Example Protocol for the *In Vitro* Estrogen Receptor (ER) Competitive Binding Assay Using Rat Uterine Cytosol (RUC)

1.0 Purpose of Assay: This assay is used to determine the relative binding affinities of test substances for the estrogen receptor, which is comprised of the ERα and ERβ subtypes, compared to 17β-estradiol. The primary purpose for this assay is as a screening tool to detect substances with possible estrogenic or anti-estrogenic properties. This example protocol is intended to serve as a guide for producing laboratory specific protocols using this and related assays.

2.0 Terminology:

DES: Diethylstilbestrol

DMSO: Dimethyl sulfoxide

^{3}H-17β-estradiol: 17β-estradiol radiolabeled with tritiated thymidine

HAP: Hydroxylapatite

TEDG buffer: 10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, pH 7.4

3.0 Preparation of Rat Uterine Cytosol

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

3.1 TEDG Buffer

Prepare buffer; dithiothreitol is added immediately prior to use.

3.2 Collect Uteri

Collect uteri from female rats ovariectomized seven to ten days prior to being humanely killed. Quickly trim fat and mesentery from the uterus. Weigh and record

the weight of each uterus. Uteri may be used immediately or rapidly frozen in liquid nitrogen, and stored at -80°C for up to three months.

Note: Consistency for all assays should be maintained with respect to the age and strain of the animals used.

3.3 Uterine Cytosol

3.3.1 Weigh trimmed uterus and place in ice-cold TEDG buffer at a ratio of 0.1 g of tissue per 1.0 mL TEDG buffer. Homogenize the tissue using an appropriate homogenizer (5-second bursts).

Note: Cool the homogenizer probe prior to homogenizing each sample by placing the probe in ice-cold TEDG buffer. The homogenization tube should be kept in an ice-cold water bath during the homogenizing process.

3.3.2 Transfer the homogenate to pre-cooled centrifuge tubes and centrifuge for 10 minutes at 2,500 x g at 4°C. The pellet contains the nuclear fraction and the supernatant the ER containing cytosol.

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

3.3.4 Combine the cytosol containing ER supernatants from uteri collected the same day and aliquot for immediate use in ER binding assay or for storage at –80°C.

Note: The cytosol can be stored frozen at –80°C for 1 month prior to use in ER binding assay. Do not thaw and re-freeze the cytosol.

3.3.5 Determine the protein content for each batch of cytosol using an appropriate method.

Note: The dithiothreitol in the buffer is not compatible with the Pierce BCA Protein Assay. Typical protein values are 4 -7 mg/mL.

4.0 Standardization of ER Competitive Binding Assays

Prior to routinely conducting the ER competitive binding assays, the methods should be standardized within each laboratory. This can be accomplished in two steps. First, a series of saturation radioligand binding assays should be conducted to demonstrate ER specificity and saturation. Nonlinear regression analysis of these data (e.g., McPherson, 1985; c1997; Motulsky, 1995) and subsequent Scatchard plots will document ER binding affinity (K_d) and the number of receptors (B_{max}). Second, a series of ER competitive binding assays should be conducted using substances (e.g., 17β-estradiol, DES, estrone) with known affinities for the ER. Comparison of IC_{50} values (e.g., the concentration of a substance that inhibits ^{3}H-17β-estradiol binding by 50%) from these assays with reported values in the literature will assist in documenting that the methods are appropriate for routine use in the laboratory.

4.1 Saturation Radioligand Binding Assay: ER saturation binding experiments measure total, non-specific, and specific binding of increasing concentrations of ^{3}H-17β-estradiol under conditions of equilibrium. 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 (e.g., K_d = 0.05 to 0.1 nM and a linear Scatchard plot).

Although several saturation radioligand assays may need to be conducted before an optimal saturation curve, K_d, and B_{max} are achieved, a good starting point is to use enough cytosol to provide 50 to 100 µg protein per assay tube. 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 a non-linear regression analysis (e.g., McPherson, 1985; c1997; Motulsky, 1995) with a final display of the data as a Scatchard plot. Rat uterine cytosol

prepared using this protocol will typically yield a K_d of 0.05 to 0.1 nM and B_{max} of 36 -44 fmol ER/100 µg protein (equivalent to 0.072 to 0.088 nM ER, respectively, when 100 µg protein used in total assay volume of 0.5 mL).

