Appendix B5

Protocol for the Estrogen Receptor Competitive Binding Assay Using Rat Uterine Cytosol

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Purpose of Assay: This assay can be used to determine the relative binding affinities of environmental chemicals for the estrogen receptor (ERα, ERβ) as compared to 17β-estradiol. Data produced using this assay can be used (1) as a screening tool to detect chemicals with possible estrogenic or anti-estrogenic properties; and (2) for development of Quantitative Structure Activity Relationship models to predict the ability of a chemical to bind to the ER.

Distribution of protocol: A final version of this protocol will be distributed as a guide to multiple laboratories, some with previous experience in conducting receptor binding assays, and others with limited or no experience. This protocol is intended to serve as a guide by providing sufficient information to successfully conduct the assay, yet not being totally definitive so that labs already proficient in conducting the assay would be prevented from using well-documented procedures currently in use in their labs.

Terminology:

E2: estradiol, 17β-estradiol, inert estradiol

^{3}H-E2: radiolabeled estradiol, [2,3,6,7,16,17-^{3}H(N)]-estradiol

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

HAP: Hydroxylapatite

DES: Diethylstilbestrol

I. Preparation of Rat Uterine Cytosol

a. Prepare TEDG buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, pH 7.4). Dithiothreitol should be added just prior to use.

b. Uterine cytosol should be prepared using uteri from female rats ovariectomized 7 -10 days prior to being killed. Consistency should be maintained for all assays with respect to the age and strain of the females. Fat and mesentary should be quickly trimmed from the uterus. Weigh and record weight of each uterus. Uteri may be used immediately or rapidly frozen on dry ice or in liquid nitrogen, and stored at -80°C for up to 3 months.

c. Prepare uteri for homogenization using ice-cold TEDG buffer at a ratio of 0.1 g of tissue (e.g., use trimmed tissue necropsy weight obtained in step 1b) per 1.0 mL TEDG buffer.

d. Homogenize the tissue using a Polytron homogenizer (5-sec bursts). *Note:* Probe of polytron should be cooled prior to homogenizing each sample by placing the probe in ice-cold TEDG buffer. If possible, the homogenization tube should be kept in an ice-cold water bath during the homogenizing process.

e. Transfer the homogenate to pre-cooled centrifuge tubes and centrifuge for 10 min. at 2,500 x g at 4°C. (The pellet will contain the nuclear fraction and the supernatant will be used for the cytosolic preparation).

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

g. Combine the supernatant (i.e., cytosol containing ER) and aliquot for immediate use in ER binding assay or for freezing at -80°C. *Note:* cytosol can be frozen for 1 month prior to use in ER binding assay. Do not thaw and re-freeze the cytosol.

h. Determine the protein content for each batch of cytosol using the BioRad Protein Assay Kit (BioRad Chemical Division, Richmond, CA). *Note:* The dithiothreitol in the buffer is not compatible with the Pierce BCA Protein Assay. Typical protein values are 4 -7 mg/mL.

II. Standardization of Methods for ER Binding Assay

Prior to routinely conducting the ER competitive binding assays, the methods should be standardized within each laboratory. This may 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 and subsequent Scatchard plots will document ER binding affinity (k_d) and number (B_{max}). Second, a series of ER competitive binding assays should be conducted using chemicals with known affinities for the ER, such as inert E2, DES, estrone. Comparison of IC_{50}s (e.g., the concentration of a chemical that inhibits ^{3}H-E2 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.

A. Saturation Radioligand Binding Assay. ER saturation binding experiments measure total, non-specific and specific binding of increasing concentrations of ^{3}H-E2 under conditions of equilibrium. A graph of specific ^{3}H-E2 binding vs. 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-E2 to a single, high affinity binding site (e.g, K_d = 0.05 - 0.1 nM).

