INTERLABORATORY REPORT

Task Order 06:

Second Inter-Laboratory Validation of the Estrogen Receptor Binding Assay
(Rat Uterine Cytosol)

Task 7 and Option:

Test Coded Chemicals

RTI Project 0210114.006

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ENDOCRINE DISRUPTOR SCREENING PROGRAM (EDSP)
LABORATORY ASSAY VALIDATION SERVICES
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RTI INTERNATIONAL

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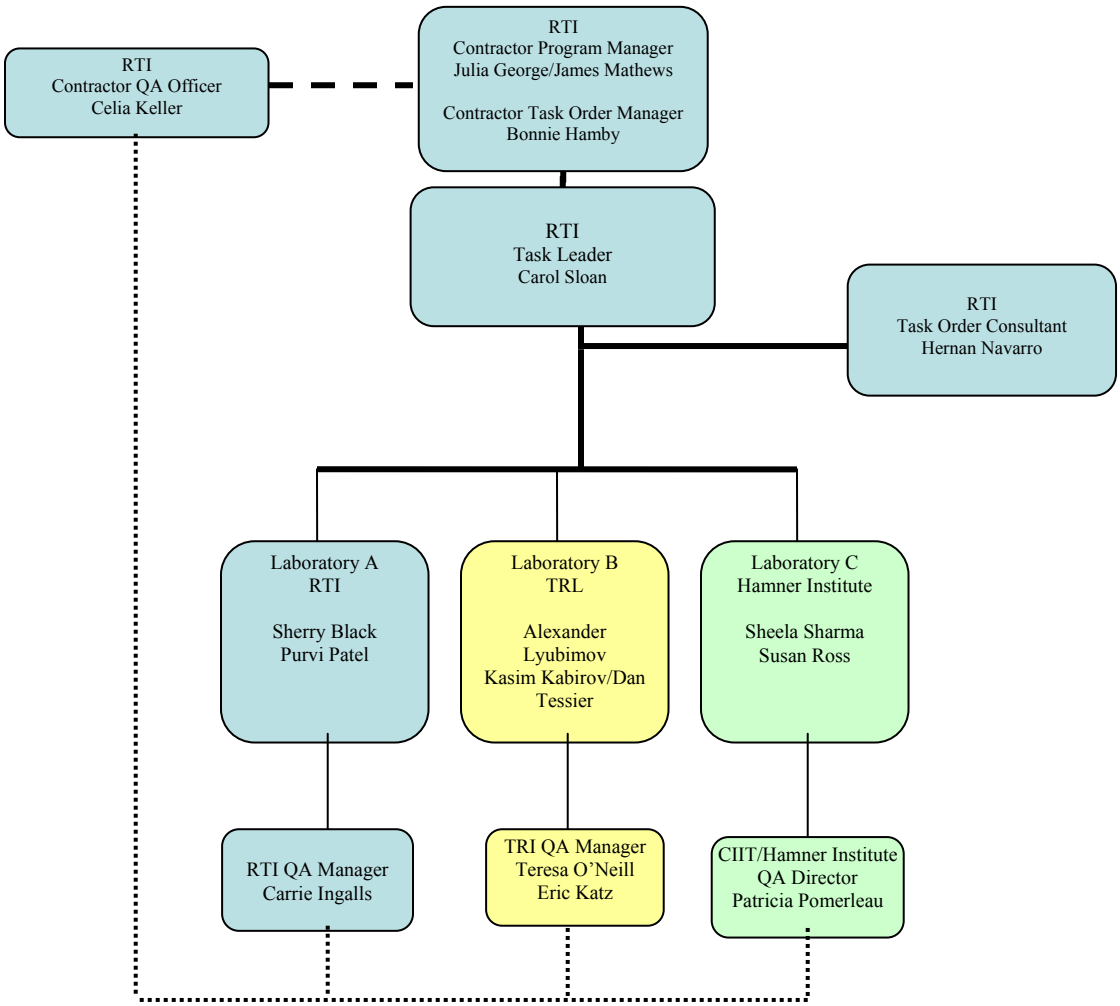
1.0 Introduction

The purpose of this task is to establish whether competent laboratories with no training in this specific protocol can obtain acceptable results for the 43 coded chemicals, positive chemical (17ß-estradiol), and weak positive control (norethynodrel) within criteria for acceptable performance by using the written protocol provided by USEPA.

2.0 Organizational Chart



Organizational Chart for Second Inter-Laboratory Validation of the Estrogen Receptor Binding Assay (Rat Uterine Cytosol)

3.0 Cytosol Preparation

The rat uterine cytosol (RUC) was prepared by the laboratories according to the EPA furnished protocol (Appendix I). The rats were purchased from three different animal vendors (Table 1).