An example of a saturation assay worksheet using increasing concentrations of radioligand is provided in **Table 1**.

Note: For this example, a stock solution of unlabeled 17β-estradiol is prepared in absolute ethanol, with all serial dilutions prepared in assay buffer. All concentrations of ^{3}H-17β-estradiol are prepared in assay buffer.

Table 1 Typical 17β-Estradiol Saturation Assay

Tube Number	^{3}H-17β-Estradiol			Unlabeled 17β-Estradiol			Buffer	Cytosol
	Initial Conc. (nM)	Vol. (µL)	Final Conc. (nM)	Initial Conc. (nM)	Vol. (µL)	Final Conc. (nM)	Vol. (µL)	Vol. (µL)
1	0.3	50	0.03	-			350	100
2	0.3	50	0.03	-			350	100
3	0.3	50	0.03	-			350	100
4	0.6	50	0.06	-			350	100
5	0.6	50	0.06	-			350	100
6	0.6	50	0.06	-			350	100
7	0.8	50	0.08	-			350	100
8	0.8	50	0.08	-			350	100
9	0.8	50	0.08	-			350	100
10	1.0	50	0.1	-			350	100
11	1.0	50	0.1	-			350	100
12	1.0	50	0.1	-			350	100
13	3.0	50	0.3	-			350	100
14	3.0	50	0.3	-			350	100
15	3.0	50	0.3	-			350	100
16	6.0	50	0.6	-			350	100
17	6.0	50	0.6	-			350	100
18	6.0	50	0.6	-			350	100
19	10	50	1	-			350	100
20	10	50	1	-			350	100
21	10	50	1	-			350	100
22	30	50	3	-			350	100

Tube Number	^{3}H-17β-Estradiol			Unlabeled 17β-Estradiol			Buffer	Cytosol
	Initial Conc. (nM)	Vol. (μL)	Final Conc. (nM)	Initial Conc. (nM)	Vol. (μL)	Final Conc. (nM)	Vol. (μL)	Vol. (μL)
23	30	50	3	-			350	100
24	30	50	3	-			350	100
25	0.3	50	0.03	30	50	3	300	100
26	0.3	50	0.03	30	50	3	300	100
27	0.3	50	0.03	30	50	3	300	100
28	0.6	50	0.06	60	50	6	300	100
29	0.6	50	0.06	60	50	6	300	100
30	0.6	50	0.06	60	50	6	300	100
31	0.8	50	0.08	80	50	8	300	100
32	0.8	50	0.08	80	50	8	300	100
33	0.8	50	0.08	80	50	8	300	100
34	1.0	50	0.1	100	50	10	300	100
35	1.0	50	0.1	100	50	10	300	100
36	1.0	50	0.1	100	50	10	300	100
37	3.0	50	0.3	300	50	30	300	100
38	3.0	50	0.3	300	50	30	300	100
39	3.0	50	0.3	300	50	30	300	100
40	6.0	50	0.6	600	50	60	300	100
41	6.0	50	0.6	600	50	60	300	100
42	6.0	50	0.6	600	50	60	300	100
43	10	50	1	1000	50	100	300	100
44	10	50	1	1000	50	100	300	100
45	10	50	1	1000	50	100	300	100
46	30	50	3	3000	50	300	300	100
47	30	50	3	3000	50	300	300	100
48	30	50	3	3000	50	300	300	100
49	0.3	50	0.03	^{3}H-17β-estradiol only, for determining total dpms				
50	0.3	50	0.03	^{3}H-17β-estradiol only, for determining total dpms				
51	0.3	50	0.03	^{3}H-17β-estradiol only, for determining total dpms				
52	0.6	50	0.06	^{3}H-17β-estradiol only, for determining total dpms				
53	0.6	50	0.06	^{3}H-17β-estradiol only, for determining total dpms				
54	0.6	50	0.06	^{3}H-17β-estradiol only, for determining total dpms				
55	0.8	50	0.08	^{3}H-17β-estradiol only, for determining total dpms				
56	0.8	50	0.08	^{3}H-17β-estradiol only, for determining total dpms				
57	0.8	50	0.08	^{3}H-17β-estradiol only, for determining total dpms				
58	1.0	50	0.1	^{3}H-17β-estradiol only, for determining total dpms				
59	1.0	50	0.1	^{3}H-17β-estradiol only, for determining total dpms				

