ANDROGEN RECEPTOR BINDING
(RAT VENTRAL PROSTATE CYTOSOL)

Standard Evaluation Procedure

ENDOCRINE DISRUPTOR SCREENING PROGRAM
U.S. Environmental Protection Agency
Washington, DC 20460

October 2011

Table of Contents

I. INTRODUCTION

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 890 890.1150 Androgen Receptor Binding Assay 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 valid. 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. ANDROGEN RECEPTOR BINDING ASSAY

A. Purpose of the Assay

The Androgen Receptor Binding Assay identifies chemicals that have the potential to interact with the androgen receptor (AR) *in vitro*. Androgens are sex hormones that play critical roles in male sexual differentiation, development and maturation that also have some role in

female development and physiology. The biological action of androgens is mediated through their interaction with the AR; androgens have no hormonal activity in the absence of a functional AR. Androgens bind to the AR, which subsequently dimerize initiating a cascade of events which result in physiological responses such as male sexual differentiation, maintenance of the male sexual characteristics, spermatocyte production, and prostate gland development and growth. This assay uses AR isolated from rat ventral prostates. There is a high degree of DNA sequence conservation in the AR across mammalian phylogenetic lines (Kelce *et al*., 1998; Betney and McEwan, 2003). Therefore, substances that bind the AR from rats are presumed to be capable of binding the AR in humans.

Because AR binding is the initiating step in the cascade of androgen-mediated effects, assays have been developed to measure competitive inhibition of this binding step by environmental compounds. This screening assay measures the receptor-binding affinity of chemicals by evaluating their ability to displace a bound reference androgen, usually 5α-dihydrotestosterone (DHT) or R1881, a synthetic androgen. Such interference with normal androgen binding has the potential to interfere (*i.e*., compete) with normal androgen activity *in vivo* by acting as either an agonist producing androgen-like effects, or as an antagonist, which prevents or blocks the normal actions of androgens. Although the assay identifies compounds that compete for AR binding *in vitro*, it cannot distinguish between agonist and antagonist activity.

B. Study Design

The AR Binding Assay described in OCSPP Guideline 890.1150 is a radioligand binding assay. 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 AR isolated from rat ventral prostate cytosol preparations is present in reasonable numbers and is functioning with appropriate affinity for the radio-labeled reference ligand. The Guideline recommends that proper saturation binding of the reference ligand to the AR be demonstrated with each batch of rat ventral prostate cytosol (the source of the AR for this assay) before using the cytosol to conduct competitive binding assays. The Saturation Binding Experiment measures the binding (at equilibrium) of various concentrations of the radioligand (*i.e.*, [^{3}H]R1881; a synthetic radio-labeled androgen with high affinity for the AR similar to the natural androgen, DHT) both with and without the presence of 100-fold higher concentrations of unlabeled (inert) ligand. Counts or disintegrations per minute (dpms) derived from increasing concentrations of [^{3}H]R1881 binding in the absence of unlabeled ligand indicate the total binding (TB). In addition to binding to the AR, the radioligand may also bind non-specifically to other sites. Dpms derived from increasing concentrations of [^{3}H]R1881 binding in the presence of 100-fold excess inert R1881 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 or K_d). (see OCSPP Guideline 890.1150 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 AR. Details of the assay are provided in the Test Guideline. Briefly, the experiment is 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 to allow AR 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 AR will be retained in the HAP pellet). Unbound (free) ligand is discarded in the supernatant.

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

In addition to the unknowns to be tested, each run of test chemicals also includes the strong positive control (inert R1881), weak positive control (*e.g.* dexamethasone) 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.

C. Evaluation of Androgen Receptors Preparations

The source of the AR for this assay is the rat ventral prostate cytosol (obtained from Sprague Dawley rats). The Saturation Binding Experiment may be used to determine the optimal protein concentration for binding, and to ensure that the AR is present in reasonable concentrations and is functioning with appropriate affinity for the reference androgen R1881. 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 three adequate saturation binding runs, each containing three replicates at each 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, the 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 Androgen Receptor Binding Assay be performed with the technical (purest) form of the chemical intended for commercial use. The Guideline recommends that specifications of the test material be clearly indicated in the study report and include the following: CAS Number, molecular formula, molecular weight, source, lot number, purity, storage conditions, and the identity of any contaminants present at concentrations ≥1%.

B. Radioligand ([3H]-R1881)

The Guideline recommends the following information be reported for the radioligand used in the study: supplier, catalog number, batch number and CAS number, number and locations of radiolabels, specific activity on date of production, date of production, and results of any purity determinations.