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 - 100 µg protein per assay tube. The concentration for ^{3}H-E2 should range from 0.03 - 3.0 nM in a total assay volume of 0.5 mL. Non-specific binding should be determined by using 100 x the concentration of radiolabeled E2. Analysis of these data should use a non-linear regression analysis such as RADLIG and LIGAND (KELL, BioSoft, Ferguson, MO), 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 - 0.1 nM and B_{max} of 36 -44 fmol ER/100 ug protein (e.g, 0.072 - 0.088 nM ER 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 shown below. *Note:* For this example, a stock solution of inert E2 should be prepared in ethanol, with all serial dilutions prepared in assay buffer. All concentrations of ^{3}H-E2 should be prepared in assay buffer.

Typical Estradiol Saturation Assay									
	^{3}H-E2			***Inert E2***			***Buffer***	***Cytosol***	
Tube #	***Initial [ ] nM***	***Vol (µl)***	***Final [ ] nM***	***Initial [] nM***	***Vol (µl)***	***Final [ ] nM***	***Vol (µl)***	***Vol (µl)***	***DPM***
1	0.3	50	0.03	-			350	100	
2	0.3	50	0.03	-			350	100	
3	0.6	50	0.06	-			350	100	
4	0.6	50	0.06	-			350	100	
5	0.8	50	0.08	-			350	100	
6	0.8	50	0.08	-			350	100	
7	1.0	50	0.1	-			350	100	
8	1.0	50	0.1	-			350	100	
9	3.0	50	0.3	-			350	100	
10	3.0	50	0.3	-			350	100	
11	6.0	50	0.6	-			350	100	
12	6.0	50	0.6	-			350	100	
13	10	50	1	-			350	100	
14	10	50	1	-			350	100	
15	30	50	3	-			350	100	
16	30	50	3	-			350	100	
17	0.3	50	0.03	30	50	3	300	100	
18	0.3	50	0.03	30	50	3	300	100	
19	0.6	50	0.06	60	50	6	300	100	
20	0.6	50	0.06	60	50	6	300	100	
21	0.8	50	0.08	80	50	8	300	100	
22	0.8	50	0.08	80	50	8	300	100	
23	1.0	50	0.1	100	50	10	300	100	
24	1.0	50	0.1	100	50	10	300	100	
25	3.0	50	0.3	300	50	30	300	100	
26	3.0	50	0.3	300	50	30	300	100	
27	6.0	50	0.6	600	50	60	300	100	
28	6.0	50	0.6	600	50	60	300	100	
29	10	50	1	1000	50	100	300	100	
30	10	50	1	1000	50	100	300	100	
31	30	50	3	3000	50	300	300	100	
32	30	50	3	3000	50	300	300	100	
33	0.3	50	0.03	^{3}H- E2 only, for determining total dpms					
34	0.3	50	0.03	^{3}H- E2 only, for determining total dpms					
35	0.6	50	0.06	^{3}H- E2 only, for determining total dpms					
36	0.6	50	0.06	^{3}H- E2 only, for determining total dpms					
37	0.8	50	0.08	^{3}H- E2 only, for determining total dpms					

38	0.8	50	0.08	^{3}H- E2 only, for determining total dpms	
39	1.0	50	0.1	^{3}H- E2 only, for determining total dpms	
40	1.0	50	0.1	^{3}H- E2 only, for determining total dpms	
41	3.0	50	0.3	^{3}H- E2 only, for determining total dpms	
42	3.0	50	0.3	^{3}H- E2 only, for determining total dpms	
43	6.0	50	0.6	^{3}H- E2 only, for determining total dpms	
44	6.0	50	0.6	^{3}H- E2 only, for determining total dpms	
45	10	50	1	^{3}H- E2 only, for determining total dpms	
46	10	50	1	^{3}H- E2 only, for determining total dpms	
47	30	50	3	^{3}H- E2 only, for determining total dpms	
48	30	50	3	^{3}H- E2 only, for determining total dpms	