Tube Number	^{3}H-17β-Estradiol			Unlabeled 17β-Estradiol			Buffer	Cytosol
	Initial Conc. (nM)	Vol. (μL)	Final Conc. (nM)	Initial Conc. (nM)	Vol. (μL)	Final Conc. (nM)	Vol. (μL)	Vol. (μL)
60	1.0	50	0.1	^{3}H-17β-estradiol only, for determining total dpms				
61	3.0	50	0.3	^{3}H-17β-estradiol only, for determining total dpms				
62	3.0	50	0.3	^{3}H-17β-estradiol only, for determining total dpms				
63	3.0	50	0.3	^{3}H-17β-estradiol only, for determining total dpms				
64	6.0	50	0.6	^{3}H-17β-estradiol only, for determining total dpms				
65	6.0	50	0.6	^{3}H-17β-estradiol only, for determining total dpms				
66	6.0	50	0.6	^{3}H-17β-estradiol only, for determining total dpms				
67	10	50	1	^{3}H-17β-estradiol only, for determining total dpms				
68	10	50	1	^{3}H-17β-estradiol only, for determining total dpms				
69	10	50	1	^{3}H-17β-estradiol only, for determining total dpms				
70	30	50	3	^{3}H-17β-estradiol only, for determining total dpms				
71	30	50	3	^{3}H-17β-estradiol only, for determining total dpms				
72	30	50	3	^{3}H-17β-estradiol only, for determining total dpms				

Abbreviations: Conc. = concentration; Vol. = volume; dpms = disintegrations per minute

4.2 ER Competitive Binding Assay: 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 total ^{3}H-17β-estradiol binding versus the concentration (log units) of the competitor. The concentration of the test substance that inhibits 50% of the maximum ^{3}H-17β-estradiol binding is the IC_{50} value. Preliminary experiments should evaluate the effect of the ER concentration of the cytosol, assay volume, and ^{3}H-17β-estradiol concentration on the IC_{50} calculation using unlabeled 17β-estradiol. A good starting point for the ER competitive binding assay is to use enough cytosol to provide 50 to 100 μg protein per assay tube, with 0.5 -1.0 nM ^{3}H-17β-estradiol in a total assay volume of 0.5 mL. Once assay conditions have been optimized, additional ER

competitive binding assays should be conducted to test substances with known affinities for the ER. Such substances include tamoxifen, ethynyl estradiol, coumestrol, and estrone as positive ER binding substances, and R1881 (methyltrienolone) as the negative ER binding substance. Data for the unlabeled 17β-estradiol standard curve and each validation substance should be plotted as the percent ^{3}H-17β-estradiol bound versus the molar concentration (log) of competitor. Estimates of IC_{50} values should be determined using appropriate nonlinear curve fitting software (e.g., McPherson, 1985; c1997; Motulsky, 1995). Since the IC_{50} value is a property of the experiment and the K_i a property of the receptor and the test substance, the K_i value should be provided, as well as the RBA value. The K_i value is calculated using the Cheng -Prusoff equation (Cheng and Prusoff, 1973).

When conducting this assay as a screening test for substances with an ability to bind to the ER, concurrent negative, solvent, and positive controls are included in each experiment. The negative control provides assurance that the solvent does not interact with the test system. The solvent should be tested at the highest concentration that is added with the test substance. A positive control substance (e.g., tamoxifen, coumestrol) is included to demonstrate the sensitivity of each experiment and to allow for an assessment of variability in the conduct of the assay across time. Recommended concentrations of the test substance and positive controls to use are from 1 x 10^{-9} to 1 x 10^{-3} M, in log increments.