Table 1. Comparison of RUC Preparation

	RTI (Lab A)	TRL (Lab B)	Hamner (Lab C)
Source of rats	Charles River	Taconic	Harlan
Age of rats (days) at ovariectomy	94/94	84-90	88-93
Days after ovariectomy that uteri were removed	8/7	8-10	8
Strain of rats	Sprague-Dawley	Sprague-Dawley	Sprague-Dawley
Rats ovariectomized at source	yes	yes	yes
Number of cytosol preparations	2	5	3
Date ovariectomized	1/14/08, 3/4/08	2/8/08, 2/13/08, 2/22/08, 5/28/08	2/19/08
Uteri removed at source and frozen	no	no	yes
Date uteri removed	1/22/08, 3/11/08	2/18/08, 2/21/08, 2/22/08, 3/3/08, 6/6/08	2/27/08
Dates cytosol made	1/23/08, 3/19/08	2/18/08, 2/21/08, 2/22/08, 3/3/08, 6/6/08	2/29/08, 4/20/08, 6/12/08
Protein concentration of cytosol batches (mg/mL)	2.2, 2.3	1.16, 3.16, 2.69, 2.75, 2.52	8.09, 6.67, 2.5

3.1 Problems Encountered

RTI scientists noticed that some of the animals still had ovarian tissue present, which was removed from the animal along with the uterus. All uteri were kept in separate bags after collection and flash freezing, and they were identified as to animal number and weight. It is not known if this had any effect on the binding experiments that were conducted with the uteri, although there was an attempt to avoid the use of these particular uteri in the assays. Since Hamner bought the uteri removed from the ovariectomized females, therefore they would have no way of knowing if the surgeries had been complete. RTI scientists also noticed that some

uteri still appeared to be stimulated by endogenous estrogen when uteri were removed at eight days after the ovariectomy, which would suggest that the period should be longer than 8 days after ovariectomy when removing uteri.

The protein content of two of the three cytosol batches prepared at Hamner appeared to be much higher than those of the other laboratories or those from Task 6 of this task order. It is not known what effect this had on the overall assays performed using those batches of cytosol compared to those of the other laboratories.

4.0 Laboratory Performance

4.1 Results

The laboratories were instructed to follow the protocol provided and demonstrate 3 runs that met the performance criteria that were stated in the protocol (Appendix I). Summaries of the number of runs performed by the three participating laboratories for each of the first 23 coded chemicals are presented in Tables 2-4. Some chemicals required many more runs to meet the goal of three acceptable (meeting the performance criteria) runs. For the option of 20 coded chemicals, the laboratories were to run as many runs as were possible in the remaining time on the task order. RTI ran multiple runs on all chemicals, TRL did on several chemical and Hamner ran each chemical code once.

The prism graphs for the laboratory designated acceptable runs are presented in Appendix II for all 43 coded chemicals. The option actually consisted of 4 duplicates of five individual chemicals. The assay resulted in similar results for some of the chemicals, such as bisphenol A, norethynodrel, and equol, which were positive and octyltriethoxysilane which was clearly negative. Some chemicals resulted in assay results that were scattered and made interpretation and classification by the laboratories more difficult.

Table 2. Run Table for RTI

RTI							
Chemical Code	**No. of Runs Performed**	**Codes of Runs (2008) Acceptable Runs are in Green**					**No. of Acceptable Runs as Determined by Laboratory**
1	5	1-1	1-2	1-3	1-4	1-5	3
2	3	2-1	2-2	2-3			3
3	5	3-1	3-2	3-3	3-4	3-5	3
4	4	4-1	4-2	4-3	4-4		3
5	4	5-1	5-2	5-3	5-4		3
6	4	6-1	6-2	6-3	6-4		3
7	4	7-1	7-2	7-3	7-4		3
8	5	8-1	8-2	8-3	8-4	8-5	3
9	3	9-1	9-2	9-3			3
10	3	10-1	10-2	10-3			3
11	3	11-1	11-2	11-3			3
12	3	12-1	12-2	12-3			3
13	4	13-1	13-2	13-3	13-4		3
14	3	14-1	14-2	14-3			3
15	3	15-1	15-2	15-3			3
16	4	16-1	16-2	16-3	16-4		3
17	3	17-1	17-2	17-3			3
18	3	18-1	18-2	18-3			3
19	3	19-1	19-2	19-3			3
20	3	20-1	20-2	20-3			3
21	3	21-1	21-2	21-3			3
22	4	22-1	22-2	22-3	22-4		3
23	5	23-1	23-2	23-3	23-4	23-5	3