C. Reference Standard Ligand (Unlabeled R1881)

It is recommended that information on the supplier, purity and CAS number of the unlabeled R1881 be provided in the DER.

D. Controls (Weak Positive 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. Additionally, it is recommended that the name and final concentration of the solvent used in the assay tubes be reported in the DER.

Note: No negative control chemical is recommended in this Test Guideline (OCSPP 890.1150). This differs from the Estrogen Receptor Binding Assay Test Guideline (OCSPP 890.1250) which recommends both a negative control chemical and a solvent control.

E. Androgen Receptor

1. Strain/Species

The AR Binding Assay was optimized using AR from rat ventral prostate cytosol from Sprague Dawley rats. Therefore, it is recommended that this specific strain be utilized as the source of AR (USEPA, 2007).

2. Cytosol Preparation

To reduce variation in the assay results, the Agency recommends that the rat prostate cytosol be prepared following the protocol specified by the OCSPP 890.1150 Guideline. Briefly, the ventral prostate tissues are collected from castrated Sprague Dawley rats (60 to 90 days old; 90-day old is preferred to provide optimal AR protein expression). Castration results in a transient increase in AR which peaks at 24 hours, therefore it is important to collect the ventral prostate tissue as close to 24 hours after castration as possible. This will ensure that there is sufficient AR to conduct the assay. Castration also eliminates endogenous androgen prior to harvesting the tissue for AR. The cytosol preparations from homogenized prostate tissues can be pooled, aliquoted and stored at –80ºC. 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 prostate or prostate cytosol is from a commercial source, it is recommended that information on the supplier and storage conditions be reported in the DER.

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 0.25 nM [^{3}H]-R1881 in a final volume of 0.3 mL per tube. The optimal protein concentration that binds 25-35% of the total radioactivity added is typically appropriate for the Saturation Binding Experiment (typically 1.2 mg protein/assay).

(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]-R1881 is bound to the AR causing the concentration of the unbound (free) [^{3}H]-R1881 to differ significantly from the concentration of [^{3}H]-R1881 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]-R1881 in a final volume of 0.3 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 1.2 mg 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. The concentration of solvent in the assay tubes should be reported, and should not alter the sensitivity or the reliability of the assay. It is recommended that evidence of insolubility be reported in the DER as well as 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 0.685 to approximately 1.57 nM,
- B_{max} are 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 (Scatchard, 1949).
- The data points (graphed in a Scatchard-type 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 20%
- The saturation binding curve is generally expected to show little variability between replicates, and would typically be expected 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]
Test chemical Top (% binding)	80 to 115
R1881 fitted curve parameters	
Top (% binding)	82 to 114
Bottom (% binding)	-2.0 to 2.0
Hill Slope	-1.2 to -0.8
Weak positive control (dexamethasone) fitted curve parameters	
Top (% binding)	87 to 106
Bottom (% binding)	-12 to 12
Hill Slope	-1.4 to -0.6

a These values represent ranges from the validation study. It may be helpful if an additional run be made when a run does not fall within these ranges, particularly if that run differs from the other 2 runs.

Additional guidance for the competitive binding experiment includes:

- Curves for the both radioinert R1881 and the weak positive control typically would generally be expected to show that increasing concentrations of compound displace [^{3}H]-R1881 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).
- The Guideline recommends 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 show high displacement of radioligand).
- The percent binding at this top plateau should generally be expected to be within 25 percentage points of the value for solvent control or the lowest concentration of the R1881 standard for that run.
- Examination across the runs would typically be expected to indicate consistency of the Hill slope, placement along the X-axis, and top and bottom plateaus.

I. Statistical Evaluations

For each test run, the four parameter concentration response models are fitted to the concentration response data for each chemical by nonlinear regression analysis. The model fits results in parameter estimates and associated standard errors as well as estimates of residual variability. These are used for inferences about the concentration response model parameters and for statistical comparisons between the test chemical and the standard within a run, among runs within test laboratory, and across test laboratories. Nonlinear regression analysis can be carried out using PRISM Version 4 or 5 software or general purpose statistical systems such as SAS. (EPA does not have a recommendation on whether to use weighted or non-weighted least squares, as that question is still under discussion at EPA. An interim recommendation is to carry out non-weighted fits for the AR assay.) For each test chemical, multiple runs are carried out. For each run, the DER should report the estimates of the following parameters:

- B, the bottom plateau
- T, the top plateau
- β, the "hill slope" (β is necessarily negative) (Hill, 1910).
- $Log_{10}IC_{50}$, the logarithmic concentration at which E(Y) = 50%
- $Log_{10}RBA$, ($log_{10}(IC_{50,std}/ IC_{50,test})$)

For parameters reported from the Saturation Binding Assay (K_d and B_{max}) and Competitive Binding Assay (log IC_{50} and RBA), the DER should include the mean and standard deviation calculated for each run, and the mean and standard error that was determined from the three runs. The methods for statistical analysis should be summarized in the DER, along with an assessment as to whether they were appropriate.