4.3 Standardized *In Vitro* ER Competitive Binding Assay Acceptance Criteria

4.3.1 Saturation Assays. In general, when evaluating data from ER saturation assays, the following points should be considered:

- As increasing concentrations of ^{3}H-17β-estradiol were used, did the specific binding curve reach a plateau (e.g., was maximum specific binding reached indicating saturation of ER with ligand)?

- Did the data produce a linear Scatchard plot (e.g., 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])?
- Is the K_d within an acceptable range (e.g., 0.05 to 0.1 nM)?

  Note: Literature values for K_d using rat uterine cytosolic preparations have varied from 0.05 to 0.5 nM. The variation in K_d may be a reflection of different laboratories using radiolabeled estradiol with a wide range of specific activity (e.g., ^{3}H-17β-estradiol versus ^{125}I-17β-estradiol). In addition, publications by Salomonsson et al. (1994) and Kuiper et al. (1997, 1998) suggest that a lower Kd may be observed when assay conditions minimize ligand depletion, and that slightly different K_d values exist for ERα and ERβ.
- Are the standard errors for the K_d or B_{max} excessive? If the ratio of either the standard error (SE) of the K_d to the K_d, or the SE of the B_{max} to the B_{max} is much larger than 20%, then the methods for the assay should be re-evaluated.
- Is non-specific binding excessive? The value for non-specific binding should be less than 50% of the total binding.

4.3.2 Competitive Binding Assays. In general, the assay should demonstrate that increasing concentrations of unlabeled 17β-estradiol can compete with a single concentration of ^{3}H-17β-estradiol for binding to the ER. Specific questions to evaluate are as follows:

- As a safeguard against ligand depletion, was the total maximal binding no greater than 10% of the amount of ^{3}H-17β-estradiol added per assay tube?

- Were the K_i and IC_{50} values for unlabeled 17β-estradiol reasonable? The IC_{50} value for unlabeled 17β-estradiol should be approximately equal to the molar concentration of ^{3}H-17β-estradiol used in the assay tube plus the K_d (determined by nonlinear analysis and Scatchard plot of data obtained from saturation radioligand binding assays).
- Were the K_i, IC_{50}, and RBA values for the substance used to validate the performance of the assay reasonable based on published and historical data?
- Was the negative control substance unable to inhibit binding of the radiolabeled 17β-estradiol?

5.0 ER Competitive Binding Assay: Working Protocol

5.1 Preparation of Assay Buffer

Prepare TEDG buffer without dithiothreitol, adjust to pH 7.4 and store at 4°C. Add dithiothreitol immediately prior to use in assay.

5.2 Preparation of ^{3}H-17β-Estradiol

Store at 4 to 5°C in the original container. Obtain the highest specific activity (SA) available from the vendor.

Note: The SA should be adjusted for decay over time.

Dilute the radiolabeled 17β-estradiol with TEDG buffer. Each assay tube should contain 0.5 to 1 nM final concentration of ^{3}H-17β-estradiol.

5.3 Solvent and Positive Controls

When testing substances for their ability to bind to the ER, concurrent negative, solvent, and positive controls should be included in each experiment. The negative control provides assurance that the solvent does not interact with the test system.

The solvent should be tested at the highest concentration that is added with the test substance. A positive control substance is included to demonstrate the sensitivity of each experiment and to allow for an assessment of variability in the conduct of the assay across time. A positive control substance (e.g., tamoxifen, coumestrol) is included to demonstrate the sensitivity of each experiment and to allow for an assessment of variability in the conduct of the assay across time. The suggested concentration range of the test substance and positive control to test is from 1×10^{-9} to 1×10^{-3} M, in log increments.

5.4 Selection of Receptor Concentration and Assay Volume

Receptor concentration of the cytosol and assay volume per assay tube should be adjusted to minimize the likelihood of ligand depletion (e.g., 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 significantly differ from the concentration of ^{3}H-17β-estradiol that was originally added to the assay tube [Hulme and Birdshall, 1992]). A general rule is to optimize the assay conditions so that the ratio of the total ^{3}H-17β-estradiol bound in the absence of competitor, to the total ^{3}H-17β-estradiol added to each assay tube, is no more than 10%. Decreasing the amount of cytosolic protein and/or increasing the assay volume will generally lower this ratio. Serial dilutions of the cytosol to obtain 50 to 150 µg protein per assay tube in a total assay volume of 500 µL is a good starting point for determining the optimal ER concentration.