Table 3. Run Table for Hamner

HAMNER							
Chemical Code	**No. of Runs Performed**	**Dates of Runs (2008) Acceptable Runs are in Green**					**No. of Acceptable Runs as Determined by the Laboratory**
12	5	3/5	3/6	3/10	3/17	3/24	3
19	4	3/5	3/6	3/10	3/11		3
11	3	3/5	3/6	3/10	3/11		3
21	3	3/11	3/12	3/17			3
15	3	3/11	3/12	3/17			3
22	3	3/26	3/27	3/31			3
16	5	3/18	3/26	3/27	4/2	6/11	3
5	3	3/26	3/27	3/31			3
17	5	4/2	4/3	4/7	4/8	4/10	3
9	8	4/2, 4/3, 4/7	6/13, 6/19	6/25	7/1	7/2	3
4	4	4/3	4/7	4/8	4/9		3
14	6	4/8	4/9	4/10	4/14	4/16, 4/17	3
20	5	4/9	4/10	4/14	4/16	4/17	3
3	16	4/14, 4/15, 5/7	5/15, 5/16, 5/18	5/23, 5/27, 5/28	6/12, 6/13, 6/16, 6/19	6/23, 6/24, 6/25	3
23	8	4/15, 5/1	5/7, 5/15	5/16, 5/27	5/28	6/11	3
1	5	4/15	5/1	5/7	5/18	5/22	3
8	5	5/1	5/7	5/19	5/22	5/27	3
2	10	5/19, 5/28	6/2, 6/3	6/7, 6/9	6/17, 6/18	6/23, 6/24	3
10	9	5/21, 6/2	6/3, 6/7	6/9, 6/16,6/17	6/23	6/24	3
18	3	6/4	6/5	6/6			3
13	4	5/21	5/29	5/30	6/6		3
7	4	6/2	6/3	6/5	6/6		3
6	4	6/2	6/3	6/5	6/6		3

Table 4. Run Table for TRL

TRL							
Chemical Code	**No. of Runs Performed**	**Run Date / Run Identifier**					
		1	**2**	**3**	**4**	**5**	
12	5	3/11/2008 TTN001	3/27/2008 TTN003	4/2/2008 RN001	4/16/2008 TTN006	6/11/2008 RN013	
19	6	3/11/2008 TTN001	4/14/2008 TTN005	4/9/2008 RN002	4/21/2008 TTN007	5/14/2008 TTN017	5/12/2008 TTN016
11	6	3/17/2008 TTN002	4/7/2008 TTN004	4/16/2008 TTN006	5/12/2008 RN008	5/14/2008 TTN017	5/12/2008 TTN016
21	6	3/27/2008 TTN003	4/14/2008 TTN005	4/2/2008 RN001	4/16/2008 TTN006	5/14/2008 TTN017	5/12/2008 TTN016
15	6	3/17/2008 TTN002	4/7/2008 TTN004	4/9/2008 RN002	4/16/2008 TTN006	5/14/2008 TTN017	5/12/2008 TTN016
22	4	4/17/2008 RN003	4/21/2008 TTN007	4/22/2008 TTN008	4/23/2008 TTN009		
16	3	4/21/2008 TTN007	4/22/2008 TTN008	4/23/2008 TTN009	4/30/2008 TTN012		
5	3	4/21/2008 TTN007	4/22/2008 TTN008	4/23/2008 TTN009			
17	5	4/17/2008 RN003	4/22/2008 TTN008	4/23/2008 TTN009	4/28/2008 TTN010	5/5/2008 TTN013	
9	5	4/28/2008 TTN010	4/29/2008 TTN011	4/30/2008 TTN012	5/5/2008 TTN013	5/7/2008 TTN014	5/21/08 RN011
4	6	4/17/2008 RN003	4/28/2008 TTN010	4/29/2008 TTN011	4/30/2008 TTN012	5/5/2008 TTN013	5/7/2008 TTN014
14	5	4/28/2008 TTN010	4/29/2008 TTN011	5/5/2008 TTN013	5/7/2008 TTN014	5/21/08 RN011	
20	5	4/29/2008 TTN011	4/30/2008 TTN012	5/7/2008 TTN014	5/8/2008 TTN015	6/12/2008 TTN018	5/21/08 RN011
3	5	5/8/2008 TTN015	5/13/2008 RN009	5/29/08 RN012	6/12/2008 TTN018	6/16/2008 TTN019	
23	5	5/8/2008 TTN015	5/13/2008 RN009	5/29/08 RN012	6/12/2008 TTN018	6/16/2008 TTN019	
1	5	5/8/2008 TTN015	5/20/2008 RN010	5/29/08 RN012	6/12/2008 TTN018	6/16/2008 TTN019	5/21/08 RN011
8	6	5/7/2008 RN007	5/12/2008 RN008	5/20/2008 RN010	5/29/08 RN012	6/11/2008 RN013	6/16/2008 TTN019
2	5	5/7/2008 RN007	5/12/2008 RN008	5/13/2008 RN009	5/20/2008 RN010	6/11/2008 RN013	
10	4	5/7/2008 RN007	5/12/2008 RN008	5/13/2008 RN009	5/20/2008 RN010		

TRL (continued)							
Chemical Code	**No. of Runs Performed**	**Run Date / Run Identifier**					
		1	**2**	**3**	**4**	**5**	
18	3	4/24/2008 RN004	5/1/2008 RN005	5/6/2008 RN006			
13	4	5/1/2008 RN005	5/6/2008 RN006	5/7/2008 RN007	6/11/2008 RN013		
7	3	4/24/2008 RN004	5/1/2008 RN005	5/6/2008 RN006			
6	3	4/24/2008 RN004	5/1/2008 RN005	5/6/2008 RN006			
All criteria met				Criteria: For estradiol and norethynodrel curves Bottom plateau between -5.0 and 1.0 Top plateau between 90.0 and 110.0 Hill slope between -1.1 and -0.7 SD for estradiol less than 5.0 SD for norethynodrel less than 5.7			
Some criteria out of range by < 5%							
Criteria not met							
Bad assay; e.g., all low DPM, aberrant control curve							

After the runs were completed the laboratories were required to classify the total 43 chemicals as positive, negative or equivocal binders. Criteria were stated in the protocol to guide the laboratories in classification of the chemicals.

