Protocol for the In Vitro Estrogen Receptor Saturation Binding and Competitive Binding Assays Using Rat Uterine Cytosol

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1.0 Purpose of the assay

This assay will be used to provide information on the ability of a compound to interact with the estrogen receptors (ERs) isolated from the rat uterus. The 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. It therefore may not be appropriate for use in quantitative structure-activity relationship model development for estrogen receptor binding without further refinement. The 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.

2.0 Brief description of the assay

The expression of a hormone's activity begins when it binds to its specific receptor and initiates a cascade of events leading to a physiological response. The estrogen receptor (ER) is located inside target cells, in or near the nucleus, and hormone-bound ER interacts with specific sites in the genome of the cell to control production of mRNA. The hormone-binding domain (HBD) of the estrogen receptor is highly specific compared to receptors for other steroid classes. It is highly conserved across species. Environmental chemicals that compete with endogenous estrogens have the potential to either induce hormone-dependent transcriptional activity (agonist) or block normal hormone function by preventing the endogenous hormone from binding to the receptor (antagonist). Thus a test of a compound's ability to bind to ER constitutes a direct, simple evaluation of its estrogenic potential in thousands of vertebrate species.

The assay described in this protocol measures the ability of a radiolabeled ligand (17β-estradiol) to interact with the ER in the presence of increasing concentrations of a test chemical. Rat uterine cytosol containing ER is incubated in test tubes with increasing concentrations of a test substance and an aliquot of radiolabeled [^{3}H]-estradiol. If the test substance interacts with the receptor's HBD, less radioligand can bind so an active competitor produces a descending dose-response plot. Similarly, compounds that do not displace radiolabeled estradiol from ER would be presumed to be devoid of estrogen-related activity. The assay has a long history of use within the research community for rapid and relatively inexpensive detection of chemicals with ER binding capability (ICCVAM 2002).

3.0 Safety and operating precautions

Laboratories are reminded to follow all standard operating procedures and other applicable safety measures provided by their institutions for the handling and disposal of radioactive materials, as well as for other occupational health and safety concerns.

All studies utilizing animals should be approved, prior to implementation, by the laboratory's Institutional Animal Care and Use Committee (IACUC) or its equivalent.

4.0 Terminology

Term	*Meaning*
$[^3H]E_2$	17β-Estradiol radiolabeled with tritium
ddH_2O	Double distilled water
DTT	Dithiothreitol
E_2	17β-Estradiol (inert estradiol)
HAP	Hydroxyapatite
PMSF	Phenylmethylsulfonyl fluoride
TEDG buffer	Tris, EDTA, DTT, glycerol buffer
Tris	Tris(hydroxymethyl)aminomethane
replicate	One of multiple tubes that contain the same contents at the same concentrations and are assayed concurrently within a single run. In EPA's protocol, each concentration of test substance is tested in triplicate; that is, there are three replicates that are assayed simultaneously at each concentration of test substance.
run	A complete set of concurrently-run tubes that provides all the information necessary to characterize binding of a test chemical to the receptor (viz., total $[^3H]$-17β-estradiol added to the assay tube, maximum binding of $[^3H]$-17β-estradiol to the estrogen receptor, nonspecific binding, and total binding at various concentrations of test substance). A run could consist of as few as one tube (i.e., replicate) per concentration, but since EPA's protocol requires assaying in triplicate, one run consists of three tubes per concentration. In addition, EPA's protocol requires three independent (i.e., non-concurrent) runs per chemical.

5.0 Equipment and materials

5.1 Equipment

- Stir/hot plates
- Pipettes
  Mechanical, variable volume pipette
    Must be calibrated on a regular basis. Check volumes on a high sensitivity scale; for example, 10 µl = 10 µg using distilled water. Pipettes needed include:
    0.5 to10 µl
    2 to 20 µl
    20 to 200 µl
    100 to 1000 µl
    2 to 10 ml
  Repeating pipettes
    0.1 to 2.5 ml
  Programmable pipettes
    0.5 to 2.5 ml
- Balance, analytical
- Tissue homogenizer
  (e.g., Polytron PT 35/10)
- Multi-tube vortex
- Rotator(s) and drums
  for incubation in cold box (e.g., Cel-gro Tissue Culture Rotator, Barnstead International Lab-Line catalog number 1640)
- Refrigerated general laboratory centrifuge
  capacity approximately 300 tubes, with buckets for 12 by 75 mm tubes at 4º C
- High-speed refrigerated centrifuge
  (up to 30,000 x g) (e.g., Beckman Optima™)
- Refrigerated ultra-centrifuge
  capable of 105,000 x g at 4º C (e.g., Beckman Optima™)
- pH meter with Tris-compatible electrode
  with traceable standards (pH 4, 7, and 10)
- Scintillation counter with traceable standards
- Ice bath tubs and buckets
- Pump dispenser
  2 each 1-5 ml and 1 each 5-25 ml
- Freezer -80º C, freezer -20º C, refrigerator 4º C
- Traceable thermometers
  for monitoring refrigerator and freezer temperatures
    - 80º C freezer (temperature recorded daily during the business week and monitored for off-hour emergencies)
    - 20º C freezer (temperature recorded daily during the business week and monitored for off-hour emergencies)

4º C refrigerator (temperature recorded daily during the business week and monitored for off-hour emergencies)

- Microtiter plates
- Microplate reader, capable of running Bradford protein assays (e.g., BioRad Model 550)
- Tube racks

5.2 Reagents

(ACS reagent grade or better)

- DTT, Dithiothreitol, CAS 3483-12-3, Mol. Wt. 154.3
- Dyes for protein assay (e.g., BioRad Protein Assay Dye, catalog # 500-0006, BioRad Chemical Division, Richmond, CA)
- Radio-inert 17β-estradiol (E_2), CAS 50-28-2, Mol. Wt. 272.4
- Radiolabeled 17β-estradiol ([^{3}H]E_2), CAS 50-28-2, Mol. Wt. 272.4 (obtain highest specific activity available). (e.g., PerkinElmer NEN, catalog # NET 517, Estradiol, [2,4,6,7,16, 17β-^{3}H(N)]- Specific Activity: 110-170 Ci (4.07-6.29 TBq)/mmol)
- Dimethyl sulfoxide (DMSO), CAS 67-68-6, Mol. Wt. 78.13
- EDTA disodium salt dihydrate, Ethylenediaminetetraacetic acid, CAS 6381-92-6, Mol. Wt. 372.2
- Ethyl alcohol (Ethanol), 200 Proof USP, CAS 64-17-5
- Glycerol 99%, CAS 56-81-5, Mol. Wt. 92.10
- HAP, Hydroxyapatite, CAS 1306-06-5 (e.g., Fluka dry form "Fast Flow", BioRad slurry, or fine powder from Sigma. Note that the performance of HAP may vary depending on source.)
- Norethynodrel, CAS 68-23-5, Mol. Wt. 298.4; test chemical for assay standardization, weak positive ligand
- PMSF, Phenylmethylsulfonyl fluoride, CAS 329-98-6, Mol. Wt. 174.2
- Octyltriethoxysilane, CAS 2943-75-1, Mol. Wt. 276.49; negative control ligand
- Scintillation cocktail (e.g., PerkinElmer Optifluor, catalog # 6013199)
- Tris Base, Tris(hydroxymethyl)aminomethane, CAS 77-86-1, Mol. Wt. 121.1
- Tris-HCl, Tris(hydroxymethyl)aminomethane hydrochloride, CAS 1185-53-1, Mol. Wt. 157.6
- HCl, Hydrochloric acid, CAS 7647-01-0, Mol. Wt. 36.46
- NaOH, Sodium hydroxide, CAS 1310-73-2, Mol. Wt. 40.0
- Accurate pH standards, commercial grade, including pH 4, 7, and 10

5.3 Supplies

- 20 ml polypropylene scintillation vials
- 12 x 75 mm round-bottom siliconized or silanized borosilicate glass test tubes (e.g., PGC Scientifics, catalog # 79-6326-44)
- 1000 ml graduated cylinders
- 500 ml Erlenmeyer flasks
- Pipette tips

- Gloves

5.4 Software

5.4.1 Nonlinear curve-fitting software

Select a statistical package capable of analyzing saturation and competitive binding data. K_d and B_{max} should be analyzed using nonlinear regression and then graphed as a Scatchard plot. For example:

GraphPad Prism	(GraphPad Software Inc., San Diego, CA)
KELL (includes Radlig and Ligand)	(Biosoft, Cambridge, UK)
SAS	(SAS Institute Inc., Cary, NC)

5.4.2 Spreadsheet software

For example, Microsoft Excel or compatible.

6.0 Preparation of buffer solutions

Unless otherwise specified, prepare buffers at least one day before assay.

6.1 Stock solutions

May be used for up to 3 months.

6.1.1 200 mM EDTA stock solution

Dissolve 7.444 g disodium EDTA in a final volume of 100 ml ddH_2O = 200 mM. Store at 4º C.

6.1.2 100 mM PMSF stock solution

Dissolve 1.742 g PMSF in a final volume of 100 ml ethanol = 100 mM. Store at 4º C.

Note: *The PMSF stock solution is highly toxic.*

6.1.3 1M Tris stock buffer

Add 147.24 g Tris-HCl + 8.0 g Tris base to 800 ml ddH_2O in a volumetric flask and allow to cool to 4º C. Once cool, adjust pH to 7.4 and bring the final volume to 1.0 liter. Store at 4º C.

6.1.4 2X TEG buffer

20 mM Tris, 3 mM EDTA, 20% glycerol, pH 7.4

To make 100 ml, add the following together in this order:

70 ml ddH_2O
2.0 ml Tris (1 M) stock solution
20 ml glycerol
1.5 ml EDTA (200 mM) stock solution

Note: *Cool to 4° C before adjusting to pH 7.4, then bring volume to 100 ml with ddH_2O, and store at 4° C. (Tris buffers have temperature dependent pK_a values. Be sure to cool the buffer before adjusting the pH!)*

6.2 Working assay buffer (TEDG+PMSF buffer)

10 mM Tris, 1.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 10% glycerol, pH 7.4
This buffer solution is prepared daily as needed.

To make 100 ml, add the following together in this order:

50 ml 2X TEG buffer (prepared as above and cooled to 4°C)
15.43 mg DTT (add immediately before use)
1.0 ml PMSF (100 mM) (prepared as above and cooled to 4º C. Add to TEDG buffer immediately before use.)
Bring to 100 ml with cold (4º C) ddH_2O.

Note: *Add DTT and PMSF immediately prior to use; keep all solutions at 4º C at all times.*

7.0 Preparation of rat uterine cytosol

7.1 Collection of uteri

Note: *Consistency for all assays should be maintained with respect to the age and strain of the animals used. The performance criteria are based on Sprague-Dawley rats. Rapid processing at 4° C is necessary to minimize degradation of the estrogen receptor.*

Collect uteri from Sprague-Dawley female rats (85 to 100 days of age at time of kill) ovariectomized seven to ten days prior to being humanely killed. (See Appendix D: Uterine dissection diagram for obtaining estrogen receptors for the ER binding assay.) Work quickly to avoid desiccation and degradation while processing uteri. Immediately after dissecting a uterus, quickly trim fat and mesentery from it. Weigh and record the blotted weight of each uterus. Uteri may be placed in ice cold TEDG buffer + PMSF for immediate use, or placed in storage container(s) and rapidly frozen in liquid nitrogen for storage at -80° C for up to six months.

Pre-dissected uteri can be purchased from a supplier. If so, the following information should be provided to the supplier:

- rat strain should be Sprague-Dawley,
- animals must be ovariectomized 7-10 days prior to dissection of uteri,
- uteri from animals of similar ages must be provided (85-100 days old at kill),
- the recorded blotted weight of uteri immediately following dissection shall be provided,
- the supplier must guarantee that the uteri were flash frozen immediately following dissection and weighing.

Upon receipt at the laboratory performing the receptor binding assay, there should be an immediate check to make sure there has been no thawing during shipping.

7.2 Preparation of uterine cytosol

Note: *It is important to conduct all steps in this section at 4° C to prevent protein degradation. To ensure minimal heating during homogenization, cool the homogenizer probe prior to homogenizing each sample by placing the probe in ice-cold TEDG + PMSF buffer. The homogenization tube should be kept in an ice-cold water bath during the homogenizing process.*

1) Weigh trimmed uterus and place in ice-cold TEDG buffer + PMSF at a ratio of 0.1 g of tissue per 1.0 ml TEDG + PMSF buffer. Homogenize the tissue using a Polytron (PT 35/10) homogenizer for 3 to 5 bursts (~5 seconds per burst).

2) Transfer the homogenate to pre-cooled centrifuge tubes and centrifuge for 10 minutes at 2,500 x g at 4° C. The supernatant contains the ER.

3) Transfer the supernatant to pre-cooled ultracentrifuge tubes and centrifuge at 105,000 x g for 60 minutes at 4° C. Discard the pellet.

4) Keeping cytosol ice-cold, combine the cytosol supernatants containing ER prepared that day.

5) Determine the 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. Be sure to report the calculations and results of the protein determination in the final report to EPA. Protein concentration will vary for each batch of cytosol prepared. However, when preparing the cytosol as recommended in this section, use of 20 rats (approximately 3.8 - 4.8 grams uterine tissue) typically yields 60 - 90 mg total cytosolic protein (13 to 28 mg protein in cytosol per gram of tissue).

