include any justification provided in the study report for use of a weak positive chemical other than norethynodrel. Additionally, the DER should include the name and final concentration of the solvent used in the assay tubes to ensure that this value falls below the maximum allowable solvent concentration of 3% for ethanol or 10% for DMSO. These limits are placed on solvent concentrations because of known interference of higher solvent concentrations with this assay.

E. Estrogen Receptor

1. Strain/Species

The ER Binding Assay was optimized using ER from Rat Uterine Cytosol (RUC) from Sprague Dawley rats. Therefore, it is recommended that this specific strain be utilized as the source of ER.

2. Cytosol Preparation

To reduce variation in the assay results, the Agency recommends that the Rat Uterine Cytosol be prepared following the protocol specified by the OCSPP 890.1250 Guideline. Briefly, the uterine tissues are collected from Sprague-Dawley rats (85-100 days of age) approximately 7-10 days after ovariectomy. The cytosol preparations from homogenized uterine tissue can be pooled, aliquoted and stored at -80 ºC for up to 90 days. If it is necessary to use a cytosol that is more than 90 days old, it is recommended that a saturation binding experiment be conducted to check the K_d and B_{max} of the receptor to ensure that the receptor is performing as expected. The protein concentration of the cytosol preparation is determined for each batch of the cytosol. If uterus or uterine cytosol is from a commercial source, the DER should include information on the supplier and storage conditions.

3. Standardization of Receptor Concentration

Different batches of cytosolic preparations will contain different concentrations of active receptor. To optimize the performance of the assay and to ensure consistency between experiments, the Guideline recommends that the receptor concentration be standardized for both the Saturation Binding Experiment and the Competitive Binding Experiment.

(a) Standardization of Receptor for Saturation Binding Experiment:

To determine the optimal protein concentration, serial dilutions of cytosol protein are tested using 0.03 nM [^{3}H]-estradiol in a final volume of 0.5 mL per tube. The optimal protein concentration that binds 25-35% of the total radioactivity added is appropriate for the Saturation Binding Experiment (typically 50 ± 10 µg protein/assay tube).

(b) Standardization of Receptor for Competitive Binding Experiment:

The receptor concentration of the cytosol is typically adjusted to minimize the likelihood of ligand depletion. Ligand depletion occurs when a high percentage of the [^{3}H]-estradiol is bound to the ER causing the concentration of the unbound (free) [^{3}H]-estradiol to differ significantly from the concentration of [^{3}H]-estradiol that was originally added to the assay tube. To determine the optimal protein concentration, serial dilutions of cytosol protein are tested using 1.0 nM [^{3}H]-estradiol in a final volume of 0.5 mL per tube. The optimal protein concentration that binds 10-15% of the total radioactivity added is typically appropriate for the Competitive Binding Experiment (typically 50 ± 10 µg protein/assay tube).

F. Test Chemical Concentration Selection

The large range of test chemical concentrations (10^{-10} to 10^{-3} M, or up to limit of solubility) used in the competitive binding assay is expected to provide sufficient data to allow full characterization of the competitive binding curve, determination of the IC_{50}, calculation of the RBA, and accurate classification of the interaction. The Agency recommends that a justification be provided in the study report (and summarized in the DER) if concentrations other than those specified in the guideline are used in the assay. The preferred solvent is ethanol, followed by water or DMSO. These three solvents are commonly used in *in vitro* assays and many

compounds can be effectively solubilized in at least one of these solvents. It is recommended that the concentration of solvent in the assay tubes be reported in the DER as well as any evidence of insolubility and the means by which solubility was evaluated (e.g., microscopy, nephelometer).

G. Saturation Binding Experiment Performance Guidance

The reviewer should evaluate the conduct of the saturation binding experiment. The Agency recommends the following guidance be considered when evaluating the saturation binding experiments:

- The identification and treatment of outliers should be explained.
- The values for K_d are generally expected to lie within the range from approximately 0.03 to approximately 1.5 nM,
- B_{max} are typically expected to lie within the range from approximately 10 to 150 fmol/100 µg protein,
- The K_d and B_{max} values are typically expected to be similar across runs.
- A well-conducted experiment generally yields a linear Scatchard plot.
- The data points of the Scatchard plot would generally not be expected to describe a curve that is substantially convex or concave.
- Non-specific binding is typically expected to be less than 50% total binding even at the highest concentration of radioligand.
- The saturation binding curve is typically expected to show little variability between replicates, and to cross the x-axis at the origin.
- Specific binding would typically be expected to plateau (i.e., reach saturation) within the range of concentrations tested.
- Variability between replicates for total binding should not be excessive.