4.2 Discussion of Chemical Classifications by Laboratories

There was general agreement across laboratories for the chemicals that were classified as positive in the first 23 chemicals (Table 5). RTI and Hamner had all of the strong and moderately positive chemicals (17β-estradiol, 17-ethynylestradiol, DES, meso-Hexestrol, genistein, norethynodrel, equol, zearalenone, and tamoxifen) listed as positive. Of the chemicals in these categories, TRL classified 17β-estradiol, DES, and tamoxifen as equivocal.

For the negative chemicals, RTI and Hamner had corticosterone listed as positive, and TRL had it as equivocal when run in the 23-chemical portion of the task order. This is especially interesting since it was also one of the optional chemicals and was correctly classified as negative by RTI and Hamner in all but one case where it was called equivocal by RTI. TRL had called it equivocal as part of the 23 chemicals, and called it negative twice and equivocal twice in the optional portion. Progesterone and octylreiethoxysilane were classified as negative by all three laboratories. Atrazine was classified correctly as negative by Hamner and TRL but positive by RTI since one of their runs had parameters that classified it as positive.

The weak chemicals are highlighted in Table 5 and had various classifications by the laboratories. Butyl paraben and bisphenol A were classified as positive by all of the laboratories. Benz(a)anthracene was classified as negative by all of the laboratories. Nonyphenol (mixture), 4-n-heptylphenol, and enterolactone were positive, negative and equivocal for RTI, Hamner, and TRL, respectively. Kepone was positive in two laboratories and equivocal in the third. 5α-dihydrotestosterone was negative in two laboratories and equivocal in the third. O,p'-DDT was negative in two laboratories and positive in the third.

R1881 that was designed to be a negative control for the assay proved to be positive for the control runs, and as a coded chemical it was positive for RTI and Hamner and equivocal for TRL.

For the other four chemicals used as optional chemicals (Table 6), dexamethosone was classified correctly as negative by RTI and Hamner on all occasions and negative once, equivocal twice and positive once by TRL. HPTE was classified positive in all instances by RTI and Hamner and positive twice by TRL and twice equivocal by them. Genistein was classified positive on all runs by RTI and Hamner and positive for three runs by TRL and negative on one of their runs. Norethindrone was classified as positive for all runs by RTI and TRL and positive for three runs for Hamner and negative on one run by them.

Table 5. Classification of Chemicals

Code	Compound	Affinity	Laboratories		
			RTI	Hamner	TRL
1	17β-Estradiol	Strong	Positive	Positive	Equivocal
2	17-Ethynylestradiol	Very strong	Positive	Positive	Positive
3	DES	Very strong	Positive	Positive	Equivocal
4	Meso-Hexestrol	Strong	Positive	Positive	Positive
5	Genistein	Moderate	Positive	Positive	Positive
6	Norethynodrel	Moderate	Positive	Positive	Positive
7	Butyl paraben (n-butyl 4-hydroxybenzoate)	Weak	Positive	Positive	Positive
8	Nonylphenol (mixture)	Weak	Positive	Negative	Equivocal
9	o,p'-DDT	Weak	Negative	Positive	Negative
10	Corticosterone	Negative	Positive	Positive	Equivocal
11	Equol	Moderate	Positive	Positive	Positive
12	Zearalenone	Strong	Positive	Positive	Positive
13	Tamoxifen	Strong	Positive	Positive	Equivocal
14	5α-Dihydrotestosterone	Weak	Negative	Negative	Equivocal
15	Bisphenol A	Weak	Positive	Positive	Positive
16	4-n-heptylphenol	Weak	Positive	Negative	Equivocal
17	Kepone (Chlordecone)	Weak	Positive	Positive	Equivocal
18	Benz(a)anthracene	Weak	Negative	Negative	Negative
19	Enterolactone	Weak	Positive	Negative	Equivocal
20	Progesterone	Negative	Negative	Negative	Negative
21	Octyltriethoxysilane	Negative	Negative	Negative	Negative
22	Atrazine	Negative	Positive	Negative	Negative
23	R1881	Negative	Positive	Positive	Equivocal
27	Corticosterone,4		Negative	Negative	Equivocal
28	Dexmethasone,1		Negative	Negative	Negative
29	Norethindrone,2		Positive	Positive	Positive
30	Dexamethasone,3		Negative	Negative	Equivocal
31	Corticosterone,3		Equivocal	Negative	Equivocal

Table 5. Classification of Chemicals (continued)