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).*

6) Aliquot protein cytosol (1 to 2 ml aliquots) either for immediate use in ER binding assay or for storage at -80° C.

Note: *The cytosol can be stored frozen at -80° C for 90 days prior to use in ER binding assay. Thaw each aliquot of cytosol on ice no more than 60 minutes before using in assay. Do not thaw and re-freeze the cytosol, and do not thaw at room temperature.*

Note: *Recombinant receptor is not acceptable in this protocol.*

8.0 Demonstrating acceptable performance in cytosol preparation and laboratory techniques

Prior to routinely conducting the ER competitive binding assays, the cytosol must be shown to be performing correctly in the laboratory in which it will be used. This can be accomplished in two steps as follows:

1) Conduct a saturation radioligand binding assay to demonstrate ER specificity and saturation. Nonlinear regression analysis of these data (e.g., BioSoft; McPherson, 1985; Motulsky, 1995) and the subsequent Scatchard plot should document ER binding affinity of the radioligand (K_d) and the number of receptors (B_{max}) for a particular batch of uterine cytosol.

2) Conduct a competitive binding assay using 17β-estradiol and norethynodrel, which have known affinities for the ER using the protocols below. Comparison of IC_{50} values (i.e., the concentration of a substance that inhibits [^{3}H]-17β-estradiol binding by 50%) from these assays with expected values will assist in documenting that the laboratory is performing the assay correctly.

Each assay (saturation and competitive binding) consists of three runs, and each run contains three replicates.

Considerations for evaluating saturation binding assays are given in Section 9.6.2. Criteria for acceptable performance of known standards in the competitive binding assay are discussed in Section 10.7.3. Before running unknown test chemicals, a lab must meet the performance criteria for each of the standards (17β-estradiol and norethynodrel) in order to indicate that a technician is capable of performing the assay correctly and consistently.

At least one successful Saturation Binding Assay must be performed each time a new batch of cytosol is used in Competitive Binding Assays.

9.0 ER Saturation Binding Assay: Working Protocol

The Saturation Binding Assay measures total and non-specific binding of increasing concentrations of [^{3}H]-17β-estradiol under conditions of equilibrium. From these values, specific binding can be calculated. At each concentration within one run, EPA requires three concurrent replicates. EPA requires three non-concurrent runs.

9.1 Preliminary steps

9.1.1 Summary of preparations for the Saturation Binding Assay

The day before the binding assay

a) Prepare assay buffer (TEG stock solution).
b) Prepare calculations for dilution of radioisotope (i.e., calculations for dilutions in Tables 1 & 2); determine number of tubes needed.
c) Label and set up the tubes in racks for the radiolabeled 17β-estradiol and the unlabeled 17β-estradiol.
d) Prepare and wash a 60% HAP slurry solution in TEDG + PMSF buffer.

The morning of the binding assay

a) Prepare the [^{3}H]-17β-estradiol dilutions for saturation binding (Table 1).
b) Prepare the unlabeled 17β-estradiol dilutions (Table 2).
c) Prepare the dilution of the uterine cytosol.

Following completion of the binding assay

a) Record raw data output from scintillation counter into spreadsheet.
b) Analyze data to determine if the runs are acceptable.
c) If performance criteria are not met, determine potential areas for error and repeat experiment.

Summary table of assay conditions

		Saturation Binding Assay Protocol
Source of receptor		Rat uterine cytosol
Concentration of radioligand (as serial dilutions)		0.03 – 3 nM
Concentration of inert ligand (100 x [radioligand])		3 – 300 nM
Concentration of receptor		50 µg protein/tube*
Temperature		4º C
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10 %
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM

*Protein concentration may need to be adjusted. See section 9.1.5.

9.1.2 Preparation of assay buffer

Prepare TEG stock solution, adjust to pH 7.4 and store at 4° C for up to 3 months. Immediately before using in assay, add DTT and PMSF. See section 6.2 and Appendix A: Buffer preparation worksheet.

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

Prepare on the day of the assay.
Store [^{3}H]-17β-estradiol at -20° C in the original container.

Before preparing the serial dilutions of the [^{3}H]-17β-estradiol for the saturation binding assay, the SA (specific activity) should be adjusted for decay over time. To calculate the specific activity on the day of the assay, use the following equation:

$$SA_{adjusted} \text{ (Fraction isotope remaining)} = SA * e^{-K_{decay} * Time}$$

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, and is equal to 1.54×10^{-4} /day
- Time = days since the date on the stock bottle from the manufacturer.

Alternatively, these calculations can be made on the "QuickCalcs" webpage from GraphPad: http://www.graphpad.com/quickcalcs/radcalcform.cfm.

[^{3}H]-17β-Estradiol is usually shipped from vendor in ethanol. Prepare dilutions of the [^{3}H]-17β-estradiol in TEDG + PMSF buffer to achieve the concentrations noted in column E of Table 1. Siliconized or silanized glass tubes should be used when preparing serial dilutions.

To calculate the amount of stock [^{3}H]-17β-estradiol to add to buffer to make the stock dilutions (Column E) necessary for the final concentration in Column F:

1) Convert the adjusted specific activity from Ci/mmole to nM. The manufacturer usually packages a specific concentration of Ci/ml and will give this information on the package (for example, often 1.0 mCi/ml in ethanol). If $SA_{adjusted}$ = X Ci/mmole, and Y = concentration of radiolabel, then X Ci/mmole is converted to nM 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

2) Prepare a primary stock in TEDG + PMSF buffer. For example, since the highest concentration in Column E is 30 nM, a stock concentration that is 300 nM would be appropriate.

In this example, one ml was chosen as the amount of stock solution to prepare. A different volume could have been chosen.

How many µl of radioligand at Y/X *10^6 nM stock concentration will equal 300 nM in 1 ml? Use the equation

Z µl ((Y/X) * 10^6 nM) = 1000 µl (300 nM).

Therefore, Z µl = 1000 µl (300 nM) / ((Y/X) * 10^6 nM)

For example, if Y=1.0 mCi/ml and the adjusted specific activity is X=140 Ci/mmole, then Z=42 µl [^{3}H]-17β-estradiol plus sufficient TEDG + PMSF buffer to bring to 1 ml will yield 300 nM [^{3}H]-17β-estradiol.

(Dilution calculations can be double-checked on the "QuickCalcs" webpage from GraphPad: http://www.graphpad.com/quickcalcs/ChemMenu.cfm)

3) Dilutions can be made according to the table below (use TEDG buffer + PMSF for dilutions) by adding the stock (300 nM) or previous dilutions (H8 – H2) at a volume listed in Column B to a volume of buffer listed in Column C to equal the final volume in Column D at a diluted [^{3}H]-17β-estradiol concentration listed in Column E. All dilutions are to be kept at 4° C on ice. Dilutions in Table 1 include enough volume for one run (all three curves: total [^{3}H]-17β-estradiol binding, non-specific [^{3}H]-17β-estradiol binding, and hot tubes), with three replicates at each concentration.

4) The final solutions made for Column E can then be used, adding 50 µl to the respective assay tubes (in a final volume of 500 µl) to obtain the final assay concentrations in Column F.

Note: Table 1, like all the dilution schemes in this protocol, is an example of how dilutions may be made and is provided only for your convenience. Other dilution schemes may be used as long as the final concentrations in the ER assay tubes (shown in Column F) are the ones specified in the protocol, ***and*** *the solvent concentration in the final mix does not exceed the limit specified in Section 10.2.1. The dilution scheme must be documented in the final report.*

Table 1. Example of dilution procedure for radiolabeled 17β-estradiol

Column A	*Column B*		*Column C*		*Column D*		*Column E*	*Column F*
Tube #	**Volume of stock to add for diluted concentration**	**+**	**Volume of buffer to add**	**=**	**Total volume of diluted [^{3}H]-17β-estradiol**	**at**	**Diluted [^{3}H]-17β-estradiol concentration**	**Final [^{3}H]-17β-estradiol concentration (nM) in ER assay tube***
H8	Use 200 µl of stock [^{3}H]-17β-estradiol (300 nM)	+	1800 µl	=	2.0 ml	at	30 nM	3 nM
H7	Use 600 µl of dilution H8 (30nM)	+	1200 µl	=	1.8 ml	at	10 nM	1 nM
H6	Use 1200 µl of dilution H7 (10 nM)	+	800 µl	=	2.0 ml	at	6.0 nM	0.6 nM
H5	Use 1000 µl of dilution H6 (6 nM)	+	1000 µl	=	2.0 ml	at	3.0 nM	0.3 nM
H4	Use 600 µl of dilution H5 (3 nM)	+	1200 µl	=	1.8 ml	at	1.0 nM	0.1 nM
H3	Use 1200 µl of dilution H4 (1 nM)	+	300 µl	=	1.5 ml	at	0.8 nM	0.08 nM
H2	Use 750 µl of dilution H3 (0.8nM)	+	250 µl	=	1 ml	at	0.6 nM	0.06 nM
H1	Use 500 µl of dilution H2 (0.6nM)	+	500 µl	=	1 ml	at	0.3 nM	0.03 nM

** When 50 µl of each standard (Column E) is added to the ER assay tube, the final concentration will be as indicated (Column F) when the total volume in the ER assay tube is 500 µl.*

9.1.4 Preparation of 17β-estradiol for non-specific binding tubes

Use amber glass vials or equivalent when preparing stock and series dilutions.

- Make a stock solution (300 µM): weigh out 4.085 mg of 17β-estradiol (M.W. 272.4) in a 100 ml volumetric cylinder. Dissolve and bring final volume to 50 ml with absolute ethanol, final concentration = 0.0817 mg/ml (300 µM). Mix well.

- Make a working solution by pipetting 0.1 ml of the 300 µM stock and mix with 0.9 ml absolute ethanol in an appropriate glass vial, final concentration = 0.00817 mg/ml (30 µM).

- Make serial dilutions: A series of unlabeled 17β-estradiol concentrations should be prepared in buffer to achieve the final concentrations shown in Table 2. The final concentration of unlabeled 17β-estradiol in the individual NSB assay tubes should be 100 × the concentration of the radiolabeled [^{3}H]-17β-estradiol concentration in the corresponding H tubes noted in Table 1. Dilution volumes in Table 2 are made for the non-specific binding saturation curve with three replicates per dose.

Table 2. Example of dilution procedure for unlabeled 17β-estradiol

olumn A	*Column B*		*Column C*		*Column D*		*Column E*	*Column F*
Tube #	**Volume of stock to add for diluted concentration**	**+**	**Volume of buffer to add**	**=**	**Total volume of diluted 17β-estradiol**	at	**Diluted 17β-estradiol concentration**	**Final 17β-estradiol concentration (nM) in ER assay tube***
HC8	Use 100 µl of working solution unlabeled 17β-estradiol (30 µM)	+	900 µl	=	1 ml	at	3.0 µM	300 nM
HC7	Use 300 µl of dilution HC8 (3.0 µM)	+	600 µl	=	900 µl	at	1.0 µM	100 nM
HC6	Use 600 µl of dilution HC7 (1.0 µM)	+	400 µl	=	1 ml	at	0.6 µM	60 nM
HC5	Use 500 µl of dilution HC6 (0.6 µM)	+	500 µl	=	1 ml	at	0.3 µM	30 nM
HC4	Use 600 µl of dilution HC5 (0.3 µM)	+	1200 µl	=	1800 µl	at	0.1 µM	10 nM
HC3	Use 800 µl of dilution HC4 (0.1 µM)	+	200 µl	=	1 ml	at	0.08 µM	8 nM
HC2	Use 750 µl of dilution HC3 (0.08 µM)	+	250 µl	=	1 ml	at	0.06 µM	6 nM
HC1	Use 500 µl of dilution HC2 (0.06 µM)	+	500 µl	=	1 ml	at	0.03 µM	3 nM

**When 50 µl of each standard (Column E) is added to the ER assay tube, the final concentration will be as indicated (Column F) when the total volume in the ER assay tube is 500 µl.*

9.1.5 Standardization of receptor concentration

Having too much receptor in the assay tube can lead to gross violation of the assumption, necessary for simple analysis of the data, that the concentration of free radioligand remains essentially unchanged when some of the radioligand binds to the receptor. Having too little receptor in the assay tube, on the other hand, can result in so little radioligand bound that measurement of the signal (i.e., decays per minute) becomes unreliable. Also, too little protein in the tube can lead to disintegration of the centrifuged pellet and consequent loss of bound radioligand when the assay tube is decanted. Since the receptor concentration (per µg of cytosolic protein) varies between different batches of rat uterine cytosol, it is not possible to specify a standard cytosolic protein concentration that will be appropriate to use in all cases. Instead it is typical to determine, for each batch of cytosolic protein, the amount of protein that should be added in order to obtain the optimal level of receptors in the assay tube. For the saturation assay, the optimal protein concentration binds 25 -35% of the total radiolabeled estradiol that has been added to the tube. To ensure that this percent range of radioligand is bound at the lowest concentration of radioligand added to the assay, the 0.03 nM concentration shall be used to make this determination for the saturation binding assay.