H. Competitive Binding Experiment Performance Guidance

To ensure that the Competitive Binding Experiment functioned properly, it is recommended that each run be evaluated using the following criteria (Table 1).

TABLE 1. Competitive Binding Assay Performance Criteria [a]	
Criterion	**Tolerance Limit(s)** [a]
Radioinert 17β-estradiol fitted curve parameters	
Log_e(residual Std. Dev.)[b]	≤2.35
Top (% binding) [c]	94 to 111
Bottom (% binding)	-4 to 1
(Hill) Slope ($log_{10}(M)^{-1}$)	-1.1 to -0.7
Weak Positive control (norethynodrel) fitted curve parameters	
Log_e(residual Std. Dev.)	≤2.60
Top (% binding)	90 to 110
Bottom (% binding)	-5 to 1
(Hill) Slope ($log_{10}(M)^{-1}$)	-1.1 to -0.7
Solvent concentration	
Ethanol	≤3%
DMSO	≤10%
Negative control (octyltriethoxysilane) does not displace more than 25% of [^{3}H]-17β-estradiol from the ER on average across all concentrations	≤25%

a These values were derived from inter-laboratory validation studies reported in the Integrated Summary Report (2009b). Performance criteria are defined as the tolerance bounds that include 80% of the acceptable runs with 95% confidence, for each of the binding curve parameters (top, bottom, slope, residual standard deviation, and in the case of the weak positive control, the RBA, across all laboratories.

b. $Log_e(S_{yx})$ (i.e., Log_e(Residual Std. Dev)) is the natural log of the standard deviation of the residuals remaining after fitting the one-site competitive binding model as described in the Estrogen Receptor – Rat Uterine Cytosol Test Guideline. There is usually one residual for each replicate at each concentration, and the $log_e(S_{yx})$ is calculated for each run. The value is given in base e; the corresponding $log_{10}(S_{yx})$ is 1.0206.

c If the top plateau for estradiol is significantly above the upper performance criterion, then curves for all chemicals in the run may be normalized using binding of estradiol at the lowest concentration in the reference curve as 100%.

Additional considerations for the competitive binding experiment include:

- Curves for the both 17β-estradiol and the weak positive control typically would generally be expected to show that increasing concentrations of compound displace [^{3}H]-17β-estradiol in a manner consistent with one-site competitive binding, as indicated by a descent from 90% to 10% binding over approximately an 81-fold increase in concentration (i.e., covering approximately 2 log units).
- It is recommended that all test chemicals be tested over a concentration range that fully defines the top of the curve (it is generally not sufficient to test only one or two concentrations that shows high displacement of radioligand).
- The percent binding at this top plateau generally would be expected to be within 25 percentage points of the value for solvent control or the lowest concentration of the estradiol standard for that run.
- Examination across the runs should generally indicate consistency of the Hill slope, placement along the X-axis, and top and bottom plateaus.

I. Statistical Evaluation

For the Saturation Binding Assay, the guideline recommends total binding and non-specific binding data be modeled using non-linear regression methods (Motulsky, 1995) carried out using Graph Pad Prism software (Motulsky, 2003, 2007) or other general purpose statistical programs such as SAS (SAS Institute, Inc., 2003). EPA has used Graph Pad Prism v. 5 for non-linear

regression, incorporating automatic outlier elimination implemented by using the ROUT method (Motulsky and Brown, 2006) in Prism v. 5 with a Q value of 1.0. It is recommended that receptor binding data modeling correct for ligand depletion (Swillens, 1995).

For the Competitive Binding Assay, the guideline recommends non-linear regression be used to fit a curve (for 17β-estradiol, the positive control, and the test substance) to the Hill equation formula (Hill, 1910) which incorporates log IC_{50} as a parameter to be estimated. Prior to fitting, it may be appropriate to eliminate data points at high concentrations that are 10 or more percentage points higher (i.e., less displacement) than the minimum at a lower concentration. The intent of this step is to remove the right leg of U-shaped curves, which may be due to insolubility of the test chemical. This is applied only if the minimum value for the curve is below 80% binding of radioligand (i.e., > 20% displacement).

For parameters reported from the Saturation Binding Assay (K_d and B_{max}) and competitive binding assay (log IC_{50} and RBA), it is recommend that the mean and standard deviation be calculated for each run, and mean and standard error be determined from the three runs. The reviewer should summarize the methods for statistical analysis in the DER, along with the reviewer's assessment as to whether the statistical methods used were appropriate.