Code	Compound	Affinity	Laboratories		
			RTI	Hamner	TRL
32	HPTE,1		Positive	Positive	Positive
33	HPTE,2		Positive	Positive	Equivocal
34	Genistein,3		Positive	Positive	Negative
35	Dexmethasone,4		Negative	Negative	Positive
36	Norethindrone,1		Positive	Negative	Positive
37	Genistein,2		Positive	Positive	Positive
38	Corticosterone,1		Negative	Negative	Negative
39	Norethindrone,4		Positive	Positive	Positive
40	Genistein,4		Positive	Positive	Positive
41	Genistein,1		Positive	Positive	Positive
42	HPTE,3		Positive	Positive	Equivocal
43	HPTE,4		Positive	Positive	Positive
44	Norethindrone,3		Positive	Positive	Positive
45	Dexmethasone,2		Negative	Negative	Equivocal
46	Corticosterone,2		Negative	Negative	Negative

Table 6. Classification of Optional Chemicals (Arranged by Chemical)

Code	Chemical	Affinity	Laboratories		
			RTI	Hamner	TRL
45	Dexmethasone,2	Negative	Negative	Negative	Equivocal
28	Dexmethasone,1		Negative	Negative	Negative
30	Dexamethasone,3		Negative	Negative	Equivocal
35	Dexmethasone,4		Negative	Negative	Positive
32	HPTE,1	Positive	Positive	Positive	Positive
33	HPTE,2		Positive	Positive	Equivocal
42	HPTE,3		Positive	Positive	Equivocal
43	HPTE,4		Positive	Positive	Positive
37	Genistein,2	Positive	Positive	Positive	Positive
34	Genistein,3		Positive	Positive	Negative
40	Genistein,4		Positive	Positive	Positive
41	Genistein,1		Positive	Positive	Positive
46	Corticosterone,2	Negative	Negative	Negative	Negative
27	Corticosterone,4		Negative	Negative	Equivocal
31	Corticosterone,3		Equivocal	Negative	Equivocal
38	Corticosterone,1		Negative	Negative	Negative
29	Norethindrone,2	Positive	Positive	Positive	Positive
36	Norethindrone,1		Positive	Negative	Positive
39	Norethindrone,4		Positive	Positive	Positive
44	Norethindrone,3		Positive	Positive	Positive

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5.0 ER Binding Assay Suggestions

5.1 Assay Protocol

In general, RTI felt the assay procedures were well described. However, there were too many confirmatory assays run that added time and cost to the project. For example, three trials of saturation binding assays were required for every new cytosolic preparation, and E2 and NOR control curves were run with every set of unknowns. The goal should be to run the minimum number of confirmatory assays that permit one to monitor assay performance.

1. **Conduct abbreviated pilot studies.** Screen all compounds in triplicate at 10 μM and only characterize those compounds that inhibit [^{3}H]estradiol binding by 50% or more. In the present experiments, this would have successfully identified all non-negative binders and indicated which compounds would have benefited from adjustment to lower concentrations. This should significantly cut down on the number of reruns required and the cost of characterizing compounds.
2. **The Kd determinations should only be done one time**, as this value should not vary as long as the assay conditions are unchanged. Bmax could change with different preparations, so each new cytosolic preparation could be simply checked by running several total and NSB samples and adjusting the amount of assay protein accordingly.
3. **Consider not running the standards with every assay**. Perhaps limit to running E2 and NOR once with each new cytosolic preparation. If standards are run with each assay, then suggest running E2 and NOR only at their respective IC_{20} and IC_{80} values.
4. **Stability of materials.** Clearly note that the storage conditions specified in the protocol for labile chemicals take precedent over those supplied by the supplier. Stability of the [^{3}H]estradiol preparation should be determined in advance of these investigations, and a maximum interval of use defined. Vials sealed under inert atmosphere and containing an amount anticipated for one month of use would help assure radiolabel quality. Standardization of receptor preparation and maximal storage interval should be defined in advance. Solutions of other key labile reagents, such as dithiothreitol, should be made fresh daily.
5. **Solubility of chemicals**. (All labs) Problems arose when chemicals were placed into solution and then precipitated out when exposed to the cold conditions required for conduct of the assays. This occurred at various concentrations for the different

laboratories. The protocol should allow for trying various solvents per chemical and explain more clearly that different concentrations can be used when necessary

6. **Stating types or brands of reagents and supplies to use in the assays directly in the protocol or on a supply list.** This was mentioned by all laboratories to RTI during the course of the studies. There was an initial problem with not being able to locate sources of siliconized tubes and a substitution had to be made. Also the sources for HAP were a problem since they didn't always have it on hand and the labs all finally settled on using a prepared slurry from BioRad. RTI was asked by the labs to furnish a supply list but only made suggestions when problems arose. Perhaps a "suggested supply list" would be helpful to laboratories performing the assay in the future.
7. **There were places in the protocol where the wording could have been clearer.** TRL would have liked a clearer description of the uterine tissue/cytosol preparation. They were the only laboratory that removed the uteri and prepared the cytosol in the same day. This resulted in having to prepare smaller, more numerous batches (Table 1).
   RTI noted that the Prism software provides for outlier omission but the protocol didn't make it clear if that could be used. All laboratories felt that data interpretation was ambiguous.
8. RTI and Hamner felt that the fact that the **small amount (10µL) of test chemical and standard** used in the assay was difficult to pipet in a manner that would not introduce variation in the assay results.