To determine the optimal protein concentration, test serial amounts of protein per tube, using 0.03 nM radiolabeled estradiol in a final volume of 0.5 ml. The concentration of protein that binds 25-35% of the total radioactivity added is appropriate for use in the saturation assay. 50 +/- 10 µg protein/assay tube is generally expected to provide total binding in the appropriate range, although it may be prudent to test a wider range. Note: Use of less than 35 µg protein per assay tube is usually not advisable since it can result in the loss of pellets during the separation of bound estradiol from free estradiol (see Section 9.4).

Once the appropriate concentration of cytosolic protein has been determined, dilute the cold (but thawed, if previously frozen) cytosol with cold (4º C) TEDG + PMSF assay buffer so that the concentration of protein is reduced from the concentration determined in Section 7.2 to the stock concentration chosen above. Be sure to keep the cytosol at 4º C at all times (even while thawing) to minimize degradation of the receptor. Discard any unused cytosol; do not refreeze it.

9.2 Preparation of ER Saturation Binding Assay tubes

- Label 12 x 75 mm round-bottom siliconized or silanized assay tubes (glass) in triplicate. An example of a saturation assay tube layout worksheet is provided in Appendix B: Data entry and analysis worksheets.

Note: *Tubes 1-24 receive assay buffer, serial dilutions of [^{3}H]-17β-estradiol and protein cytosol; tubes 25-48 receive serial dilutions of [^{3}H]-17β-estradiol, inert 17β-estradiol and protein cytosol; and tubes 49-72 (identified as hot) represent serial dilutions of the [^{3}H]-17β-estradiol that should be delivered directly into scintillation vials. The volume of each component added to tubes is indicated in Table 3, below, and in more detail in the saturation assay tube layout in Appendix B.*

Note: *Make sure that the tubes and contents are at 4° C prior to the addition of the uterine cytosol, to prevent degradation of the estrogen receptor.*

Table 3. Saturation Binding Assay additions

Tubes 1-24	*Tubes 25-48*	*Tubes 49-72*	*Constituent*
TB	NSB	Hot	
350 µl	300 µl	--	TEDG + PMSF assay buffer
50 µl	50 µl	50 µl	[^{3}H]-17β-estradiol (as serial dilutions)
--	50 µl	--	Inert 17β-estradiol (as serial dilutions, 100x the labeled)
100 µl	100 µl	--	Uterine cytosol (diluted to the appropriate concentration)
500 µl	500 µl	50 µl	Total volume in each assay tube

TB = Total binding ([^{3}H]-17β-estradiol bound to receptors)
NSB = Non-specific binding ([^{3}H]-17β-estradiol and 100-fold-greater cold 17β-estradiol bound to receptors)
Hot = [^{3}H]-17β-estradiol alone in the tubes for dpm determination at each concentration

- Vortex assay tubes quickly but completely after additions are completed. Be sure that tubes do not warm above 4º C during vortexing.

Note: *Make sure that all components are concentrated at the bottom of tube. If any of the liquid remains on the side of the tube, centrifuge assay tubes for 1 minute at 600 x g (4º C) to concentrate fluid at bottom of tube.*

- Incubate assay tubes at 4º C for 16 to 20 hours. Assay tubes should be placed on a rotator during the incubation period.

9.3 Preparation of 60% HAP slurry

- Prepare HAP slurry one day before use. Prepare an adequate amount of HAP slurry for the number of tubes in the next day's run. The amounts of HAP given below (powder or hydrated product) will generally yield enough slurry for 70-100 assay tubes, so this amount should be adequate for a typical saturation binding assay run with estradiol (72 tubes, see Appendix B). Prepare the dry powder HAP by adding 10 g HAP powder to 100 ml TEDG + PMSF buffer and gently mixing. If using the hydrated HAP product, mix gently to re-suspend the HAP and add ~25 ml of slurry to a 100 ml graduated cylinder for the washing process.

- Add additional TEDG + PMSF buffer to a final volume of 100 ml (if hydrated HAP), cap the container, and refrigerate (4º C) for at least 2 hours.

- Aspirate or decant the supernatant and re-suspend the HAP in fresh TEDG + PMSF buffer (4º C) to 100 ml. Mix gently. Again, allow the HAP to settle for ~2 hours (4º C) and repeat the wash step to ensure that the TEDG + PMSF buffer saturates the HAP.

- After the last wash, let the HAP slurry settle overnight (at least 8 to 10 hours at 4º C).

- The next day (i.e., the day on which the HAP slurry will be used in the assay), note the volume of HAP on the graduated cylinder, aspirate or decant the supernatant, and re-suspend the HAP to a final volume of 60% HAP and 40% cold TEDG + PMSF buffer (based on the overnight settled HAP volume). For example, if the HAP settles to the 50 ml mark on the graduated cylinder, add 33.3 ml TEDG + PMSF. The HAP slurry should be well-suspended and ice-cold when used in the separation procedure, and it should be maintained as a well-suspended slurry during aliquoting.

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

Note: *To minimize dissociation of bound [^{3}H]-17β-estradiol from the ER during this process, it is extremely important that the buffers and assay tubes be kept ice-cold and that each step be conducted quickly. A multi-tube vortexer is necessary to process tubes efficiently and quickly.*

- Remove ER assay tubes from rotator and place in an ice-water bath. Using a repeating pipette, quickly add 250 microliters of ice cold HAP slurry (60% in TEDG + PMSF buffer, well mixed prior to using) to each assay tube.

- Vortex the tubes for ~10 seconds at 5-minute intervals for a total of 15 minutes with tubes remaining in the ice-water bath between vortexing.

Note: *This is best accomplished by vortexing an entire rack of tubes at once using a multi-tube vortexer. It is important to continue to keep the assay tubes cold.*

- Following the vortexing step, add 2.0 ml of the cold (4º C) TEDG + PMSF buffer, quickly vortex, and centrifuge at 4º C for 10 minutes at 1000 x g.

- After centrifugation, immediately decant and discard the supernatant containing the free [^{3}H]-17β-estradiol. The HAP pellet will contain the estrogen-receptor-bound [^{3}H]-17β-estradiol.

Note: *This step can be accomplished quickly by placing the assay tubes in a decanting tube rack. All tubes in the rack can be decanted at once. With the tubes still inverted, blot against clean absorbent pad (paper towel). Watch carefully to prevent any of the HAP pellets from running down the side of the assay tube, which may occur if the protein concentration in the cytosol is quite low. Immediately place the tubes back in the ice bath.*

- Add 2.0 ml ice-cold TEDG + PMSF buffer to each assay tube and vortex (~10 sec) to resuspend the pellet. Work quickly and keep the assay tubes cold. Centrifuge again at 4º C for 10 minutes at 1000 x g.

- Quickly decant and discard the supernatant and blot tubes as above. Again, make sure no pellet runs down the side of the tube. Repeat the wash and centrifugation steps once more. This will be the third and final wash.

- After the final wash, decant the supernatant. Allow the assay tubes to drain briefly for ~0.5 minute.

- At this point, the separation of the free [^{3}H]-17β-estradiol and ER-bound [^{3}H]-17β-estradiol has been completed. The assay tubes may be left at room temperature.

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

- Add 1.5 ml of absolute ethanol to each assay tube. Allow the tubes to sit at room temperature for 15 to 20 minutes, vortexing for ~10 seconds at 5-minute intervals. Centrifuge the assay tubes for 10 minutes at 1000 x g.

- Pipet a 1.0 ml aliquot into 20 ml scintillation vials containing 14 ml scintillation cocktail, being careful not to disturb the centrifuged pellet. Cap and shake vial.

- Place vials in scintillation counter for determination of Disintegrations Per Minute (DPMs)/vial with quench correction.

Note: *Since a 1.0 ml aliquot is used for scintillation counting, the DPMs should be adjusted to account for the total radioactivity in 1.5 ml (i.e., DPMs/1 ml x 1.5 ml total = Total DPMs bound in experiment).*

9.6 Data analysis

9.6.1 Terminology

9.6.1.1 Total [^{3}H]-17β-estradiol

Radioactivity in DPMs added to each assay tube. (DPMs in the defined volume of the tube can be converted to concentration of [^{3}H]-17β-estradiol.)

9.6.1.2 Total binding

Radioactivity in DPMs in the tubes that have only [^{3}H]-17β-estradiol available to bind to the receptor. There is one total-binding tube per concentration of [^{3}H]-17β-estradiol (per replicate).

9.6.1.3 Non-specific binding (NSB)

Radioactivity in DPMs in the tubes that contain 100-fold excess of unlabeled over labeled 17β-estradiol. There is one NSB tube per concentration of [^{3}H]-17β-estradiol (per replicate).

9.6.1.4 Specific binding

Total binding minus non-specific binding.

9.6.1.5 K_d

Affinity of the radioligand ([^{3}H]-17β-estradiol) for the estrogen receptor. Unit is nM.

9.6.1.6 B_{max}

Maximum number of receptors bound. Unit is fmol ER/100 μg cytosol protein.

9.6.2 General considerations

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 should reach a plateau for maximum specific binding indicative of saturation of the ER with the radioligand. In addition, analysis of the data should document 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).

The amount of cytosolic protein to add to each tube depends on estrogen receptor concentration, and is determined for each batch of cytosol. As stated in section 9.1.5, the concentration of protein that binds 25-35% of the total radioactivity added is appropriate for use in the saturation assay. It is generally in the range 35 to 100 µg protein in a total assay volume of 0.5 ml. The concentration for [^{3}H]-17β-estradiol should range from 0.03 to 3.0 nM in a total assay volume of 0.5 ml. Non-specific binding should be determined by adding unlabeled 17β-estradiol at 100x the concentration of radiolabeled 17β-estradiol.

Analysis of these data should use non-linear regression analysis (e.g., BioSoft; McPherson, 1985; Motulsky, 1995) using both the total binding and non-specific binding data points (not the calculated specific binding values), automatic outlier elimination using the method of Motulsky and Brown (2006) (implemented in Prism 5 software as the ROUT procedure) with a Q value of 1, and correction for ligand depletion via the method of Swillens (1995), being sure to enter the appropriate incubation volume (default units are ml) and specific activity (default units are dpm/fmol when data are entered as dpm) as constraints. Display the data points on a Scatchard plot but instead of plotting the straight line that best fits the data points, display the straight line that describes the B_{max} and K_d determined by the Swillens analysis (i.e., the line with X-intercept at B_{max} and slope of $-1/K_d$). Rat uterine cytosol prepared using this protocol will typically yield a K_d of 0.03 to 1.5 nM and B_{max} of 10 to 150 fmol ER/100 microgram protein.

An example of a saturation assay worksheet using increasing concentrations of radioligand is provided in Appendix B: Data entry and analysis worksheets. The worksheet shows how the tubes could be ordered for analysis, and provides space for data entry. The analysis provided in the example is specific to the test conditions noted in the worksheet; the user is responsible for modifying the worksheet appropriately if, for example, a different concentration of protein is used, different concentrations of radioligand are tested, etc. An example of a run entered into and analyzed by GraphPad Prism software for curve-fitting is also provided in the file "example saturation data.pzf".

Other general considerations, which are being provided as guidance rather than as specific performance criteria, include the following:

- Did the data produce a linear Scatchard plot? Non-linear plots generally indicate a problem with the assay such as ligand depletion [concave plot] or incorrect assessment of non-specific binding [convex plot].

- Are the runs consistent? That is, are the standard errors of the mean for the K_d or B_{max} excessive?

- Is non-specific binding excessive? In general, the value for non-specific binding should be less than 50% of the total binding at the highest concentration.

If there are significant deviations from any of these points, it would be appropriate to repeat the saturation experiment with appropriate adjustments.

9.7 Test report

The test report must include, but is not limited to, the following information:

9.7.1 Radioactive ligand ([^{3}H]-17β-estradiol)

- Name, including number and position of tritium atom(s)
- Supplier, catalogue number, and batch number
- Specific Activity (SA) and date for which that SA was certified by supplier
- Concentration as received from supplier (Ci/mmol)
- Concentrations tested (nM). If dilutions were prepared using a scheme different from Table 1, provide an analogous table showing how dilutions were made.

9.7.2 Radioinert ligand (17β-estradiol)

- Supplier, batch number, catalog number, CAS number, and purity
- Concentrations added to NSB tubes (nM). If dilutions were prepared using a scheme different from Table 2, provide an analogous table showing how dilutions were made.

9.7.3 Estrogen receptor

- Source of rat uterine cytosol. If from a commercial source, the supplier must be identified. Include strain and age (at necropsy) of rats from which uteri were taken, and number of days between ovariectomy and removal of the uterus.
- Isolation procedure.
- Protein concentration of ER preparation. Provide details of the protein determination method, including the manufacturer of the protein assay kit, data from the calibration curves, and data from the protein determination assay.
- Method and conditions of transport and storage of ER, if applicable.

9.7.4 Test conditions

- Protein concentration used.
- Total volume per assay tube during incubation with receptor.

- Incubation time and temperature.
- Notes on any abnormalities during separation of free radiolabeled estrogen.
- Notes on any problems in analysis of bound radiolabeled estrogen.
- Statistical methods used when estimating K_d and B_{max}.

9.7.5 Results

For each run, provide at least the following. Be sure to include a run identifier on each product. When preparing graphs, use the same axis length and range on all comparable graphs to facilitate comparisons across runs.