B. ER Competitive Binding Assay. An ER competitive binding assay measures the binding of a single concentration of ^{3}H-E2 in the presence of increasing concentrations of a test chemical. The competitive binding curve is plotted as total ^{3}H-E2 binding vs. the concentration (log units) of the competitor. The concentration of the test chemical that inhibits 50% of the maximum ^{3}H-E2 binding is the IC_{50}. Preliminary experiments should evaluate the effect of the ER concentration of the cytosol, assay volume and ^{3}H-E2 concentration on the IC_{50} calculation using inert E2. A good starting point for the ER competitive binding assay is to use enough cytosol to provide 50 - 100 µg protein per assay tube, with 0.5 -1.0 nM ^{3}H-E2 in a total assay volume of 0.5 mL. Suggested concentrations for test chemicals with a high affinity for the ER are 1×10^{-11} to 1×10^{-7} M; and 1×10^{-10} to 3×10^{-4} M for chemicals expected to have a lower binding affinity for the ER. Once assay conditions have been optimized, additional ER competitive binding assays should be conducted to compare chemicals with known affinities for the ER, such as DES, estrone, and ethynyl estradiol (positive controls), and the androgen agonist, R1881 (negative control). (See Pages 13 -14 of this protocol for Example Worksheet: ER Competitive Binding Assay). Data for the inert E2 standard curve and each test chemical should be plotted as the percent ^{3}H-E2 bound versus the molar concentration (log) of competitor. Estimates of IC_{50} should be determined using appropriate nonlinear curve fitting software such as GraphPad Prism (GraphPad Software, Inc., San Diego, CA). (See Pages 10 -12 of this protocol for additional comments on data analysis).

C. Checklist for Standardizing ER Binding Assays within Laboratory

i. Saturation Assays. If conducting the ER assay is new to the laboratory, several publications cited in the reference section of this protocol will be extremely useful when evaluating the data (e.g., Book edited by Hulme et. al., and the manuals from GraphPad Prism and Biosoft KELL). In general, when evaluating data from the ER saturation assays, the following points should be considered.

- As increasing concentrations of ^{3}H-E2 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., Nonlinear plots generally indicate a problem with the assay such as ligand depletion (concave plot) or incorrect assessment of non-specific binding (convex plot), etc.).

- Is the K_d within an acceptable range (e.g., 0.05 - 0.1 nM)? *Note:* Literature values for K_d using uterine cytosolic preparations have varied from 0.05 - 0.5 nM. The variation in K_d may be a reflection of different labs using radiolabeled estradiol with a wide range of specific activity (^{3}H-E2 vs ^{125}I-E2). In addition, publications by Salomonsson et al. (1994) and Kuiper et al. (1997, 1998) suggest that a lower Kd may be observed when assays conditions minimize ligand depletion, and that slightly different K_ds exist for ERα and ERβ.
- Are the standard errors for the K_d or B_{max} too high? Divide the standard error (SE) of the k_d by the k_d, and the SE of the B_{max} by the B_{max}. If either ratio is much larger than 20%, then the methods for the assay should be re-evaluated (GraphPad Prism Manual, 1999).
- Is non-specific binding too high? The value for non-specific binding should be less than 50% of the total binding (GraphPad Prism Manual, 1999).

ii. Competitive Binding Assays. Again, if the assay is new to the laboratory, it is suggested that the publications cited in the reference section be utilized to facilitate adequate evaluation of the data. In general, the assay should demonstrate that increasing concentrations of inert E2 can compete with a single concentration of ^{3}H-ER 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-E2 added per assay tube?
- Was the IC_{50} for inert E2 reasonable? (e.g The IC_{50} for inert E2 should be approximately equal to the molar concentration of ^{3}H-E2 used in the assay tube plus the K_d (determined by nonlinear analysis and Scatchard plot of data obtained from saturation radioligand binding assays).
- Are the data easily replicated with respect to IC_{50}s for inert E2, and selected test chemicals?

III. Estrogen Receptor Competitive Binding Assay: Working Protocol

1.0 Preparation of Assay Buffer

TEDG Buffer (10 mM Tris, 1.5 mM EDTA, 1.0 mM dithiothreitol, 10% glycerol).
Prepare buffer without dithiothreitol, adjust to pH 7.4 and store at 4^o C.
Add dithiothreitol just prior to use in assay.

2.0 Preparation of Trace

[2,3,6,7,16,17-^{3}H(N)]-estradiol (^{3}H-E2)
New England Nuclear (DuPont) No. NET-517
Store at 4 - 5 ^{o} C in original container.
Specific Activity (SA) may change with lot.
Obtain the highest specific activity available from the vendor.
Example: Lot # 33639215, Certification Date 5/16/01;
SA = 118 Ci/mmol (261,960 DPM/pmol).
Concentration: 1.0 mCi/mL
Note: SA should be adjusted for decay over time.