5.2 Data Analysis

1. **Initial Data Manipulations.** All the data and data manipulations should be in one file. The Excel/Prism file system made it difficult to determine where the graphed values came from. We suggest inputting the raw data into Prism and using its transformation functions to calculate specific binding, log concentration and percent bound.
2. **Curve Fitting.** We suggest analyzing data together from the same runs and using shared top and bottom in the fit. Doing so could reduce the number of out-of-specification assays. If it is clear that high percent bound values are the result of low counts in the total binding samples, then permit the data to be normalized using the "Normalize" function in Prism. This will not change the percent error or the IC50; that is, it will not

improve or degrade the data. However, it should increase the number of assays that are within specifications.

3. For ease in evaluating the data, an informative, consistent file naming protocol should be used, with links between Excel and Prism files.

6.0 ACKNOWLEDGEMENTS

We appreciate the long hours and many weekends spent by the three participating laboratories: RTI International, Hamner Research Institutes, and Toxicology Research Laboratory and to Ms. Sherry Black who graciously compiled the composite graphs for this report.

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Appendix I

Protocol

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

Endocrine Disruptor Screening Program
U.S. Environmental Protection Agency
April 2007

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1.0 Purpose of the assay

This assay is used to determine the ability of a compound to compete with 17β-estradiol for binding to the estrogen receptors (ERs) isolated from the rat uterus. The quantitative measures desired from the assay are the IC_{50} (the concentration of test chemical needed to displace half of the radiolabeled ligand from the receptor) and the relative binding affinities (RBAs) of test substances for the estrogen receptor (relative to 17β-estradiol). The primary purpose for this assay is as a screening procedure to detect substances that can bind to the estrogen receptors in an isolated rat uterine cytosolic fraction.

2.0 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.

3.0 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
R1881	Methyltrienolone (negative control)
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.

4.0 Equipment and materials

4.1 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 4° 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 4° 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 -80° C, freezer -20° C, refrigerator 4° C
- Traceable thermometers
  for monitoring refrigerator and freezer temperatures
  - 80° C freezer (temperature recorded daily during the business week and monitored for off-hour emergencies)
  - 20° C freezer (temperature recorded daily during the business week and monitored for off-hour emergencies)

4° 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

4.2 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 ($[^3H]E_2$), 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, hydrated, CAS 1306-06-5
- 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
- R1881 (Methyltrienolone), CAS 965-93-5, Mol. Wt. 284.4; 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

4.3 Supplies

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

4.4 Software

4.4.1 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)
Statistical Analysis System (SAS Institute Inc., Cary, NC)

4.4.2 Spreadsheet software

For example, Microsoft Excel or compatible.

5.0 Preparation of buffer solutions

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

5.1 Stock solutions

May be used for up to 3 months.

5.1.1 200 mM EDTA stock solution

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

5.1.2 100 mM PMSF stock solution

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

Note: The PMSF stock solution is highly toxic.

5.1.3 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 4° C. Once cool, adjust pH to 7.4 and bring the final volume to 1.0 liter. Store at 4° C.

5.1.4 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!)

5.2 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 4° C. Add to TEDG buffer immediately before use.)
Bring to 100 ml with cold (4° C) ddH_2O.

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

6.0 Preparation of rat uterine cytosol

6.1 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) 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 of age),
- 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.

6.2 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 4 -7 mg/ml. Be sure to report the calculations and results of the protein determination in the final report to EPA.

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.*

7.0 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 8.6 but there are no specific performance criteria. Criteria for acceptable performance of known standards in the competitive binding assay are discussed in Section 9.7.3. 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.

8.0 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.

8.1 Preliminary steps

8.1.1 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 meet performance criteria.
c) If performance criteria are not met, determine potential areas for error and repeat experiment.

Summary table of assay conditions

		Saturation Binding Assay Protocol
Type 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		4° 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 reduced if ligand depletion is observed with 50 µg protein/tube.

8.1.2 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 paragraph 5.2 and/or Appendix A: Buffer preparation worksheet.

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

Prepare on the day of the assay.
Store [^{3}H]-17β-estradiol at 4° 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:

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

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 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.

Table 1. Preparation procedure for radiolabeled 17β-estradiol dilution

Column A	*Column B*		*Column C*		*Column D*		*Column E*	*Column F*
Tube #	**Volume of stock to add for diluted**	**+**	**Volume of**	**=**	**Total volume of diluted [^{3}H]-**	**at**	**Diluted [^{3}H]-17β-estradiol**	**Final [^{3}H]-17β-estradiol**

	concentration		buffer to add		17β-estradiol		concentration	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.*

8.1.4 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 secondary stock by pipetting 1 ml of the 300 µM stock and mix with 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 assay 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. Preparation procedure for unlabeled 17β-estradiol dilution

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 17β-estradiol	at	Diluted 17β-estradiol concentration	Final 17β-estradiol concentration (nM) in ER assay tube*

HC8	Use 100 µl of stock 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.*

8.1.5 Standardization of receptor concentration

Initially, approximately 50 micrograms of protein (rat uterine cytosol) per assay tube shall be used in the assay as calculated from the results of the protein content determination (Section 6.2). If greater than approximately 15% of radiolabeled ligand specifically binds and/or the Scatchard plot is extremely non-linear, consider reducing protein concentration (i.e., estrogen receptor number).