- Date of run, number of days since SA certification date, and adjusted SA on day of run.
- A graph of total, specific, and non-specific binding across the range of concentrations tested. Plot each data point (one per replicate) as well as the fitted curves for total, specific, and non-specific binding. (Since specific binding is not plotted automatically when the analysis using only the total binding and non-specific binding data is used, you may need to analyze the specific binding data separately. This analysis is only to guide the eye, so it is not critical whether robust regression with outlier removal is used. However, be sure not to use the K_d or B_{max} values from this analysis.)
- A graph of measured concentrations in the total [^{3}H]-17β-estradiol tubes (see Appendix B).
- Scatchard plot, using nM for the units of the specific bound (X) axis. Show each data point. Instead of showing the best-fit line through the data points, plot the line based on the Swillens correction for ligand depletion (i.e., the line based on the B_{max} and K_d estimated from the data when the Swillens correction is used). Include the values for K_d (in nM) and B_{max} (in both nM and fmol/100 μg protein), estimated when ligand depletion is accounted for, on this graph.
- Raw data (decays per minute) for each tube (see Appendix B).

In addition, provide the following information summarizing the information from all of the runs on one batch of cytosol, for each batch of cytosol:

- A graph showing total, non-specific, and specific binding for all runs done on that batch. Be sure to differentiate runs by color and date. Do not plot data points or any other indicator of variability.
- A table of K_ds and B_{max}s for all runs on that batch.

9.7.6 Conclusion

Give the estimated K_d and standard error of the mean of the radioligand ([^{3}H]-17β-estradiol) and the estimated B_{max} and standard error of the mean for each batch of cytosol prepared and briefly note any reasons why confidence in these numbers should be high or low.

10.0 ER Competitive Binding Assay: Working Protocol

The Competitive Binding Assay measures the binding of a single concentration of [^{3}H]-17β-estradiol in the presence of increasing concentrations of a test substance. At each concentration within one run, EPA requires three concurrent replicates. EPA requires three non-concurrent runs for each chemical tested.

10.1 Preliminary steps

10.1.1 Summary of preparations for the Competitive Binding Assay

The day before the binding assay

a) Prepare assay buffer (TEG stock solution).
b) Perform calculations for radioisotope decay and dilution.
c) Perform calculations for cytosolic protein dilution.
d) Perform calculations for estradiol dilutions, norethynodrel dilutions and test chemical dilutions (Table 4, Table 5, and Table 6).
e) Perform calculations for number of tubes in the run (Section 10.3.1).
f) Label and set up tubes for standard curve dilutions (see Table 4, Table 5).
g) Label and set up the tubes in racks for the test chemicals (Table 6).
h) Prepare and wash a 60% HAP slurry solution in TEDG + PMSF buffer.
i) Optional: Test the solubility of the test chemical in the chosen solvent.

The morning of the binding assay

a) Prepare the [^{3}H]-17β-estradiol dilutions for competitive binding.
b) Prepare the negative and positive control dilutions.
c) Prepare the reference standard dilutions (Table 5).
d) Prepare the test chemical dilutions (Table 6).
e) Prepare all solutions that go into the test reaction (Table 7).

Following the binding assay

a) Record raw data output from scintillation counter into spreadsheet.
b) Determine if the run meets the performance criteria.

Note: All of the dilution schemes in this protocol are examples *of how dilutions may be made and are provided only for your convenience. Other dilution schemes may be used as long as the final concentrations in the assay tube are the ones specified in the protocol,* **and** *the solvent concentration in the final mix does not exceed the limit specified in Section 10.2.1. The dilution scheme must be documented in the final report.*

Summary table of assay conditions:

		Competitive Binding Assay Protocol
Source of receptor		Rat uterine cytosol
Concentration of radioligand		1.0 nM
Concentration of receptor		50 µg protein/tube*
Concentration of test substance (as serial dilutions)		100 pM to 1 mM**
Temperature		4º C
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10 %
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM

* Receptor concentration may need to be adjusted. See Section 10.1.5.
** Range and spacing of test substance concentrations may need to be adjusted depending on solubility

10.1.2 Preparation of assay buffer

Prepare TEG buffer without DTT and PMSF, adjust to pH 7.4 and store at 4° C for up to 3 months. Add DTT and PMSF immediately prior to use in assay. See Section 6.2 and Appendix A: Buffer preparation worksheet.

10.1.3 Optional: Solubility test

If the limit of solubility of the test chemical in the chosen solvent is not known, it may be advisable to prepare the highest concentration that will be tested to see if the chemical will precipitate out in cold assay buffer. Prepare a small quantity of the highest concentration of test chemical in the chosen solvent (see sections 10.2.1 and 10.2.3), then add 10 µl of this concentration to 490 µl *cold* (4º C) buffer. Vortex gently. *Note: Incubation for the length of the assay (16-20 hours), also at 4º C, is optional.* Examine the tube carefully by visual inspection for evidence of precipitation. Observation under a dissecting scope or monitoring absorbance (650 nm) with a spectrophotometer are useful approaches for detecting precipitation. If precipitation is noted, it may be appropriate to try a different dilution scheme (e.g., starting from a lower stock concentration yet still maintaining the same final concentration if possible) or a different solvent. If the chemical is not soluble at the highest concentration recommended in the protocol while keeping the solvent concentration below the maximum specified (see section 10.2.1), a lower concentration of test chemical should be prepared (e.g., ½ log lower) and tested.

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

Prepare on the day of the assay.

Note: *The Specific Activity should be adjusted for decay over time (see below).*

Dilute the [^{3}H]-17β-estradiol with TEDG + PMSF buffer so that each assay tube contains 1.0 nM final concentration of [^{3}H]-17β-estradiol. The following detailed

steps demonstrate how this is done:

1) Before preparing the dilution of the [^{3}H]-17β-estradiol for the competitive binding assay, the SA (specific activity) should be adjusted for decay over time. To calculate the specific activity on the day of the assay, use the following equation:

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

   where

   - SA is the specific activity on the packaging date (both are printed on the stock bottle from the manufacturer).
   - K_{decay} is the decay constant for tritium and is equal to 1.54×10^{-4} /day
   - Time = days since the date on the stock bottle from the manufacturer.

Alternatively, these calculations can be made on the "QuickCalcs" webpage from GraphPad: http://www.graphpad.com/quickcalcs/radcalcform.cfm.

2) [^{3}H]-17β-Estradiol is usually shipped from the vendor in ethanol. Prepare the stock dilution of the [^{3}H]-17β-estradiol in TEDG + PMSF buffer. To calculate the amount of stock [^{3}H]-17β-estradiol to add to the dilution (for a final concentration of 1 nM in 500 µl assay tube volume) use the following steps:

   a) Convert the adjusted specific activity from Ci/mmole to nM. The manufacturer usually packages a specific concentration of Ci/ml and will give this information on the package (for example, 1.0 mCi/ml in ethanol). If $SA_{adjusted}$ = X Ci/mmole, and Y = concentration of radiolabel, then X Ci/mmole is converted to nM 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

   b) Prepare a 50 nM diluted stock of the [^{3}H]-17β-estradiol so that 10 µl in a total volume of 500 µl per assay tube will give a final concentration of 1 nM. (A 50-fold dilution of a 50 nM diluted stock of [^{3}H]-17β-estradiol takes place if 10 µl is added to the assay tube total volume of 500 µl to give a final concentration of 1 nM.) For example, if the amount of 50 nM stock solution that is needed is 1.5 ml:

      i. if the radioligand concentration from the manufacturer is Y/X *10^6 nM (as calculated above), then how many µl of radioligand at this concentration will equal 50 nM diluted stock [^{3}H]-17β-estradiol in 1.5 ml TEDG + PMSF buffer? Use the equation

Z µl ((Y/X) * 10^6 nM) = 1500 µl (50 nM)

Therefore, Z µl = 1500 µl (50 nM) / ((Y/X) * 10^6 nM)

For example, adding Z=10.5 µl of purchased [^{3}H]-17β-estradiol which has a concentration of Y=1.0 mCi/ml and an adjusted specific activity of X=140 Ci/mmole and bringing the volume to 1.5 ml with TEDG + PMSF buffer will yield 1.5 ml of 50 nM [^{3}H]-17β-estradiol. (NOTE: these numbers are just an example. Actual numbers depend on the radioligand concentration provided by the manufacturer.)

The total volume of diluted [^{3}H]-17β-estradiol that is required for a run (1.5 ml in the example) depends on the number of chemicals being assayed in that run, the number of concentrations per chemical, etc. The amount you need may be different from the example.

(If there is any question about how to calculate the dilution, it can be done on the "QuickCalcs" webpage from GraphPad: http://www.graphpad.com/quickcalcs/ChemMenu.cfm .)

c) Keep the 50 nM [^{3}H]-17β-estradiol on ice until standards, test chemicals, and assay tubes are prepared.

10.1.5 Standardization of receptor concentration and assay volume

Before performing a competitive binding assay, the receptor concentration of the cytosol is normally adjusted to minimize the likelihood of ligand depletion. Ligand depletion occurs when a high percentage of the [^{3}H]-17β-estradiol is bound to ER causing the concentration of the unbound (free) [^{3}H]-17β-estradiol to differ significantly from the concentration of [^{3}H]-17β-estradiol that was originally added to the assay tube [Hulme and Birdsall, 1992]. For the competitive binding assay, the optimal amount of cytosolic protein added should contain enough receptor to bind no more than 10 - 15% of the radiolabeled estradiol that has been added to the tube. To determine the optimal protein concentration, determine the percent of radiolabeled estradiol bound in serial amounts of protein per tube, using 1.0 nM radiolabeled estradiol in a final volume of 0.5 ml. (Note that 1.0 nM is the final concentration of radiolabeled estradiol used in all of the competitive binding assay tubes, and is different from the concentration used for the protein determination for the saturation binding assay, section 9.1.5.) 50 +/- 10 µg protein/assay tube is generally expected to provide total binding in the appropriate range, although it may be prudent to test a wider range. **Note: Use of less than 35 µg protein per assay tube is usually not advisable since it can result in the loss of pellets during the separation of bound estradiol from free estradiol (see Section 10.5)** It would be appropriate to choose concentrations surrounding the concentration that was found to be acceptable in the saturation binding assay.

Dilute the cold (but thawed, if previously frozen) cytosol with cold (4º C) TEDG + PMSF assay buffer, so that the concentration of protein is reduced from the concentration determined in Section 7.2 to the stock concentration chosen above. Be sure to keep the cytosol at 4º C at all times (even while thawing) to minimize degradation of the receptor. Discard any unused cytosol; do not refreeze it.

10.2 Preparation of controls, reference standard, and test chemicals

10.2.1 Solvent, negative, and weak positive controls

When testing substances for their ability to bind to the ER, concurrent solvent (vehicle), negative, and weak positive controls should be included in each experiment (i.e., each run; a run may include several test chemicals). The solvent control indicates that the solvent does not interact with the test system. The negative control (octyltriethoxysilane) provides assurance that the assay as run does not report binding for chemicals that do not bind to the ER. A weak positive substance (norethynodrel) is included to demonstrate the sensitivity of each experiment and to allow an assessment of variability in the conduct of the assay across time.

Solvent control

Choose the best solvent for the test chemical from among dimethylsulfoxide (DMSO), ethanol, or water[1]. When using ethanol or DMSO, the solvent should be tested at the same concentration as is found in the final test chemical assay tubes. The maximum % of ethanol allowed in assay tubes is 3%. The maximum % of DMSO allowed in assay tubes is 10%. These limits are placed on solvent concentration because of known interference of higher solvent concentrations with the assay. All assay tubes should contain equal amounts of the solvent.

Note: If using ethanol, use screw-cap tubes or take other measures to minimize evaporation. This applies to all tubes, not just solvent control tubes.

Negative control

The final concentration range to test for the negative control is from 1 x 10^{-10} to 1 x 10^{-3} M, in log increments. This range and spacing is the same as the default range and spacing for test chemicals. The assay tubes for the negative control can therefore be prepared by following the guidance in Section 10.2.3, "Serial dilutions of test substance". The molecular weight of octyltriethoxysilane is 276.5 grams/mole, so to make 1 ml of a 100 mM stock solution, add 27.65 mg of octyltriethoxysilane to 900 µl of solvent (either ethanol or DMSO, depending on what was used for the test chemical) and bring the volume to 1 ml with the same solvent. Dilutions are prepared as in Table 6.

[1] The solvent used for a test chemical must also be used for the reference chemical (inert 17β-estradiol) and the control chemicals (norethynodrel and octyltriethoxysilane) unless the solvent is water. That is, if the test chemical is run in ethanol, the reference chemical and controls must be run in ethanol; if the test chemical is run in DMSO, the reference chemical and controls must be run in DMSO. If the test chemical is run in water, the controls should be run in ethanol.

Weak positive control

The final concentration range to test for the positive control is from 1 x $10^{-8.5}$ to 1 x 10^{-4} M, spaced as shown in Table 4 .

Example of preparation procedure for positive control curve

Note: *Use amber glass vials or equivalent when preparing stock and series dilutions.*

- Make a fresh stock solution (10 mM): Accurately weigh out 29.84 mg of norethynodrel (M.W. 298.4) into 9 ml of solvent in a volumetric flask. Add sufficient solvent to bring the final volume to 10 ml. Mix well to ensure that the norethynodrel is fully dissolved. The final concentration is 10 mM.