IV. STUDY INTERPRETATION

A. Saturation Binding Experiment

ER saturation binding experiments measure total and non-specific binding of increasing concentrations of [^{3}H]-17β-estradiol under conditions of equilibrium. From these measurements, specific binding at each concentration can be calculated. A graph of specific [^{3}H]-17β-estradiol binding versus radioligand concentration would generally be expected to reach a plateau for maximum specific binding, indicating saturation of the ER with the radioligand.

Total binding is defined as the radioactivity in dpms bound in the centrifuge pellet in the tubes that have only [^{3}H]-17β-estradiol available to bind to the receptor. Non-specific binding is the radioactivity bound in the centrifuge pellet in the tubes that contain 100-fold excess of unlabeled over labeled 17β-estradiol. Data are subjected to non-linear regression using the total binding and non-specific binding data points, automatic outlier elimination, and correction for ligand depletion, to fit the following model, where Y = total binding, α = the ratio between nonspecifically bound ligand and free ligand, and X = concentration of [^{3}H]-17β-estradiol:

Swillens Equation[1]:
$$Y = \frac{B_{\max} * X}{X + K_d} + (\alpha * X)$$

Specific binding, the difference between total and non-specific binding, is calculated not measured. Graphs of the three runs should typically be included in the DER, depicting total, non-specific, and specific binding, with binding (in dpm) on the y-axis and concentration of

[1] Note: This equation was corrected according to the Corrections and Clarifications on Technical Aspects of the EDSP Tier 1 Assays (OCSPP Test Guideline Series 890)

[^{3}H]-17β-estradiol on the x-axis. It is recommended that data points be shown so that variability can be evaluated visually. The graph is intended to allow a rough determination of whether the specific binding curve reached a plateau (i.e., the binding sites were saturated). It is recommended that a Scatchard plot showing all three Scatchard lines and all data points also be included. The graphs should generally be evaluated for variability of data points, similarity of K_d and B_{max} across runs, and linearity of the data within each run. Typically the data should indicate the binding of the [^{3}H]-17β-estradiol to a single, high-affinity binding site (i.e., K_d = 0.03 to 1.5 nM and a linear Scatchard plot) and an appropriate number of receptors available for binding (B_{max} = 10-150 fmol ER/100 μg cytosol protein). In a Scatchard plot (Scatchard, 1949), specific binding is shown on the x-axis (usually labeled "Bound") and the ratio of specific binding of 17β-estradiol to free 17β-estradiol (usually labeled "Bound/Free") is shown on the y-axis. In these plots, B_{max} is the x-intercept and K_d is the negative reciprocal of the slope. However, the Scatchard plot is not the most accurate technique to use for estimating K_d and B_{max}. The Agency strongly recommends that nonlinear regressions be used to calculate K_d. Additionally, the reviewer should include a table indicating the values for K_d and B_{max} for the three runs in the DER.

B. Competitive Binding Assay

In the discussion below, it is important to distinguish the IC_{50} from the EC_{50}. The IC_{50} is the concentration at which 50% of the radioligand is bound to the receptor, while the EC_{50} is the concentration at which binding of the radioligand is halfway between the top plateau and the bottom plateau. The IC_{50} and EC_{50} coincide only when the top plateau is at 100% and the bottom plateau is at 0%. Some modeling programs may calculate only the EC_{50}, or may calculate the EC_{50} but call it the IC_{50}. The Agency prefers the IC_{50} because in general it allows comparison of binding strengths of different compounds, while the EC_{50} may not allow the comparison.

If the radioligand and the inhibitor both bind reversibly to the same single binding site on the receptor, then specific binding at equilibrium follows a four parameter relation between percent bound (Y) and inhibitor concentration (X). The concentration response relationship is described by a sigmoid curve (a variation of the Hill equation), where T = top plateau of the curve, B = bottom plateau of the curve, β = "Hill" slope, $\log_{10}(IC_{50})$ is the logarithm of the concentration at which the expected value of Y = 50%, and ε = the random variation around the concentration response relationship, with a mean of 0 and the variance a function of the expected value of Y (often modeled as a constant, σ):

$$Y = B + \frac{(T - B)}{1 + 10^{\beta(\log IC_{50} - X) + \log_{10}\left[\left(\frac{T-B}{50-B}\right) - 1\right]}} + \varepsilon$$

For a competitive inhibition curve, the percent bound decreases with increasing concentration; therefore β is always negative. Values for $\log_{10}(IC_{50})$ can be directly compared among chemicals because they always represent the same percentile of the concentration response. An ideal response by a one-site competitive binder would result in T = 100, B = 0, and β = -1. Since a test chemical that interacts with the receptor might not be a one-site competitive

binder and that multiple receptor types may be present in the RUC, the ideal values may not apply.