Since 100 µl of cytosolic preparation will be added to the final assay volume, dilute the cold (but thawed, if previously frozen) cytosol with cold (4° C) TEDG + PMSF assay buffer, so that the concentration of protein is reduced from the concentration determined in Section 6.2 to the stock concentration chosen above (initially, 50 µg/100 µl but lower if this initial concentration is found to cause ligand depletion). Be sure to keep the cytosol at 4° C at all times (even while thawing) to minimize degradation of the receptor. Discard any unused cytosol; do not refreeze it.

8.2 Preparation of ER Saturation Binding Assay tubes

- Label 12 x 75 mm round-bottom siliconized 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
100 µl	100 µl	--	Uterine cytosol (diluted to 50 µg protein/100 µl solution, or lower if required to keep ligand depletion acceptably low)
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 volume

- Vortex assay tubes quickly but completely after additions are completed. Be sure that tubes do not warm above 4° 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 (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.

8.3 Preparation of 60% HAP slurry

- Prepare HAP slurry the day before the step 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 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 mix. 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 (4° C) for at least 2 hours.

- Aspirate or decant the supernatant and re-suspend the HAP in fresh TEDG + PMSF buffer to 100 ml. Mix gently. Again, allow the HAP to settle for ~2 hours (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 4° 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% 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.

8.4 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 (4° 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 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 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. The assay tubes may be left at room temperature.

8.5 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. 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).

8.6 Data analysis

8.6.1 Terminology

8.6.1.1 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.)

8.6.1.2 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).

8.6.1.3 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).

8.6.1.4 Specific binding

Total binding minus non-specific binding.

8.6.1.5 K_d

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

8.6.1.6 B_{max}

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

8.6.2 General considerations

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 (i.e., K_d = 0.05 to 0.5 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 micrograms of 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., BioSoft; McPherson, 1985; 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 0.5 nM and B_{max} of 36 to 44 fmol ER/100 microgram protein (equivalent to 0.036 to 0.044 nM ER when 50 microgram protein is used in a total assay volume of 0.5 ml).

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 several runs entered into and analyzed by GraphPad Prism software for curve-fitting is also provided in the file "example saturation data.pzf".

8.6.3 Evaluation of results from the Saturation Binding Assay

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? I.e., was maximum specific binding reached, indicating saturation of ER with ligand?

- 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].

- Is the K_d reasonable?

Note: Literature values for K_d using rat uterine cytosol 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 ([^{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 K_d 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 SE of the K_d to the mean K_d or the SE of the B_{max} to the mean B_{max} is larger than 20%, then the assay should be re-run carefully.

- Is non-specific binding excessive? The value for non-specific binding should be less than 50% of the total binding.

- If any of the above criteria are not met, it may be useful to repeat the saturation experiment with an appropriately adjusted concentration of protein.

8.7 Test report

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

8.7.1 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)

8.7.2 Radioinert ligand (17β-estradiol)

- Supplier, batch, and catalog number
- CAS number
- Purity
- Concentrations added to NSB tubes

8.7.3 Estrogen receptor

- Type and 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.

8.7.4 Test conditions

- Composition of buffer(s) used.
- Protein concentration used.
- Total volume per assay tube.
- 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, if any.

8.7.5 Results

For each run, report at least the following. Be sure to include the run identifier on each product.

- Date of run, number of days since SA certification date, and adjusted SA on day of run.
- 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 curve (one per run for each of total, specific, and non-specific binding) on the same graph.
- Graph of measured concentrations in the total [^{3}H]-17β-estradiol tubes.
- Scatchard plot.
- Raw data (decays per minute) for each tube.
- Estimated K_d

- Estimated B_{max}

If more than one acceptable Saturation Binding Assay was run, also plot the data and curves from up to three runs on a single plot, being sure to distinguish runs (e.g., by color) and type of curve (total, specific, non-specific) (e.g., by line style).

8.7.6 Discussion

Address the items listed in Section 8.6.3, Evaluation of results from the Saturation Binding Assay.

8.7.7 Conclusion

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

9.0 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.

9.1 Preliminary steps

9.1.1 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 (Tables 4 and 5).
e) Perform calculations for number of tubes in the run (Section 9.3.1).
f) Label and set up tubes for standard curve dilutions (see 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.

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.

Summary table of assay conditions:

		Competitive Binding Assay Protocol
Type 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		4° 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 decreased if significant ligand depletion occurs at 50 µg protein/tube.
** Range and spacing of test substance concentrations may need to be adjusted depending on solubility

9.1.2 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 paragraph 5.2 and Appendix A: Buffer preparation worksheet.

9.1.3 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 (i.e., 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.

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.