IV. STUDY INTERPRETATION

A. Saturation Binding Experiment

AR Saturation Binding Experiments measure total and non-specific binding of increasing concentrations of [^{3}H]R1881 under conditions of equilibrium. From these measurements, specific binding at each concentration can be calculated. A graph of specific [^{3}H]R1881 binding versus radioligand concentration would typically be expected to reach a plateau for maximum specific binding, indicating saturation of the AR with the radioligand.

The Guideline defines total binding as the radioactivity in dpms bound in the centrifuge pellet in the tubes that have only [^{3}H]R1881 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 R1881. 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]R1881:

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 [3H]R1881 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 reviewer should evaluate the graphs for variability of data points, similarity of Kd and Bmax across runs, and linearity of the data within each run. Typically the data would be expected to indicate the binding of the [3H]R1881 to a single, high-affinity binding site (i.e., Kd = 0.685 to 1.57 nM and a linear Scatchard plot). In a Scatchard plot (Scatchard, 1949), specific binding is shown on the x-axis (usually labeled "Bound") and the ratio of specific binding of R1881 to free R1881 (usually labeled "Bound/Free") is shown on the y-axis. In these plots, Bmax is the x-intercept and Kd is the negative reciprocal of the slope. However, the Scatchard plot is not the most accurate technique to use for estimating Kd and Bmax. The Agency strongly recommends that nonlinear regressions be used to calculate Kd. 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 Experiment

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

[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)

concentration at which binding of the radioligand is halfway between the top plateau and the bottom plateau. The IC_{50} and EC_{50} coincide when the top plateau is at 100% and the bottom plateau is at 0%, but otherwise may differ. 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.

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.

C. Relative Binding Affinity (RBA)

Because of the potential for variation in IC_{50} values among AR binding assays, the generally accepted method for presenting and comparing the assay results is to compute the Relative Binding Affinity (RBA) of the test substance against a reference androgen (R1881). The RBA is calculated as IC_{50} of R1881 × 100 ÷ IC_{50} of test substance

D. Binder Classification

If the data fit a 4-parameter nonlinear regression model the test chemical would typically be classified as:

- **Binder:** The average curve for the test chemical across runs displaces 50% or more of the radioligand

- **Equivocal:** The average lowest portion of curves across runs is between 50% and 75% activity, or the slope of curve falls outside the range for the weak positive control (-0.6 to -1.4).

- **Non-Binder:** The average lowest portion of curves across runs is greater than 75% activity, or the data do not fit the model.

- **Untestable:** If the test compound is not soluble above 1×10^{-6} M and the binding curve does not cross 50%, the chemical is judged to be untestable.

Note: As indicated in the Corrections and Clarifications on Technical Aspects of the EDSP Tier 1 Assays (OCSPP Test Guideline Series 890) document, the classification method used in the Estrogen Receptor Binding Assay to summarize assays and classify the potential of a test chemical to interact with the receptor is also applicable to the Androgen Receptor Binding Assay. The method is described in section (k)(7)(iv) on pages 48 and 49 of OSCPP 890.1250 Estrogen Receptor Binding Assay Using Rat Uterine Cytosol.

V. ANDROGEN RECEPTOR BINDING ASSAY HAZARD CHARACTERIZATION

The Androgen Receptor Binding Assay is intended to identify xenobiotics that may interact with the androgen receptor. The assay can only detect binding to the receptor and, therefore, cannot predict transcriptional activation or distinguish between chemicals that act as androgens and those that block the receptor and act as anti-androgens. 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 using the principles described in the previous sections of this SEP, a DER will be prepared. A DER template is available that provides 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/).

Betney, R. and McEwan, I.J. (2003) Role of conserved hydrophobic amino acids in androgen receptor AF-1 function. *J. Molec Endocrinol* 31(3): 427-439.

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.

Kelce, W.R., Gray, L.E., and Wilson, E.M. (1998) Antiandrogens as environmental endocrine disruptors. Reprod Fertil Devel 10: 105-111.

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

U.S. EPA (2007). Integrated summary report for the validation of an androgen receptor binding assay as a potential screen in the endocrine disruptor screening program. Washington DC.

U.S. EPA (2009). Endocrine disruptor screening program test guidelines OPPTS 890.1150: androgen receptor binding (rat prostate cytosol). EPA 640-C-09-003. Washington DC.