5.5 Preparation of 17β-Estradiol for the Standard Curve and Non-Specific Binding (NSB)

Standard Curve: 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. Prepare serial dilutions of 17β-estradiol in absolute ethanol to achieve the final concentrations shown below. Use siliconized glass tubes when preparing the standards. **Table 2** shows recommended concentrations for the unlabeled 17β-estradiol standard curve.

5.6 Preparation of Test Substances

5.6.1 Stock Solutions: Test substances must be dissolved in water or in a solvent that is miscible with water. For substances not sufficiently water soluble, absolute ethanol or DMSO are proposed as solvents. Preference is given to absolute ethanol compared to DMSO since this solvent has been used in most of the studies conducted to date. Other solvents may be used as long as it can be demonstrated that they do not interact with the test system.

Table 2 Example of Preparation Procedure for Unlabeled 17β-Estradiol Standard Curve

Concentrations for Unlabeled 17β-Estradiol Standard Curve		
Standards	**Initial 17β-Estradiol Concentration (Molar)**	***Final 17β-Estradiol Concentration (Molar) in ER Assay Tube**
0	0 (ethanol)	0
NSB	5×10^{-6}	1×10^{-7}
S1	5×10^{-7}	1×10^{-8}
S2	5×10^{-8}	1×10^{-9}
S3	1.67×10^{-8}	3.33×10^{-10}
S4	5×10^{-9}	1×10^{-10}
S5	1.67×10^{-9}	3.33×10^{-11}
S6	5×10^{-10}	1×10^{-11}

**When 10 µL of each standard is added to the ER assay tube, the final concentration will be as indicated when the total volume in the ER assay tube is 500 µL.*

Note: Some test substances will not be soluble at this concentration, so adjustments will need to be made in the final concentration of the

serial dilution tubes depending upon the solubility characteristics of specific substances.

5.6.2 Prepare serial dilutions of each test substance in the appropriate solvent to yield the final concentrations as indicated below.

Note: For the purpose of screening, it is proposed that the upper limit dose be 1 mM and that a concentration range from 1 mM to 1 nM, in ten-fold increments, be used. If the upper limit dose must be reduced due to solubility constraints, then equivalent spacing (e.g., half-log doses) of the seven doses over the smaller dose range should be used. The serial dilutions shown in **Table 3** are based upon the addition of 10 µL of each serial dilution of the test substance in a final assay volume of 500 µL. Other ratios can be used as long as the solvent concentration does not exceed 0.2%.

Table 3 Test Substance Concentrations

Serial Dilutions of Test Substance	Initial Concentration (Molar)	*Final Concentration in ER Assay Tube (Molar)
Concentration 1	5.0×10^{-2}	1.0×10^{-3}
Concentration 2	5.0×10^{-3}	1.0×10^{-4}
Concentration 3	5.0×10^{-4}	1.0×10^{-5}
Concentration 4	5.0×10^{-5}	1.0×10^{-6}
Concentration 5	5.0×10^{-6}	1.0×10^{-7}
Concentration 6	5.0×10^{-7}	1.0×10^{-8}
Concentration 7	5.0×10^{-8}	1.0×10^{-9}

**Final Concentration of test substance in assay tube when 10 µL of Initial Concentration is used in a total volume of 500 µL*

5.7 Preparation of ER Assay Tubes

5.7.1 Label 12 x 75 mm round bottom assay tubes (siliconized glass) in triplicate with codes for the untreated negative control, the solvent control, the NSB, seven dose levels of the positive control substance, and seven dose levels of each test substance.

5.7.2 Place assay tubes in ice bath and add the following to each tube:

Components of ER RUC Binding Assay		
50	µL	Adjust amount of uterine cytosol to provide 50 to 100 µg protein/assay tube
430	µL	TEDG Assay Buffer
10	µL	^{3}H-17β-estradiol to yield final concentration of 0.5 to 1.0 nM
10	µL	Unlabeled 17β-estradiol, negative control, or test substance
500	µL	Total volume in each assay tube

5.7.3 Vortex assay tubes.

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.