- Make serial dilutions: A series of positive control concentrations should be prepared in solvent to achieve the final concentrations shown in Table 4.

Note: All of the dilution schemes in this protocol are examples of how dilutions may be made and are provided only for your convenience. Other dilution schemes may be used as long as the final concentrations in the assay tube are the ones specified in the protocol, ***and*** *the solvent concentration in the final mix does not exceed the limit specified in Section 10.2.1. The dilution scheme must be documented in the final report.*

Table 4. Example of dilution procedure for the positive control (norethynodrel)

Column A	*Column B*		*Column C*		*Column D*		*Column E*	*Column F*
Tube #	***Volume of stock to add for diluted concentration***	+	***Volume of solvent to add***	=	***Total volume of diluted positive control***	at	***Diluted positive control concentration (Molar)***	****Final positive control concentration in ER assay tube (Molar)***
P1	Use 400 µl of stock positive control (10 mM)	+	400 µl	=	800 µl	at	$5x10^{-3}$ (5 mM)	$1x10^{-4}$
P2	Use 150 µl of stock positive control (10 mM)	+	800 µl	=	950 µl	at	$1.58x10^{-3}$ (1.58 mM)	$3.16x10^{-5}$ ($=1x10^{-4\,5}$)
P3	Use 100 µl of dilution P2 (1.58 mM)	+	900 µl	=	1 ml	at	$1.58x10^{-4}$ (158 µM)	$3.16x10^{-6}$ ($=1x10^{-5\,5}$)
Intermed	Use 100 µl of dilution **P1** (5 mM)	+	900 µl	=	1 ml	at	$5x10^{-4}$ (500 µM)	(not used)
P4	Use 100 µl of Intermed (500 µM)	+	900 µl	=	1 ml	at	$5x10^{-5}$ (50 µM)	$1x10^{-6}$
P5	Use 100 µl of dilution **P3** (158 µM)	+	900 µl	=	1 ml	at	$1.58x10^{-5}$ (15.8 µM)	$3.16x10^{-7}$ ($=1x10^{-6\,5}$)
P6	Use 100 µl of dilution **P4** (50 µM)	+	900 µl	=	1 ml	at	$5x10^{-6}$ (5 µM)	$1x10^{-7}$
P7	Use 100 µl of dilution **P5** (15.8 µM)	+	900 µl	=	1 ml	at	$1.58x10^{-6}$ (1.58 µM)	$3.16x10^{-8}$ ($=1x10^{-7\,5}$)
P8	Use 100 µl of dilution P7 (1.58 µM)	+	900 µl	=	1 ml	at	$1.58x10^{-7}$ (158 nM)	$3.16x10^{-9}$ ($=1x10^{-8\,5}$)

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

10.2.2 Reference standard (17β-estradiol)

The reference standard (17β-estradiol) is 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. A standard curve using unlabeled 17β-estradiol should be prepared for each ER competitive binding assay. Final concentrations of unlabeled 17β-estradiol in the assay tubes should range from 1.0×10^{-7} to 1.0×10^{-11} M, spaced as shown in Table 5. Prepare serial dilutions of 17β-estradiol in the appropriate solvent (either ethanol or DMSO, depending on the solvent used for the test chemical) to achieve the final concentrations shown below. Use appropriate screw-cap light-sensitive containers for storage containers.

The tubes with the highest concentration of unlabeled 17β-estradiol (100 nM) have 100 x the concentration of [^{3}H]-17β-estradiol (1 nM) and therefore provide data on the level of non-specific binding of the radiolabeled estradiol. If the run includes more than one test chemical (and thus a relatively large number of assay tubes), it may be useful to include a set of non-specific binding tubes (i.e., three replicates) at the end of the run to check for drift from beginning to end of the run. Replicates at the end for solvent control, weak positive, and negative control might also be appropriate if NSB tubes are added at the end.

Example of preparation procedure for unlabeled 17β-estradiol standard curve

Note: *Use amber glass vials or equivalent when preparing stock and series dilutions.*

- Make a fresh stock solution (50 μM): Accurately weigh out 1.36 mg of 17β-estradiol (M.W. 272.4) in 9 ml of solvent in a volumetric flask. Add sufficient solvent to bring the final volume to 10 ml. The concentration is 0.136 mg/ml (500 μM). Mix well. Make a secondary stock by pipetting 1 ml of stock and mix with 9 ml solvent in an appropriate glass vial, final concentration = 0.0136 mg/ml (50 μM).

- Make serial dilutions: A series of unlabeled 17β-estradiol concentrations should be prepared in solvent to achieve the final concentrations shown in column E of Table 5. These concentrations will be further diluted in the assay tubes to yield the final concentrations shown in Column F.

Table 5. Example of dilution procedure for standard 17β-estradiol curve

Column A	*Column B*		*Column C*		*Column D*		*Column E*	*Column F*
Tube #	***Volume of stock to add for diluted concentration***	+	***Volume of solvent to add***	=	***Total volume of diluted standard 17β-estradiol***	at	***Diluted 17β-estradiol concentration (Molar)***	***Final 17β-estradiol concentration (Molar) in ER assay tube****
NSB1**	Use 100 µl of stock 17β-estradiol (50 µM)	+	900 µl	=	1 ml	at	5×10^{-6} (5 µM)	1×10^{-7}
S2	Use 100 µl of dilution NSB1 (5 µM)	+	900 µl	=	1 ml	at	5×10^{-7} (500 nM)	1×10^{-8}
S3	Use 277 µl of dilution S2 (500 nM)	+	600 µl	=	877 µl	at	1.58×10^{-7} (158 nM)	3.16×10^{-9} ($=1 \times 10^{-8.5}$)
S4	Use 100 µl of dilution **S2** (500 nM) (not S3!)	+	900 µl	=	1 ml	at	5×10^{-8} (50 nM)	1×10^{-9}
S5	Use 100 µl of dilution **S3** (158 nM) (not S4!)	+	900 µl	=	1 ml	at	1.58×10^{-8} (15.8 nM)	3.16×10^{-10} ($=1 \times 10^{-9.5}$)
S6	Use 100 µl of dilution **S4** (50 nM) (not S5!)	+	900 µl	=	1 ml	at	5×10^{-9} (5 nM)	1×10^{-10}
S7	Use 100 µl of dilution S6 (5 nM)	+	900 µl	=	1 ml	at	5×10^{-10} (500 pM)	1×10^{-11}

* Final concentration of test chemical in assay tube when 10 µl of "Column E" concentration is used in a total volume of 500 µl.
** Note that the first dilution yields a final concentration in the assay tube (100 nM) that is 100x the concentration of radiolabeled estradiol (1 nM). It thus provides both the first data point for the standard curve and the value for non-specific binding (NSB).

Note: All of the dilution schemes in this protocol are examples of how dilutions may be made and are provided only for your convenience. Other dilution schemes may be used as long as the final concentrations in the assay tube are the ones specified in the protocol, ***and*** *the solvent concentration in the final mix does not exceed the limit specified in Section 10.2.1 The dilution scheme must be documented in the final report.*

10.2.3 Serial dilutions of test substance

Each dilution is prepared in solvent to yield the final concentrations as indicated below. Examine the tube carefully by visual inspection for evidence of precipitation. Observation under a dissecting scope or monitoring absorbance (650 nm) with a spectrophotometer are useful approaches for detecting precipitation. It may be necessary to warm the stock solution of the test substance for 10 – 15 minutes in a 36° C water bath before making the dilutions. Make sure that the test substance is amenable to warmth and light (i.e., it does not degrade under these conditions) before preparing the stock solution and serial dilutions. In addition, it is important that solutions warmed to 36° C be closely watched when added to the assay tube as the temperature change to 4° C may induce the test substance to precipitate. It is important to cool the tubes to 4° C, however. Do not attempt to ensure solubility by keeping at a warmer temperature as that may adversely affect the ER.

Note: *For the purpose of screening, EPA will accept an upper limit of 1 mM and a range of test concentrations from 1 mM to 100 pM (i.e., 10^{-3} to 10^{-10} M inclusive), in ten-fold (i.e., log) increments as the default range of concentrations to test initially. If the highest concentration cannot be prepared in any of the allowable solvents (e.g., because there is precipitate in the stock solution, and adding more solvent would cause the solvent concentration in the final tube to be greater than the acceptable limit), that concentration may be omitted as long as the justification is included in the report. Other concentrations in the series should remain unchanged (viz., log-spaced on the powers of ten). If the highest concentration is omitted, an additional concentration may optionally be added at the low end of the concentration series. As few concentrations as possible should be omitted from the high-concentration end of the series in order to obtain a fully-solubilized stock solution. Evidence must be provided in the report showing measures taken at each highest-concentration-attempted to obtain full solubility, such as heating or using a different solvent.*

It is possible that a test compound will be dissolved in the stock solution but will precipitate in the final assay tube in the presence of the other reagents. Each assay tube should therefore be inspected carefully. If precipitate is noted, continue with the assay but be sure to exclude the data point from curve-fitting, and note the reason for exclusion of the data.

Finally, it is possible that the test compound is not fully soluble in the final assay tube but that the precipitate was not detected by visual inspection. For compounds which interact with the receptor, this might result in a U-shaped binding curve. Guidance on how to recognize and deal with U-shaped curves is given in Section 10.7.2.

If the default range of concentrations is insufficient to define the "top" of the curve (which may be the case if the chemical is a strong binder, for example), the concentrations tested must be shifted (or extended) to lower concentrations in order to obtain a full curve.

If there is information from other sources suggesting where the log(IC_{50}) might lie, it may be appropriate to space the dilutions more closely (but regularly) around the expected log(IC_{50}) concentration rather than to test concentrations which are known to be extreme. That is, selection of concentrations to test may depart from the default stated above if appropriate justification is given and the information supporting the initial estimate of the log(IC_{50}) is included in the report. In any case, the results must show that enough points on either side of the log(IC_{50}) were included so that the full curve, including the "top" and "bottom", is adequately characterized.

Note: *The serial dilutions shown in Table 6 are based upon the addition of 10 microliters of each serial dilution of the test substance in a final assay volume of 500 microliters. Other ratios can be used as long as the concentration does not exceed 3% ethanol or 10% DMSO of the final assay volume, and all test tubes contain equal amounts of the solvent.*

1) Calculate the grams of chemical needed to make a concentrated stock solution from which it will be easy to make dilutions. In this example, it is assumed that there are no solubility problems and that the highest concentration to be tested is 1 mM. In this case, a 100 mM stock solution would be appropriate to make. Thus, if the molecular weight of the test chemical is X g/mole, then 1 M = X g/L, and 100 mM = X g/L / 10. The volume can be adjusted to make a smaller amount of test chemical.

2) Once a stock concentration of test chemical is made, follow the dilutions in Table 6 to make the serial dilutions of test chemical for the assay. (Table 6 is an example. Other dilution schemes may be used as long as the final concentrations of test chemical are as shown, and the solvent concentration does not exceed the limits specified above. If a different dilution scheme is used, include the scheme in the final report.)

3) See section 10.2.1 concerning choice of solvent. If the highest concentration is not soluble in the first solvent, another solvent may be used. Only if none of the three allowable solvents work may the highest concentration be reduced below the upper limit. Reminder: the concentration of solvent in the final assay volume (including the ethanol in the PMSF stock and the ethanol in the radiolabeled estradiol stock) may not exceed 3% ethanol or 10% DMSO, and all tubes must contain equal amounts of solvent.

4) Add 10 µl of test chemical dilutions (TC 1-8 tubes) to the respective assay tubes to obtain competitive binding curves as described in section 10.3.2.

Table 6. Example of dilution procedure for test chemical

Column A	*Column B*		*Column C*		*Column D*		*Column F*	*Column G*
Tube #	***Volume of stock to add for diluted concentration***	+	***Volume of solvent to add***	=	***Total volume of diluted test chemical***	at	***Diluted test chemical concentration (Molar)***	****Final test chemical concentration in ER assay tube (Molar)***
TC1	Use 500 µl of stock test chemical (e.g., 100 mM^)	+	500 µl	=	1 ml	at	$5x10^{-2}$ (50 mM)	$1x10^{-3}$ (1mM)
TC2	Use 100 µl of dilution TC1 (50 mM)	+	900 µl	=	1 ml	at	$5x10^{-3}$ (5 mM)	$1x10^{-4}$ (100 µM)
TC3	Use 100 µl of dilution TC2 (5 mM)	+	900 µl	=	1 ml	at	$5x10^{-4}$ (500 µM)	$1x10^{-5}$ (10 µM)
TC4	Use 100 µl of dilution TC3 (500 µM)	+	900 µl	=	1 ml	at	$5x10^{-5}$ (50 µM)	$1x10^{-6}$ (1 µM)
TC5	Use 100 µl of dilution TC4 (50 µM)	+	900 µl	=	1 ml	at	$5x10^{-6}$ (5 µM)	$1x10^{-7}$ (100 nM)
TC6	Use 100 µl of dilution TC5 (5 µM)	+	900 µl	=	1 ml	at	$5x10^{-7}$ (500 nM)	$1x10^{-8}$ (10 nM)
TC7	Use 100 µl of dilution TC6 (500 nM)	+	900 µl	=	1 ml	at	$5x10^{-8}$ (50 nM)	$1x10^{-9}$ (1 nM)
TC8	Use 100 µl of dilution TC7 (50 nM)	+	900 µl	=	1 ml	at	$5x10^{-9}$ (5 nM)	$1x10^{-10}$ (100 pM)

^ It may be necessary to change the stock concentration depending on the properties of the test chemical.
**Final concentration of test chemical in assay tube when 10 microliters of diluted concentration is used in a total volume of 500 microliters.*

10.3 Preparation of ER Competitive Binding Assay tubes

Label 12 x 75 mm round bottom siliconized or silanized assay tubes (glass) in triplicate with codes for the untreated control, the solvent control, the non-specific binding (NSB), the negative control substance, six additional dose levels for the standard curve, eight dose levels of the weak positive substance (WP), and eight dose levels of each test substance. An example of a competitive assay tube layout using three unknown test chemicals is provided in Appendix B. It is recommended that no more than three

test chemicals be included in a run since it may be difficult to process the tubes quickly during the critical separation steps that must be performed without allowing the tubes to warm above 4º C. Evaporation of ethanol from tubes could also become a problem.