Dilute trace with TEDG assay buffer.
Each assay tube should contain 0.5 - 1 nM final concentration of ^{3}H-E2.

3.0 Selection of Receptor Concentration and Assay Volume

a. 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-E2 is bound to ER causing the concentration of the unbound (*free*) ^{3}H-E2 to significantly differ from the concentration of ^{3}H-E2 that was originally added to the assay tube. Hulme and Birdshall, 1992). A general rule of thumb is to optimize the assay conditions so that the ratio of the total ^{3}H-E2 bound in the absence of competitor, to the total ^{3}H-E2 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 - 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.

4.0 Preparation of E2 for the Standard Curve and nonspecific binding (NSB).

a. Standard Curve: A standard curve using inert E2 should be prepared for each ER competitive binding assay. Final concentrations of inert E2 in the assay tubes should range from 1.0×10^{-7} to 1.0×10^{-11} M. Prepare serial dilutions of E2 in ethanol (200 proof) to achieve the Final Concentrations shown below. Use siliconized glass tubes when preparing the standards. The following table shows recommended concentrations for the inert E2 standard curve.

Example of Preparation Procedure for Inert E2 Standard Curve

Concentrations for Inert E2 Standard Curve		
Standards	***Initial E2 Concentration (Molar)***	****Final E2 Concentration (Molar) in ER assay tube***
Negative Control	0 (Inert R1881)	0
0	0 (EtOH)	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.*		

b. Negative control: It is recommended a chemical be selected as a negative control (e.g., does not demonstrate any binding affinity for the ER), and one concentration of the chemical be

tested in each competitive binding assay. R1881, an androgen agonist, at a final concentration of 1 x 10^{-7} M is used in this protocol as the negative control.

5.0 Preparation of Test Chemicals

a. Stock Solutions: Test chemicals should be diluted in ethanol (200 proof) to 3.0 x 10^{-2} M (i.e., 30 mM). Use siliconized glass tubes when preparing dilutions. *Note:* Some test chemicals 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 specific chemical. Likewise, some chemicals may not be soluble in ethanol at all, so appropriate modifications in the ER assay should be made to accommodate any change in solvent.

b. Prepare serial dilutions of each test chemical in ethanol to yield the final concentrations as indicated below. *Note:* The serial dilutions shown in Table 2 are based upon the addition of 10 µl of each serial dilution of the test chemical in a final assay volume of 500 µl. *Caution:* No more than 0.2% ethanol should be used in the assay tubes.

Table 2 – Test Chemical Concentrations

Serial Dilutions of Test Chemical	***Initial Concentration (Molar)***	****Final Concentration in ER assay tube (Molar)***
Concentration 1	15 x 10^{-3}	3.0 x 10^{-4}
Concentration 2	5.0 x 10^{-3}	1.0 x 10^{-4}
Concentration 3	5.0 x 10^{-4}	1.0 x 10^{-5}
Concentration 4	5.0 x 10^{-5}	1.0 x 10^{-6}
Concentration 5	5.0 x 10^{-6}	1.0 x 10^{-7}
Concentration 6	5.0 x 10^{-7}	1.0 x 10^{-8}
Concentration 7	5.0 x 10^{-8}	1.0 x 10^{-9}
Concentration 8	5.0 x 10^{-9}	1.0 x 10^{-10}

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

6.0 Preparation of ER Assay Tubes

a. Label 12 x 75 mm round bottom assay tubes (siliconized glass) in duplicate as follows:

0, NSB, Neg, S1, S2, S3, S4, S5, S6
Unknown chemical 1- Concentration 1 (e.g., U1-C1, U1-C2, ... U1-C8)
Unknown chemical 2 -Concentration 1 (e.g., U2-C1, U2-C2, ...U2-C8)
Total DPMS: TC

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

Components of ER Competitive Binding Assay		
50	µL	Adjust amount of uterine cytosol to provide 50 - 100 µg protein/assay tube
430	µL	TEDG Assay Buffer
10	µL	^{3}H-E2 to yield final concentration of 0.5 - 1.0 nM
10	µL	Inert E2, negative control, or test chemical
500	µL	Total volume in each assay tube

c. 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^{o}C) to concentrate fluid at bottom of tube.)

d. Incubate assay tubes at 4^{o} C for 18 to 20 h. Assay tubes should be placed on a rotator during the incubation period.