5.7.4 Incubate assay tubes at 4°C for 18 to 20 hours. Assay tubes should be placed on a rotator during the incubation period.

5.8 Preparation of 60% HAP Slurry

5.8.1 Prepare 60% HAP slurry the day before the step to separate the bound and free ^{3}H-17β-estradiol, by adding 10 g HAP to 100 mL TEDG buffer and gently mixing. Cap the container and refrigerate (4°C) the HAP slurry overnight (8 to 10 hours). This amount of HAP will generally yield enough slurry for 70 to 100 assay tubes.

5.8.2 Aspirate the supernatant and resuspend the HAP in fresh TEDG buffer. Allow the HAP to settle and repeat the wash step.

5.8.3 After the last wash, resuspend the HAP to a final volume of 60% HAP and 40% buffer. The HAP slurry should be well suspended and ice-cold when used in the separation procedure.

5.9 Separation of Bound ^{3}H-17β-Estradiol -ER and 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.

5.9.1 Remove ER assay tubes from rotator and place in an ice-water bath. Using an Eppendorf repeating pipet, quickly add 250 µL of HAP slurry (60% in TEDG buffer, well mixed prior to using) to each assay tube.

5.9.2 Vortex the tubes at 5 minute intervals for a total of 15 minutes. *Note:* This is best accomplished by vortexing an entire rack of tubes at once. It is important to keep the assay tubes cold at this point.

5.9.3 Following the incubation period (step 5.8.2), add 2.0 mL of the TEDG buffer, quickly vortex, and centrifuge at 4°C for 10 minutes at 1000 x g.

5.9.4 After centrifugation, immediately decant the supernatant containing the free ^{3}H-17β-estradiol. The HAP pellet will contain the bound ^{3}H-17β-estradiol -estrogen receptors.

Note: This step can be accomplished quickly by placing the assay tubes in a decanting tube racks. All tubes in the rack can be decanted at once, and the tubes immediately placed back in the ice bath.

5.9.5 Add an additional 2.0 mL ice-cold TEDG buffer and vortex briefly to resuspend the pellet. Work quickly and keep assay tubes cold. Centrifuge again at 4°C for 15 minutes at 1000 x g.

5.9.6 Quickly decant and discard the supernatant. Repeat the wash and centrifugation steps once more.

5.9.7 After the final wash, decant the supernatant. Allow the assay tubes to drain briefly for 1 to 5 minutes.

Note: Watch carefully in case the HAP pellet begins to run down the side of assay tube, which may occur if protein concentration in the cytosol is quite low. At this point, the separation of the free ^{3}H-17β-estradiol and bound ^{3}H-17β-estradiol-ER has been completed. Assay tubes may be left at room temperature.

5.10 Extraction and Quantifying ^{3}H-17β-Estradiol bound to ER

5.10.1 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 at 5-minute intervals.

5.10.2 Centrifuge the assay tubes for 10 minutes at 1000 x g and 4°C.

5.10.3 Pipet an aliquot (usually 1.0 to 1.5 mL) or decant the supernatant into 20 mL scintillation vials. Add 10 mL scintillation cocktail, cap and shake vial.

Note: If 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).

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

5.11 Data Analysis

5.11.1 Terminology

- Total ^{3}H-17β-estradiol: DPMs added to each assay tube (e.g., can be converted to concentration of total ^{3}H-17β-estradiol used in the ER assay).

- Total (Maximum) Binding: DPMs in the 0 standard tubes.
- Nonspecific Binding: DPMs in the NSB standard (i.e., 100 x excess of unlabeled 17β-estradiol).
- Specific Binding: DPMs for each concentration of standard or test substance minus the mean DPM of the NSB tubes.