10.3.1 Master mixture

Calculate the number of assay tubes needed for the entire run. (For example, about 153 tubes are needed for the example in Appendix B that uses three unknowns, not including the trace tubes; round to 155 for calculations to assure sufficient amount of solutions in all assay tubes.) Prepare the combined volumes as a master mixture (demonstrated in Table 7 below using Appendix B as an example) to minimize pipetting errors between assay tubes:

Table 7. Master mixture for Competitive Binding Assay

Substance	Volume/Tube	# of tubes	Total volume	Add/assay tube
TEDG buffer + PMSF	380 µl	155	58.9 ml	
Diluted [^{3}H]-17β-estradiol (50 nM)	10 µl	155	1.55 ml	
Total			**60.45 ml**	**390 µl/tube**

Combine the above total volumes (master mixture), mix, and keep master tube on ice.

10.3.2 Individual tubes

- Add 390 µl/tube of the master mixture above and keep on ice. Prepare the standard, weak positive, negative, and test chemical as described and add to the tubes. (Adding 10 µl of the competitor/tube brings the final assay volume to 400 µl/tube.) Then, after all of the competitor (standard, weak positive, negative, or test chemical) additions have been added to the tubes, add 100 µl of cytosol to each tube for a final volume of 500 µl (see Table 8 below).

Table 8. Competitive Binding Assay additions

Volume (microliters)	*Constituent*
390	Master mixture (TEDG + PMSF assay buffer + [^{3}H]-17β-estradiol to yield final concentration of 1 nM)
10	Unlabeled 17β-estradiol, weak positive control, negative control, or test substance
100	Uterine cytosol (diluted to appropriate protein concentration as determined in Section 10.1.5)
500	Total volume in each assay tube

Note: *Make sure that the temperature of the tubes and contents are at 4º C prior to the addition of the uterine cytosol.*

- Vortex assay tubes after additions are completed.

Note: *Make sure that all components are concentrated at the bottom of tube. If any of the liquid remains on the side of the tube, centrifuge assay tubes for 1 minute at 600 x g (4° C) to concentrate fluid at bottom of tube.*

- Incubate assay tubes at 4° C for 16 to 20 hours. Assay tubes should be placed on a rotator during the incubation period.

10.4 Preparation of 60% HAP slurry

- Prepare HAP slurry the day before using it to separate the bound and free [^{3}H]-17β-estradiol. Prepare an adequate amount of HAP slurry for the number of tubes in the next day's run. The amounts of HAP given below (powder or hydrated product) will generally yield enough slurry for 175-200 assay tubes, so this amount would be just right for a typical run with estradiol, norethynodrel, octyltriethoxysilane, and non-specific tubes, plus 3 test chemicals (159 tubes, see Appendix B).

    - Prepare the dry powder HAP by adding 25 g HAP powder to 200 ml TEDG + PMSF buffer and gently mix.

    OR

    - If using the hydrated HAP product, a) mix gently to re-suspend the HAP and add ~63 ml of slurry to a 200 ml graduated cylinder for the washing process, b) add additional TEDG + PMSF Buffer to a final volume of 200 ml.

- Cap the container and refrigerate (4° C) for at least 2 hours.

- Aspirate or decant the supernatant and re-suspend the HAP in fresh TEDG + PMSF buffer to 200 ml. Mix gently. Again, allow the HAP to settle for ~2 hours at 4° C and repeat the wash step to ensure that the TEDG + PMSF buffer saturates the HAP.

- After the last wash, let the HAP slurry settle overnight (at least 8 to 10 hours at 4º C).

- The next day (i.e., the day on which the HAP slurry will be used in the assay), note the volume of HAP on the graduated cylinder, aspirate or decant the supernatant, and re-suspend the HAP to a final volume of 60% HAP and 40% cold TEDG + PMSF buffer (based on the overnight settled HAP volume). For example, if the HAP settles to the 90 ml mark on the graduated cylinder, add 60 ml cold TEDG + PMSF. The HAP slurry should be well-suspended and ice-cold when used in the separation procedure, and maintained as a well-suspended slurry during aliquoting.

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

Note: *The separation step is a potential source of significant variability in data. To minimize variability it is important to 1) keep the estrogen receptor cold* ***at all times****, and 2) ensure that even if the centrifuged pellet breaks up, none of the pieces leave the tube. To minimize dissociation of bound [^{3}H]-17β-estradiol from the ER during this process, it is extremely important that the buffers and assay tubes be kept ice-cold and that each step be conducted quickly. A multi-tube vortexer is necessary to process tubes efficiently and quickly.*

- Remove ER assay tubes from rotator and place in an ice-water bath. Using a repeating pipette, quickly add 250 microliters of ice cold HAP **slurry** (60% in TEDG + PMSF buffer, well mixed prior to using) to each assay tube.

- Vortex the tubes for ~10 seconds at 5-minute intervals for a total of 15 minutes with tubes remaining in the ice-water bath between vortexing.

Note: *This is best accomplished by vortexing an entire rack of tubes at once. It is important to continue to keep the assay tubes cold.*

- Following the vortexing step, add 2.0 ml of the cold (4º C) TEDG + PMSF buffer, quickly vortex, and centrifuge at 4° C for 10 minutes at 1000 x g.

- After centrifugation, immediately decant and discard the supernatant containing the free [^{3}H]-17β-estradiol. The HAP pellet will contain the estrogen receptor-bound [^{3}H]-17β-estradiol.

Note: *This step can be accomplished quickly by placing the assay tubes in a decanting tube rack. All tubes in the rack can be decanted at once. With the tubes still inverted, blot against clean absorbent pad (paper towel). Watch carefully to prevent any of the HAP pellets from running down the side of the assay tube, which may occur if protein concentration in the cytosol is quite low. Immediately place the tubes back in the ice bath.*

- Add 2.0 ml ice-cold TEDG + PMSF buffer to each assay tube and vortex (~10 sec) to resuspend the pellet. Work quickly and keep assay tubes cold. Centrifuge again at 4° C for 10 minutes at 1000 x g.

- Quickly decant and discard the supernatant and blot tubes as above. Again, make sure no pellet runs down the side of the tube. Repeat the wash and centrifugation steps once more. This will be the third and final wash.

- After the final wash, decant the supernatant. Allow the assay tubes to drain briefly for ~0.5 minute.

- At this point, the separation of the free [^{3}H]-17β-estradiol and ER-bound [^{3}H]-17β-estradiol has been completed. Assay tubes may be left at room temperature.

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

- Add 1.5 ml of absolute ethanol to each assay tube. Allow the tubes to sit at room temperature for 15 to 20 minutes, vortexing for ~10 seconds at 5-minute intervals. Centrifuge the assay tubes for 10 minutes at 1000 x g.

- Pipet a 1.0 ml aliquot, taking care to avoid the centrifuged pellet, into 20 ml scintillation vials containing 14 ml scintillation cocktail. Cap and shake vial.

- Place vials in scintillation counter for determination of DPMs/vial with quench correction.

Note: *Since a 1.0 ml aliquot is used for scintillation counting, the DPMs should be adjusted to account for the total radioactivity in 1.5 ml (i.e., DPMs x 1.5 = Total DPMs bound).*

10.7 Data analysis

10.7.1 Terminology

10.7.1.1 Total [^{3}H]-17β-estradiol

Radioactivity in DPMs added to each assay tube. (DPMs in the defined volume of the tube can be converted to concentration of [^{3}H]-17β-estradiol.)

10.7.1.2 Nonspecific binding

Radioactivity in DPMs in the tube that contains 100-fold excess of unlabeled over labeled 17β-estradiol NSB standard (i.e., the S0 tube = $1x10^{-7}$ M).

Note: NSB is the average of all of the NSB tubes included in a run. Include any tubes that were added at the bottom of the run.

10.7.1.3 Specific binding

Total binding minus non-specific binding.

10.7.2 Approach to Competitive Binding Assay analysis for the EDSP

In the Endocrine Disruptor Screening Program, the estrogen receptor competitive binding assay is being used only to evaluate the potential of a test substance to interact with the endocrine system. The EDSP is less concerned with proving that the interaction is, specifically, one-site competitive binding, or with accurately characterizing the strength of the binding. Nevertheless, a certain amount of quantitative analysis is necessary to ensure that the assay has been run correctly, and to aid in classifying a test chemical as a interacting with the estrogen receptor,

not interacting, or equivocal. The following paragraphs describe considerations for this analysis.

Note: Because the EDSP is not requiring clear identification of an interaction as one-site competitive binding – which could require additional saturation binding assays to prove – classification of a substance as interacting or not interacting for EDSP purposes might not be appropriate to use for structure-activity relationship analyses or other analyses where stringent classification as a one-site competitive binder may be necessary (Laws et al. 2000; Laws et al. 2006). Similarly, the "Relative Binding Affinities" estimated for the EDSP may not be appropriate for such structure-activity relationship analyses since the nature of the interaction has not been fully characterized.

An ER competitive binding assay measures the binding of a single concentration of [^{3}H]-17β-estradiol in the presence of increasing concentrations of a test substance. The competitive binding curve is plotted as specific [^{3}H]-17β-estradiol binding versus the concentration ($\log_{10}$ units) of the competitor. The concentration of the test substance that inhibits 50% of the maximum specific [^{3}H]-17β-estradiol binding is the IC_{50} value.

For the purposes of the EDSP, estimates of $\log(IC_{50})$ values should be determined using appropriate nonlinear curve fitting software to fit an unconstrained one site competitive binding model (e.g., BioSoft; McPherson, 1985; Motulsky, 1995). The relative binding affinity (RBA) should be calculated comparing the $\log(IC_{50})$ of 17β-estradiol with that of the test chemical. Be sure to calculate $\log(IC_{50})$, not $\log(EC_{50})$. That is, be sure to focus on the concentration at which 50% of binding of radiolabeled estradiol is inhibited, not simply the concentration at which the response is halfway between the maximum and minimum. Appendix C shows how to estimate $\log(IC_{50})$. As in the analysis of saturation binding data, use the method of Motulsky and Brown (2006) with a Q value of 1 for outlier elimination.

There may be cases where the raw data points describe an obviously U-shaped curve but the fitted curve, which is based on the Hill equation and does not accommodate U-shapes, masks this shape. This might happen, for example, if there is precipitation of the test chemical at high concentrations that was not noticed during preparation of the tubes. In these cases, it is appropriate to suppress the data points in the right-hand leg of the 'U" in order to fit the curve. Exclude all replicates at any concentration where the mean for the replicates displays 10 percentage points more radioligand binding (that is, 10 percentage points less radioligand displacement) than the lowest mean at a lower concentration. (For example, if the lowest mean radioligand binding at any concentration in the range 10^{-10} to 10^{-5} M is 15% and the mean at 10^{-4} M shows radioligand binding of 30%, all replicates at 10^{-4} M should be excluded from curve-fitting.) However, this rule should be applied only if the minimum value for the curve is below 80% binding. (Non-binders often display variability that

would result in discarding legitimate data points if the rule were applied without this exception.)

10.7.3 Performance criteria for the Competitive Binding Assay

The Competitive Binding Assay is functioning correctly if all of the following criteria have been met. The criteria apply to each individual run. If a run does not meet all of the performance criteria, the run must be repeated. Results for test chemicals in disqualified runs are not used in classifying the ER binding potential of those chemicals.

- Increasing concentrations of unlabeled 17β-estradiol displace [^{3}H]-17β-estradiol from the receptor in a manner consistent with one-site competitive binding. Specifically, the curve fitted to the inert-estradiol data points using non-linear regression descends from 90 – 10% over approximately an 81-fold increase in the concentration of the test chemical (i.e., this portion of the curve will cover approximately 2 log units). A binding curve that drops dramatically (e.g., from 90 - 0%) over one order of magnitude should be questioned, as should one that is U-shaped (i.e., percent bound is decreasing with increasing concentration of competitor but then begins to increase again). In both cases, something has happened to the dynamics of the binding assay and the reaction is no longer following the law of mass action. When the assay is correctly performed, inert estradiol exhibits typical one-site competitive binding behavior. The performance criteria for estradiol reflect this requirement.
- Ligand depletion is minimal. Specifically, the ratio of total binding in the absence of competitor to the total amount of [^{3}H]-17β-estradiol added per assay tube is no greater than approximately 15%.
- The parameter values (top, bottom, and slope) for 17β-estradiol, the concurrent positive control (norethynodrel), and the concurrent negative control (octyltriethoxysilane) are within the tolerance bounds provided. (See Table 9 for the acceptable ranges for the parameters.)
- 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 test 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 chemical was tested over a concentration range that fully defines the top of the curve, and the top is within 20 percentage points of either the solvent control or the value for the lowest concentration of the estradiol standard for that run.