6.0 Preparation of 60% Hydroxylapatite (HAP) Slurry

a. The day before beginning this step to separate the bound and free ^{3}H-E2, add 10 g HAP (BioRad) to 100 mL TEDG buffer and gently mix. Cap the container and place the HAP slurry in the refrigerator overnight. (This amount of HAP will generally yield enough slurry for 70 - 100 assay tubes.)

b. The next morning aspirate the supernatant and resuspend the HAP in fresh TEDG buffer. Allow HAP to settle and repeat wash. If HAP is prepared in a graduated cylinder, the amount of buffer needed to prepare a 60% HAP slurry can be estimated using the scale on the outside of the cylinder.

c. 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.

7.0 Separation of Bound ^{3}H-E2-ER and Free ^{3}H-E2

Note: To minimize dissociation of bound ^{3}H-E2 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. 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.

b. 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.)

c. Following the incubation period (step 7b), add 2.0 mL of the TEDG assay buffer, quickly vortex, and centrifuge at 4^{o}C for 10 minutes at 1000 x g.

d. At the end of the centrifugation, immediately decant the supernatant (e.g., containing the free ^{3}H-E2. The HAP pellet will contain the bound ^{3}H-E2-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.

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

f. Again quickly decant and discard supernatant. Repeat the wash and centrifugation steps once more.

g. After the final wash, decant the supernatant. Allow the assay tubes to drain briefly for 1 - 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 3H-E2 and bound ^{3}H-E2-ER has been completed. Assay tubes may be left at room temperature.

8.0 Extraction and Quantifying ^{3}H-E2 bound to ER

a. Add 1.5 ml of ethanol (100%) to each assay tube. Allow the tubes to sit at room temperature for 15 - 20 minutes, vortexing at 5 minute intervals.

b. Centrifuge the assay tubes for 10 minutes at 1000 x g. (Centrifuge can be set at 4^{o}C, but keeping the assay tubes cold is no longer critical at this point.)

c. Pipet an aliquot (usually 1.0 -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 (e.g., DPMs x 1.5 = Total DPMs bound).

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

9.0 Data Analysis

a. Terminology:

Total ^{3}H-E2: DPMs added to each assay tube
(e.g., can be converted to concentration of total ^{3}H-E2 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 inert E2)
Specific Binding: DPMs for each concentration of standard or test chemical minus the mean DPM of the NSB tubes.

b. Data Analysis:

i. IC_{50} calculation: Data for the inert E2 standard curve and each test chemical should be plotted as the percent ^{3}H-E2 bound versus the molar concentration (log) of competitor. Estimates of IC_{50} should be determined using appropriate nonlinear curve fitting software such as GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

ii. Relative Binding Affinity (RBA): The RBA for each competitor (test chemical) should be calculated by dividing the IC_{50} for E2 by the IC_{50} of the competitor and expressing as a percent (e.g., RBA for E2 =100 %).

c. Checklist for Evaluating ER Competitive Binding Assay Data

i. Inert E2 Standard Curve. The assay should demonstrate that increasing concentrations of inert E2 can displace ^{3}H-E2. The IC_{50} for E2 should be approximately equal to the molar concentration of ^{3}H-E2 plus the K_d (determined by Scatchard analysis). (Prism, GraphPad). The IC_{50}s for the inert E2 standard curve should be easily replicated.

ii. IC_{50}s for test chemicals should be easily replicated.

iii. The ratio of total binding in the absence of competitor to the amount of ^{3}H-E2 added per assay tube should not be greater than 10%.