5.11.2 Data Analysis

i. IC_{50} calculation: Data for the unlabeled 17β-estradiol standard curve and each test substance should be plotted as the percentage of ^{3}H-17β-estradiol bound versus the molar concentration (log) of competitor. Estimates of IC_{50} values should be determined using appropriate nonlinear curve fitting software.

ii. Relative Binding Affinity (RBA) values: The RBA values for each test substance and positive control is calculated by dividing the IC_{50} value for 17β-estradiol by the IC_{50} of the test substance or the positive control and expressing the value as a percent (e.g., RBA for 17β-estradiol =100 %).

iii. K_i calculation: Calculate the K_i value from the IC_{50} value using the Cheng-Prusoff (1973) equation (Cheng and Prusoff, 1973).

5.12 Assay Acceptance Criteria

5.12.1 Unlabeled 17β-estradiol Standard Curve. The assay should demonstrate that increasing concentrations of unlabeled 17β-estradiol can displace ^{3}H-17β-estradiol, and the IC_{50} value for 17β-estradiol should be approximately equal to the molar concentration of ^{3}H-17β-estradiol plus the K_d (determined by nonlinear regression and viewed by a Scatchard plot).

5.12.2 The K_d and IC_{50} values for the unlabeled 17β-estradiol standard curve should be within the confidence limits for historical data.

5.12.3 The ratio of total binding in the absence of competitor to the amount of ^{3}H-17β-estradiol added per assay tube should not be greater than 10%.

5.12.4 The K_i, IC_{50}, and RBA values for the concurrent positive control should be within the confidence limits for historical data.

5.12.5 The solvent control substance, at the concentration used, should not alter the sensitivity or reliability of the assay.

5.13 Evaluation and Interpretation of Results

A substance is classified as positive for binding to the ER if a K_i and IC_{50} values can be obtained and an RBA value can be calculated. If K_i/IC_{50} values cannot be obtained after testing to the upper limit dose or the highest dose possible, the test substance is classified as "negative" for *in vitro* ER binding. However, due to solubility constraints (for example), some test substances might induce a significant reduction in binding but without achieving at least a 50% reduction in the binding of the reference estrogen to the ER. Until additional information becomes available about the significance of this category of dose response curves, such responses should be noted and the substances classified appropriately (e.g., "equivocal") for the test.

5.14 Test Report

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

5.14.1 Test Substance

- Name, chemical structure, and CASRN, if known;
- Physical nature (solid or liquid), and purity, if known; and
- Physicochemical properties relevant to the study (e.g., solubility, stability, volatility).

5.14.2 Solvent/Vehicle

- Justification for choice of solvent/vehicle if other than water or ethanol;

- Information to demonstrate that the solvent/vehicle, if other than an established solvent, does not bind to, or otherwise affect, the ER.

5.14.3 Estrogen Receptor

- Type and source of ER (if from a commercial source, the supplier must be identified);
- Isolation procedure from tissues, method for making construct, procedure for isolating protein or construction of fusion protein if used;
- Protein concentration of ER preparation; and
- Method for storage of ER, if applicable.

5.14.4 Test Conditions

- K_d of the reference estrogen;
- Rationale for the concentration of the reference estrogen;
- Composition of buffer(s) used;
- Concentration range of test substance, with justification;
- Volume of vehicle used to dissolve test substance and volume of test substance added;
- Incubation time and temperature;
- Type and composition of metabolic activation system, if added;
- Concentration range of positive and solvent/vehicle controls;
- Method used to separate free reference estrogen, if applicable;
- Method for analyzing bound reference substance;
- Methods used to determine K_i and IC_{50} values; and
- Statistical methods used, if any.

5.14.5 Results

- Extent of precipitation of test substance;
- The solvent control response compared to the negative control;

- IC data for each replicate at each dose level for all substances, including confidence levels or other measure of intra-dose repeatability;
- Calculated K_i and IC_{50} values and confidence limits for 17β-estradiol, the positive control, and the test substance; and
- Calculated RBA values for the positive control and the test substance.

5.14.6 Discussion of the Results

- Historical K_i and IC_{50} values for reference ligand, including ranges, means, and standard deviations;
- Reproducibility of the K_i and IC_{50} values of the reference ligand, compared to historical data;
- Historical positive control data with ranges, means, and standard deviations; and
- Reproducibility of the K_i and IC_{50} values for the positive control substance, compared to historical data.

5.14.7 Conclusion

- Classification of test substance with regard to *in vitro* ER-binding activity.

5.15 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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