In addition to meeting the criteria for individual runs, examine the consistency across runs for the same chemical. Datasets that can be fit to a curve must demonstrate consistency of the top plateau level, Hill slope, and placement along the X-axis. Where a bottom is defined, the bottom must also be consistent.

Table 9. Upper and lower limits for parameters in Competitive Binding Assay curves for the standards (radioinert estradiol, norethynodrel, and octyltriethoxysilane)

Parameter	Unit	Estradiol		Norethynodrel		Octyltriethoxysilane	
		Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit
$Log_e(S_{yx})$ (i.e., Log_e(Residual Std.Dev)	--	NA	2.35	NA	2.60	NA	2.60*
Bottom plateau level	% binding	-4	1	-5	1	NA	NA
Top plateau level	% binding	94	111	90	110	NA	NA
(Hill) Slope	$log_{10}(M)^{-1}$	-1.1	-0.7	-1.1	-0.7	NA	NA

[* This value must be recalculated.]

10.7.4 Classification criteria

Classification of a test chemical is based on the results of three non-concurrent runs, each of which meet the performance criteria and taken together are consistent with each other (see Section 10.7.3). Each run is classified as "interacting", "not interacting", "equivocal", or "equivocal up to the limit of the concentrations tested", and the runs are then combined as described below.

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 chemical concentration of 10^{-6} M and one of the two following conditions hold:
a) A binding curve can be fit but 50% or less of the radiolabled estradiol is displaced by concentration 10^{-6} M.
or
b) A binding curve cannot be fit and the 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
a) the lowest point on the fitted response curve within the range of the data is above 75%.
or
b) a binding curve cannot be fitted and the lowest average percent binding among the concentration groups in the data is above 75%.

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

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

interactive: 2
equivocal: 1
not interactive: 0
equivocal up to the limit of concentrations tested: ("missing")

Chemical classification, based on the average of all the runs performed for a chemical:

interactive: average ≥ 1.5
equivocal: 0.5 ≤ average < 1.5
not interactive: average < 0.5
equivocal up to the limit of concentrations tested: "missing"

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

10.8 Test report

The test report must include, but is not limited to, the following information:

10.8.1 Test substance

- Name, chemical structure, and CAS RN (Chemical Abstract Service Registry Number, CAS#), if known
- Physical nature (solid or liquid), and purity, if known
- Physicochemical properties relevant to the study (e.g., solubility, stability, volatility)

10.8.2 Solvent/Vehicle

- Justification for choice of solvent/vehicle.
- Concentration of solvent (as percent of total volume) at each concentration of estradiol, weak positive, negative control, solvent control, and test chemical.

10.8.3 Reference estrogen (viz., inert estradiol)

- Supplier, batch, and catalog number
- CAS number
- Purity

10.8.4 Estrogen receptor

- Source of rat uterine cytosol. If from a commercial source, the supplier must be identified. Include strain and age of rats from which uteri were taken, and number of days between ovariectomy and removal of the uterus. Recombinant ER is not acceptable for this assay protocol.

- Isolation procedure.

- Protein concentration of ER preparation. Provide details of the protein determination method, including the manufacturer of the protein assay kit, data from the calibration curves, and data from the protein determination assay.

- Method and conditions of transport and storage of ER, if applicable.

10.8.5 Test conditions

- K_d of the reference estrogen. Report the K_d obtained from the Saturation Binding Assay for each batch of cytosol used.

- Concentration range and spacing of the reference estrogens tested (estradiol and weak positive).

- Concentration range and spacing of negative and solvent controls.

- Concentration range and spacing of test substance, with justification if deviating from required range and spacing.

- Dilution schemes used for preparing the concentrations of estradiol, weak positive, negative control, and test chemical. (If the schemes used in the protocol were used without modification, state that.)

- Composition of buffer(s) used.

- Incubation time and temperature.

- Notes on any abnormalities during separation of free radiolabeled estrogen.

- Notes on any problems in analysis of bound reference estrogen.

- Notes on reasons for repeating a run, if a repeat was necessary.

- Methods used to determine log(IC_{50}) values (software used, formulas, etc.).

- Statistical methods used, if any.

10.8.6 Results

- Results (viz., the dpm counts for each tube) shall be inserted into the data worksheet provided in Appendix B (or similar), adjusted as necessary to accommodate the actual concentrations, volumes, etc. used in the assay. There should be one worksheet per run.

- Date of run, number of days since Specific Activity (SA) certification date, and adjusted SA on date of run.

- Extent of precipitation of test substance.

- The solvent control response compared to the negative control.

- % Binding data for each replicate at each dose level for all substances.

- Plot the data points and the unconstrained curve fitted to the Hill equation for each run of each chemical, separately (that is, one test chemical run per graph). The data points and curves for the reference chemical, weak positive control, and negative control from that test chemical's run should also be plotted on the same graph as the test chemical.

- Plot the curves (not the data points or any other indicators of variability) for all runs of the same chemical on a separate graph. Do not plot the estradiol, weak positive, or negative control information on this graph.

- Log(IC_{50}) values for 17β-estradiol, the positive control, and the test substance (see Appendix C).

- Calculated Relative Binding Affinity values for the positive control and the test substance, relative to RBA of 17β-estradiol = 1. (The Excel spreadsheet in Appendix B may be used.) Report both the log(RBA) and the RBA.

- A record of all protocol deviations or problems encountered shall be kept and included in the final report. It should also be used to improve runs that follow.

- A summary sheet of the performance criteria measures for each run.

10.8.7 Conclusion

- Classification of test substance with regard to interaction with the estrogen receptor (interacting, equivocal, not interacting, or equivocal up to the limit of concentrations tested).

- If the test substance is interactive, estimate the RBA by averaging the RBAs obtained across the acceptable runs. Report the range of RBAs also.

10.9 Replicate studies

Generally, replicate studies are not mandated for screening assays. However, in situations where questionable data are obtained (i.e., the IC_{50} value is not well defined), replicate tests to clarify the results of the primary test would be prudent.

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Appendix A: Buffer preparation worksheet

Buffers to prepare prior to the day of the assay:

1) 200 mM EDTA Stock Solution:

	Compound	Grams/ml	Comments	Added?*
1	Disodium EDTA	7.444 g		
2	ddH_2O	80 ml	Dissolve EDTA then bring final volume to 100 ml and store at 4º C.	

2) 100 mM PMSF Stock Solution:

	Compound	Grams/ ml	Comments	Added?*
1	PMSF	1.742 g		
2	Ethanol	80 ml	Dissolve PMSF and bring final volume to 100 ml and store at 4º C.	

3) 1M Tris Stock Buffer: (make in a volumetric flask)

	Compound	Grams/ ml	Comments	Added?*
1	Tris-HCl	147.24 g		
2	Tris base	8 g		
3	ddH_2O	800 ml	Dissolve and cool to 4°C	
4			Adjust pH to 7.4	
5			Bring final volume to 1 L and store at 4°C	

* A mark may be placed in the "Added?" column when that step is completed.

4) 2X TEG Buffer (20 mM Tris, 3 mM EDTA, 20% glycerol – pH 7.4): (prepare in the listed order in a graduated cylinder for a final volume of 100 ml, can be stored at 4°C for up to 3 months)

	Compound	Grams/ ml	Comments	Added?*
1	ddH_2O	70 ml		
2	1 M Tris Stock	2.0 ml		
3	Glycerol	20 ml		
4	200 mM EDTA Stock	1.5 ml	Dissolve and cool to 4° C	
5			Adjust to pH 7.4	
6			Bring final volume to 100 ml and store at 4° C.	

Working Assay Buffer (prepare daily as needed):

1) TEDG + PMSF (10 mM Tris, 10 mM EDTA, 1mM DTT, 1 mM PMSF, 10% glycerol, pH 7.4): (Use pre-prepared and cooled TEG buffer + DTT and PMSF)

	Compound	Grams/ ml	Comments	Added?*
1	2X TEG buffer	50 ml	Made previously and stored at 4° C	
2	DTT	15.43 mg	Add immediately before use	
3	100 mM PMSF Stock	1.0 ml	Add immediately before use	
4			Bring final volume to 100 ml and store at 4° C.	

* A mark may be placed in the "Added?" column when that step is completed.

Appendix B: Data entry and analysis worksheets

The worksheets are in the Excel file "Protocol Appendix B, ER-RUC data entry templates.xls".

The data entry and analysis workbook consists of three sections:

1) Radiolabeled estradiol worksheet template
2) Saturation Binding Assay worksheet template
3) Competitive Binding Assay worksheet template

On these worksheets, there are cells requiring input from the laboratory (shaded in blue) as well as cells which already contain formulas for calculation of various useful quantities. Some cells may *appear* to require user-supplied data but are not shaded blue; these cells contain formulas which will obtain the required data from other user-supplied cells. Error messages such as "#DIV/0!" that appear in the blank template will be replaced by calculated values as data are entered.

Certain assumptions have been built into the templates which may not be appropriate for the specific situation in which a laboratory might find itself. For example, the templates are set up with the assumption that the standards and test chemicals will be tested at specific concentrations. In the case of test chemicals, the concentrations are specified as order-of-magnitude fractions of the maximum concentration. The worksheets assume that up to eight concentrations will be tested. The user will need to adjust the spreadsheets appropriately if different concentrations, or different numbers of concentrations, are tested.

The spreadsheets are meant to be used in conjunction with the non-linear curve-fitting software that will be used to fit the Hill equation, but the spreadsheets are not directly connected to that software. The output from certain cells must be transferred by the user to the non-linear curve-fitting program, and the output from curve-fitting must be transferred by the user to the appropriate cells of these worksheets. See the following files for an example using the Prism software: "example saturation data.pzf" (for the saturation binding assay) and "logIC50 template.pzf", "logIC50 example.pzf", and "logEC50 with examples.pzf" (for the competitive binding assay).

1) Radiolabeled estradiol worksheet:
   a. Replace "Laboratory Code X" with the laboratory name.
   b. The radiolabeled estradiol worksheet records the Specific Activity of the radiolabeled 17β-estradiol as of the certification date by the supplier. The values entered here are used in formulas on other sheets to calculate automatically the *adjusted* Specific Activity for the date of the run.

2) Saturation Binding Assay worksheet
   a. Cells A19:K93 show a typical layout for the tubes for a Saturation Binding Assay run.
   b. Column C codes the tubes as "hot+receptor" (H), "hot+cold+receptor" (HC), and "hot alone" (Hot), where "hot" and "cold" refer to radiolabeled and radioinert estradiol, not temperature.

c. N21:N93 is where the decay-per-minute (dpm) values for each tube are entered by the user.
d. The user should explain in Column Q if a particular tube's data should not be used (e.g., "pipetting error", "precipitate in tube", etc.); otherwise it is left blank. Note that Column P will change automatically when an entry is made in Column Q; the user does *not* need to change the value from "true" to "false".
e. The values calculated by Cells R22 through R45 are the percent of ligand depletion and are ideally below 10%. (The "Ten Percent Rule" says to keep ligand depletion below 10%.) However, ligand depletion may go as high as approximately 15% and the run will still be considered acceptable. Runs with ligand depletion higher than approximately 15% should be disqualified and run again.
f. Column DM is where the B_{max} and K_d values estimated by the non-linear curve fitting model are entered by the user. The remainder of the cells in Column DM may be filled out but are not required. Columns DL and DM are set up in the order provided by Prism so that the entire output can be cut and pasted, but only the B_{max} and K_d are required entries. Other software may be used to determine these values.

3) Competitive Binding Assay worksheet(s)
a. One sheet should be submitted per run.
b. Column O (from row 34) is where the decay-per-minute (dpm) values for each tube are entered by the user.
c. The user should explain in Column R if a particular tube's data should not be used (e.g., "pipetting error", "precipitate in tube", etc.); otherwise it is left blank. Note that Column Q will change automatically when an entry is made in Column R; the user does *not* need to change the value from "true" to "false".
d. Be sure to include, in the workbook, a decoding sheet for chemicals that links the entry in Column D with the chemical code (unless the chemical code itself is used in Column D) and the chemical name, as well as to any other relevant information such as batch number, supplier, etc. A template has not been provided for this information.
e. The value calculated by Cell Q32 is the percent of ligand depletion and is ideally below 10%. (The "Ten Percent Rule" says to keep ligand depletion below 10%.) However, ligand depletion may go as high as approximately 15% and the run will still be considered acceptable. Runs with ligand depletion higher than approximately 15% should be disqualified and run again.
f. As in the Saturation Binding Assay worksheet, columns AC and beyond have been set up so that data can easily be transferred to and from GraphPad Prism software using the example Prism files provided. Other software may be used to estimate log(IC_{50}), in which case the output may not be in the row-format shown. For this reason, only a few cells have been marked in blue as required information. Other cells in this area, while not marked blue, may be filled out at the user's discretion.

g. The block of cells to the lower right of cell AA73, described as "log(EC_{50}) results" are to be used only if Method 2 (described in Appendix C: How to estimate log(IC_{50}) using GraphPad Prism or other software) is used. The blue cells in this area will not be filled in if Method 1 is used.