IV. References

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Example worksheet: Includes a standard curve, a test chemical, and quality control measures

Date and time of assay:_____ Person conducting the assay:_____

Radioligand: ^{3}H-E2 lot number:_____, specific activity: _____Ci/mmole, _____ DPM/pmol,

Uterine Cytosol batch number _____, Protein concentration _____mg/mL ,
Amt. Protein used per assay vial: _____ug,

Date uteri harvested_____ : Date of cytosol preparation_____

Posn and tube label	*Competitor Concentration*	*Competitor*	*Initial Concentration (Molar)*	*Receptor (ul)*	*Buffer*	*Tracer*	*Competitor*	*HAP*	*Final (Molar)*
1	0	EtOH		50	430	10	10	250	
2	0	EtOH		50	430	10	10	250	
3	NSB	Inert E2	5E-6	50	430	10	10	250	1E-7
4	NSB	Inert E2	5E-6	50	430	10	10	250	1E-7
5	S1	Inert E2	5E-7	50	430	10	10	250	1E-8
6	S1	Inert E2	5E-7	50	430	10	10	250	1E-8
7	S2	Inert E2	5E-8	50	430	10	10	250	1E-9
8	S2	Inert E2	5E-8	50	430	10	10	250	1E-9
9	S3	Inert E2	1.67E-8	50	430	10	10	250	3.33E-10
10	S3	Inert E2	1.67E-8	50	430	10	10	250	3.33E-10
11	S4	Inert E2	5E-9	50	430	10	10	250	1E-10
12	S4	Inert E2	5E-9	50	430	10	10	250	1E-10
13	S5	Inert E2	1.67E-9	50	430	10	10	250	3.33E-11
14	S5	Inert E2	1.67E-9	50	430	10	10	250	3.33E-11
15	S6.	Inert E2	5E-10	50	430	10	10	250	1E-11
16	S6	Inert E2	5E-10	50	430	10	10	250	1E-11
17	Neg.	Inert R1881	5E-6	50	430	10	10	250	1E-7
18	Neg.	Inert R1881	5E-6	50	430	10	10	250	1E-7
19	u1-c1	Chemical 1	15E-3	50	430	10	10	250	3E-4
20	u1-c1	Chemical 1	15E-3	50	430	10	10	250	3E-4
21	u1-c2	Chemical 1	5E-3	50	430	10	10	250	1E-4
22	u1-c2	Chemical 1	5E-3	50	430	10	10	250	1E-4
23	u1-c3	Chemical 1	5E-4	50	430	10	10	250	1E-5
24	u1-c3	Chemical 1	5E-4	50	430	10	10	250	1E-5
25	u1-c4	Chemical 1	5E-5	50	430	10	10	250	1E-6
26	u1-c4	Chemical 1	5E-5	50	430	10	10	250	1E-6
27	u1-c5	Chemical 1	5E-6	50	430	10	10	250	1E-7
28	u1-c5	Chemical 1	5E-6	50	430	10	10	250	1E-7
29	u1-c6	Chemical 1	5E-7	50	430	10	10	250	1E-8
30	u1-c6	Chemical 1	5E-7	50	430	10	10	250	1E-8
31	u1-c7	Chemical 1	5E-8	50	430	10	10	250	1E-9
32	u1-c7	Chemical 1	5E-8	50	430	10	10	250	1E-9
33	u1-c8	Chemical 1	5E-9	50	430	10	10	250	1E-10
39	u1-c8	Chemical 1	5E-9	50	430	10	10	250	1E-10
40	0	EtOH		50	430	10	10	250	
41	0	EtOH		50	430	10	10	250	
42	NSB	Inert E2	5E-6	50	430	10	10	250	1E-7
43	NSB	Inert E2	5E-6	50	430	10	10	250	1E-7
44	Neg.	Inert R1881	5E-6	50	430	10	10	250	1E-7
45	Neg.	Inert R1881	5E-6	50	430	10	10	250	1E-7
46	Hot	Total Counts		—	—	10	—	—	
47	Hot	Total Counts		—	—	10	—	—	
48	Hot	Total Counts		—	—	10	—	—	
49	Hot	Total Counts		—	—	10	—	—	
50	Hot	Total Counts		—	—	10	—	—	