C. Relative Binding Affinity (RBA)

Because of the potential for variation in IC_{50} values among ER binding assays, the generally accepted method for presenting and comparing the assay results is to calculate the Relative Binding Affinity (RBA) of the test substance against a reference standard (17β-estradiol). The RBA is calculated as IC_{50} of 17β-estradiol × 100 ÷ IC_{50} of test substance.

D. Binder Classification

Classification of the test material is based on the average of three runs. Initially, however, it is recommended that each run be individually classified as follows:

- **Interactive** = lowest point on the fitted curve within the range of the data is less than 50% (i.e., >50% of the radiolabeled estradiol has been displaced from the ER).

- **Not interactive** = 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% (i.e., <25% of the radiolabeled estradiol has been displaced from the ER) or a binding curve cannot be fitted and the lowest average percent binding among concentration groups in the data is above 75%.

- **Equivocal up to the limit of concentrations tested** = If there are no data points at or above a test chemical concentration of 10^{-6}M and either a binding curve can be fit but ≤50% of the radiolabeled estradiol has been displaced from the ER or a binding curve cannot be fit and the lowest average percent binding among concentration groups in the data is >50%.

- **Equivocal** = A run is classified as equivocal if it does not fall into any of the categories above.

The categorical classification of each run should then be assigned a numerical value as follows:

Run Classification	Numerical Value
Interactive	2
Equivocal	1
Not interactive	0
Equivocal up to the limit of concentrations tested	"missing"

The values for each run are then averaged across runs and the chemical classified using the following ranges:

Test Material Classification	Numerical Range
Interactive	average ≥1.5
Equivocal	0.5≥ average <1.5
Not interactive	average <0.5
Equivocal up to the limit of concentrations tested	"missing"

V. ESTROGEN RECEPTOR BINDING ASSAY HAZARD CHARACTERIZATION

The Estrogen Receptor Binding Assay is intended to identify xenobiotics that may interact with the estrogen receptor. The assay can only detect binding to the receptor and, therefore, cannot predict transcriptional activation or distinguish between chemicals that act as estrogens and those that block the receptor and act as anti-estrogens. This assay is intended to be used in conjunction with other guidelines in the 890 Series to determine on a weight-of-evidence basis if a chemical interacts with components of the endocrine system.

VI. DATA EVALUATION REPORT

Once the study has been reviewed, a DER will be prepared. A DER template is available that provides additional guidance for the preparation of the DER.

VII. REFERENCES

Corrections and Clarifications on Technical Aspects of the EDSP Tier 1 Assays (OCSPP Test Guideline Series 890) in the docket; the link to this document may be found by way of the EDSP web page (http://www.epa.gov/endo/).

Hill, A. V. (1910) The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. J. Physiol. (Lond.) 40, iv-vii.

Motulsky, H.J. (1995) Analyzing data with GraphPad Prism, GraphPad Software Inc., San Diego CA, http://www.graphpad.com

Motulsky, H. (2003) GraphPad PRISM User's Guide Version 4.0, GraphPad Software, Inc. http://www.graphpad.com/prism/Prism.htm

Motulsky, H.J. and Brown, R.E. (2006) Detecting outliers when fitting data with nonlinear regression- a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics, Vol 7, pp 123-142.

Motulsky, H. (2007) GraphPad PRISM Version 5 software, San Diego, CA. http://www.graphpad.com/prism/Prism.htm

SAS, Version 9.1.3 (2003) SAS Institute, Inc., Cary, NC.

Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Ann NY Acad Sci 51: 660-672.

Swillens, S. (1995) Interpretation of binding curves obtained with high receptor concentrations: practical aid for computer analysis. Molec. Pharmacol. 47(6):1197-1203.

U.S. EPA (2009a) Endocrine disruptor screening program test guidelines OPPTS 890.1250: estrogen receptor binding (rat uterine cytosol). EPA 740-C-09-005. Washington DC.

U.S. EPA (2009b) Integrated summary report for the validation of an estrogen receptor binding assay using rat uterine cytosol as source of receptor as a potential screen in the endocrine disruptor screening program Tier 1 battery. Washington DC.