Appendix C: How to estimate log(IC_{50}) using GraphPad Prism or other software

A few words about terms: IC_{50} and EC_{50}

Please note that the terms IC_{50} (inhibitory concentration, 50%) and EC_{50} (effective concentration, 50%) refer to two different concepts. The IC_{50} is the concentration of test substance at which 50% of the radioligand is displaced from the estrogen receptor. The EC_{50} is the concentration of test substance at which binding of the radioligand is halfway between the top plateau and the bottom plateau of the curve defined (as for determination of the IC_{50}) by fitting the Hill equation to the data specific to that test substance and run. Where an IC_{50} exists, it may or may not be equal to the EC_{50}. (An IC_{50} may not always exist – the fitted curve may not cross the 50% binding level – but the EC_{50} will always exist provided the Hill equation can be fit to the data.) The IC_{50} provides the more consistent basis for evaluating interaction of the test chemical with the estrogen receptor and thus is preferred to the EC_{50} for purposes of the Endocrine Disruptor Screening Program.

Because the Hill equation describes the fraction of receptor bound by ligand as a function of the *logarithm* of the ligand concentration, we will more often refer to log(IC_{50}) and log(EC_{50}) than to the untransformed values. In these terms, when X is the concentration of test substance and Y is the % of radioligand bound to the estrogen receptor,

- log(IC_{50}) is the $\log_{10}$(X) at which Y is 50%.
- log(EC_{50}) is the $\log_{10}$(X) at which Y is (top + bottom)/2.

Examples and templates provided

There are two acceptable methods for obtaining the log(IC_{50}) from curve-fitting software. In Method 1, the software fits a curve to the data using a Hill equation formula which incorporates log(IC_{50}) as a parameter to be estimated. EPA is providing a template (log IC50 template.pzf) for use with Method 1 when GraphPad Prism is the software used[2]. This template incorporates the appropriate formula, which is not available as a standard formula in Prism. An example file populated with data is also provided (log IC50 example.pzf).

In Method 2, Prism fits a curve to the data using a form of the Hill equation in which log(IC_{50}) is not a parameter. After the curve is fit, the log(IC_{50}) is interpolated. EPA is providing an example file for Method 2 (logEC50 with examples.pzf) but is not providing a template.

[2] For those who do not have and do not intend to use GraphPad Prism, a Prism "viewer" is available at http://www.graphpad.com/prism/viewer htm . This should allow the user to follow the structure of the analysis and to follow the example.

Method 1: Fitting data to an IC_{50} formula

In this method, data are fit to a formula which directly estimates log(IC_{50}). Specifically, the formula used for curve-fitting is

Y=Bottom + (Top-Bottom)/(1+10^((LogIC50-X)*HillSlope+log((Top-Bottom)/(50-Bottom)-1)))

where X is the logarithm of the concentration of test substance and Y is the percent of radioligand bound to the receptor. LogIC50 is X at Y=50%. "Top" and "Bottom" refer to the value of Y when there is minimal binding by test chemical, and when there is maximal binding by test chemical, respectively.

The template file (log IC50 template.pzf) is set up so that data are fit to this formula. Open the data table for "Estrogen reference". The "X-Values" column holds the logs of the concentrations of reference standard (inert 17β-estradiol) while the Y1, Y2, and Y3 columns hold the triplicate values of % binding of radioligand to estrogen receptor. (Columns A, B, C, etc. can be used to for separate runs, denoted by lab identifier, run identifier, and date of run.) Note that the standard concentrations for the estrogen reference curve have already been entered into the template but can be changed if there is reason to do so. The template is set up so that once the data have been entered into the data table, Prism fits the best curve to the formula above using these data points.

Data for the weak positive control, the negative control, and test chemical are added and analyzed similarly. The instructions in the template explain how to use the data entry worksheet in conjunction with the Prism template to analyze a dataset quickly.

You can verify that Prism is using the correct settings by performing an analysis that is independent of any existing data sheet and its "family" of results and graph.

- Create a new datasheet via Insert/New Data Table (+ Graph), using the option "Create new table (choose X and Y format)". The X Column should be "Numbers (XY Graph)" and the Y Columns "3 replicates to calculate error bars." Label the columns in the resulting data table, and enter the data.
- On the Formatting toolbar, click on "Analyze" to open the "Analyze Data" window. Check "Built-in analysis" and "Curves & regression", and choose "Non-linear regression (curve-fit)".
- In the Parameters window, Equations tab, choose "More equations".
- Click "logIC50", which should be one of the options available in this file.
- (Check "Unknown from standard curve" when applicable.)
- Click the "OK" button.

See below for details on other options available in Prism.

If the data are well-behaved, Prism will display results in the "Table of Results" section of the "Results" folder. You can use data from the example runs to check that all values are as expected.

Method 2: Fitting data to an EC_{50} formula and interpolating an IC_{50}, where possible

Sometimes when using Method 1, Prism issues an error message such as "Floating point error," "Bad initial values," or "Does not converge." This can happen either when:

- there is no log(IC_{50}) that can be estimated (e.g., the bottom is greater than 50%); or
- Prism cannot handle a huge number encountered in the estimation process even if the log(IC_{50}) exists. (That is, the underlying curve, if Prism could calculate it, crosses the horizontal line corresponding to Y=50% but Prism was unable to define that curve because of computational difficulties.)

In the latter case it should be possible to estimate the log(IC_{50}) by first using Prism's built-in method for finding log(EC_{50}), then calculating the log(IC_{50}) from the point at which the fitted curve crosses the 50% line. How to do this can be seen in the file named "logEC50 with examples.pzf".

In this file, the "Sigmoidal dose-response (variable slope)" formula, one of Prism's built-in functions, is used instead of the "logIC50" formula "Sigmoidal dose-response (variable slope)" estimates log(EC_{50}) instead of log(IC_{50}). Note that for both daidzein runs in the example the model fit was successful even though the second data set (E-463-12/20/04) produces a floating point error in the "Results" table if the Method 1 template is used. By solving the following equation for X, we can get log(IC_{50}) from the log(EC_{50}) results (as long as the curve crosses Y=50%):

50 = Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

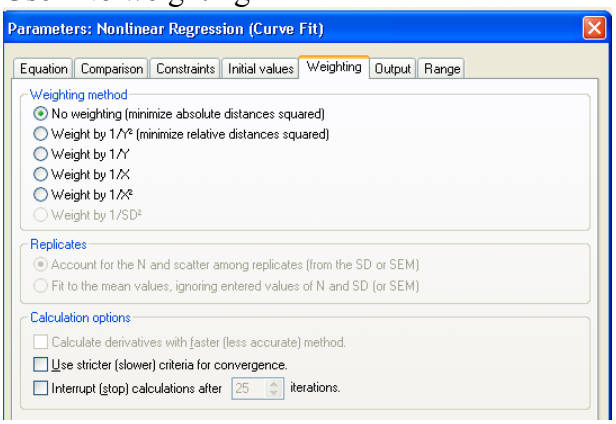
Prism can do this calculation. To set this up, we need to include a fake data point that has a "missing" X value in the data set. By including this fake data point and checking the "Unknown from standard curve" box, we can make Prism report the X value corresponding to Y = 50 % (which is the definition of log(IC_{50}) in the "Interpolated X mean values" sheet in the "Results" folder. In the example data file, the fake "data" point of Y=50% has been added to each run in the data set and the log(IC_{50}) has been estimated by Prism for each run.

This method of using Prism's built-in method for fitting the unconstrained Hill equation first and then interpolating the log(IC_{50}) has the advantage that it provides the top, bottom, slope, and log(EC_{50}) values when the log(IC_{50}) does not exist or the log(IC_{50}) model fit fails. A disadvantage is that Prism does not report the standard error of the log(IC_{50}) through this method, which it will do when we fit an explicit log(IC_{50}) model.

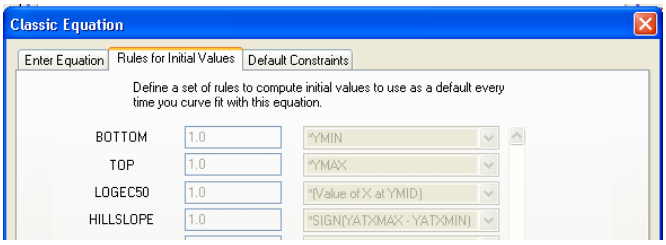
Details of options in Prism software

For either of the methods described above, various options are available in GraphPad Prism. The following options are typically used in conjunction with log(IC_{50}). These also are the default options for "Sigmoidal dose-response (variable slope)".

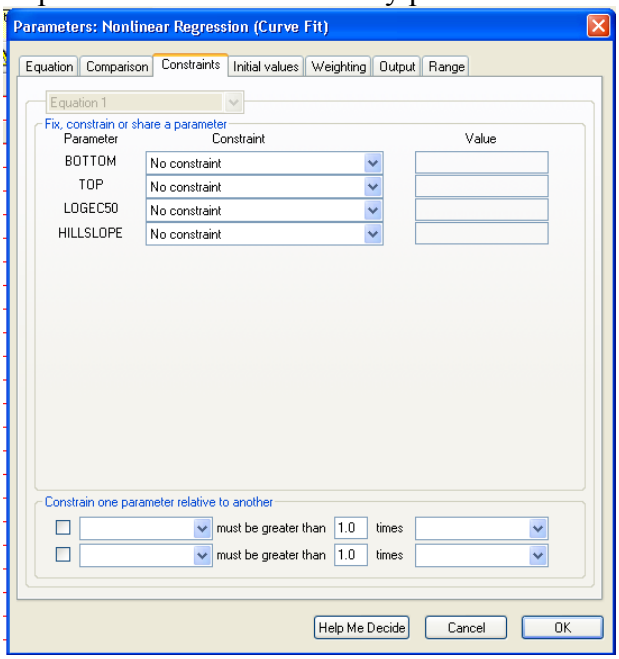
Use “No weighting”



Default initial values should work most of time.



Impose “No constraint” on any parameter. [3]



Sometimes, tweaking initial values will result in a successful fit if the defaults do not.

[3] EPA is aware that convergence can sometimes be achieved by imposing constraints on top, bottom, and/or slope. However, this method is inappropriate for EDSP purposes because it implies certainty about these values that is not inherent in the data. Top, bottom, and slope must not be constrained, even if it results in Prism not being able to fit a curve.

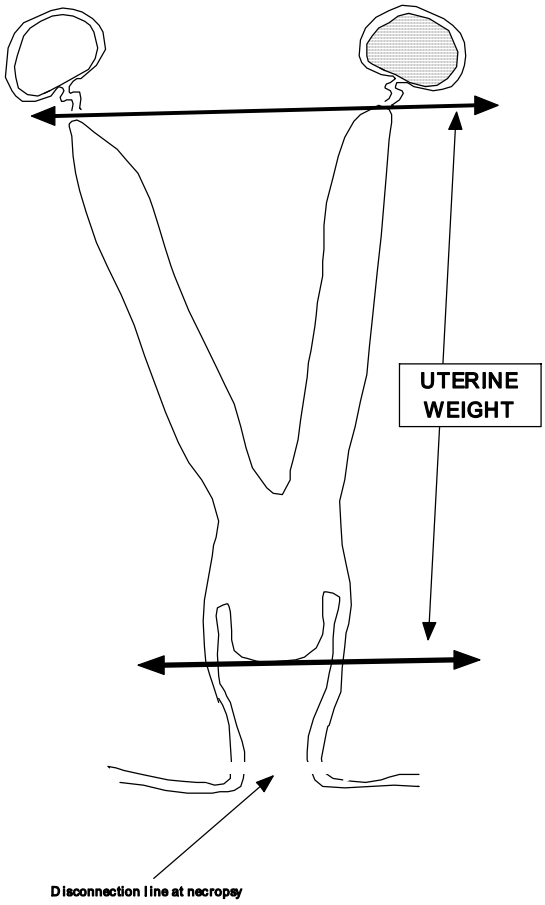
In general, a log(IC_{50}) model can be fit to (well-behaved) receptor binding data using the following model equation in conjunction with a non-linear least square procedure available in many commercial software such as SAS and Stata.

Y=Bottom + (Top-Bottom)/(1+10^((LogIC_{50}-X)*HillSlope+log((Top-Bottom)/(50-Bottom)-1)))

Statistical software packages such as Stata or SAS often are able to fit a model to a data set which Prism is unable to fit.

Appendix D: Uterine dissection diagram for obtaining estrogen receptors for the ER binding assay

The uterus (without ovaries) is carefully dissected and trimmed of fascia and fat. The vagina is removed from the uterus at the level of the uterine cervix.



The procedure is to open the pubic symphysis. Then, each ovary and uterine horn is detached from the dorsal abdominal wall. Urinary bladder and ureters are removed from the ventral and lateral side of uterus and vagina. Fibrous adhesion between the rectum and the vagina is detached until the junction of vaginal orifice and perineal skin is identified. The uterus and vagina are detached from the body by incising the vaginal wall just above the junction between perineal skin as shown in the figure. Excess fat and connective tissue are trimmed away. The vagina is removed from the uterus as shown in the figure for uterine weight measurement. Weight without the luminal fluid (blotted weight) is measured.