9.1.4 Standardization of receptor concentration and assay volume

Before performing a competitive binding assay, the receptor concentration of the cytosol and the assay volume per assay tube are 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]. Ideally, 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 should be less than 10% at equilibrium. That is, ([^{3}H]-E2 bound)/ ([^{3}H]-E2 total) ≤10%. Decreasing the amount of cytosol protein and/or increasing the assay volume will generally lower this ratio. EPA found that 50 µg of cytosolic protein per assay tube in a total assay volume of 500 µl generally provides good results. However, laboratories may find it necessary to

reduce the amount of protein added per tube to keep ligand depletion acceptably low (below approximately 15%). If ligand depletion was found to be a problem when doing the Saturation Binding Assay using 50 µg of cytosolic protein per assay tube, it would be appropriate to start with the concentration that was found to be acceptable in that assay.

Since 100 µl of cytosolic preparation will be added to the final assay volume, dilute the cold (but thawed, if previously frozen) cytosol with cold (4° C) TEDG + PMSF assay buffer, so that the concentration of protein is reduced from the concentration determined in Section 6.2 to the stock concentration chosen above (initially, 50 µg/100 µl but lower if this initial concentration is found to cause ligand depletion). Be sure to keep the cytosol at 4° 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 controls, reference standard, and test chemicals

9.2.1 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 (R1881) 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

When choosing a solvent, ethanol should always be the first choice followed if necessary by DMSO[1]. If the test chemical is known from previous information to be insoluble in ethanol at the level required for this assay, the reference for that information should be reported; the assay does not have to be run using that solvent. The solvent should be tested at the highest concentration that is added with the test substance. 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.

Negative control

The final concentration range to test for the negative control is from 1×10^{-10} to 1×10^{-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

[1] Note that the solvent used for a test chemical must also be used for the reference chemical (inert 17β-estradiol) and the control chemicals (norethynodrel and R1881) for that particular run. 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. The examples in this section assume that the solvent being used is ethanol.

be prepared by following the guidance in Section 9.2.3, "Serial dilutions of test substance". The molecular weight of R1881 is 284.4 grams/mole, so to make 1 ml of a 100 mM stock solution, add 28.44 mg of R1881 to 900 µl of absolute ethanol and bring the volume to 1 ml with absolute ethanol. Dilutions are prepared as in Table 6.

Weak positive control

The final concentration range to test for the positive control is from $1 \times 10^{-8.5}$ to 1×10^{-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 absolute ethanol in a volumetric flask. Add sufficient absolute ethanol 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 assay buffer to achieve the final concentrations shown in Table 4.

Table 4. Concentrations and volumes 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 buffer 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 microliters of diluted concentration is used in a total volume of 500 microliters.*

9.2.2 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 to achieve the final concentrations shown below. Use appropriate screw-cap light-sensitive containers for storage containers. Table 5 shows the concentrations required for the unlabeled 17β-estradiol standard curve.

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. Non-specific binding depends on only the concentration of radiolabeled estradiol used; and since in competitive binding assays this concentration is kept constant, measuring NSB once per run should suffice. However, EPA has found that there can be drift in the non-specific binding value when a large number of tubes are prepared in a single run (e.g., when more than one test chemical is assayed in a single run). Thus, NSB tubes should be prepared and included at the end of each run as well as at the beginning. Do not simply prepare the end-of-run NSB tubes with the other NSB tubes and position them at the end of the run. Instead, prepare the end-of-run NSB tubes at the end of the run.

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 absolute ethanol in a volumetric flask. Add sufficient absolute ethanol 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 absolute ethanol 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 assay buffer 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. Concentrations and volumes 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 buffer to add*	=	*Total volume of diluted standard 17β-estradiol*	at	*Diluted 17β-estradiol concentration (Molar)*	*Final 17β-estradiol concentration (Molar) in ER assay tube**
	--		--		--		0 (R1881)	0
	--		--		--		0 (EtOH)	0
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).

9.2.3 Serial dilutions of test substance

Each dilution is prepared in the appropriate solvent to yield the final concentrations as indicated below. Visually inspect the test substance (use a light box if available) to confirm that it is in solution prior to making the dilutions. 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. If the highest concentration cannot be prepared in either solvent (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 9.7.2.

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 ethanol solvent concentration does not exceed 3% (or the DMSO concentration does not exceed 10%).*

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.

3) The solvent used for the following dilutions shall follow the protocol in section 9.2.1 above, using ethanol as the preferred solvent. If the highest concentration is not soluble in ethanol, DMSO may be used. Only if neither of these solvents work may the highest concentration be reduced below the upper limit. Reminder: the concentration of ethanol in the final assay volume (including the ethanol in the PMSF stock) may not exceed 3%; the final concentration of DMSO, if used, may not exceed 10%.

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 9.3.2.

Table 6. Concentrations and volumes 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 buffer 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.*

9.3 Preparation of ER Competitive Binding Assay tubes

Label 12 x 75 mm round bottom siliconized assay tubes (glass) in triplicate with codes for the untreated control, the solvent control, the 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. Examples of competitive assay tube layouts using three, four, or five unknown test chemicals are provided in Appendix B.

9.3.1 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.

9.3.2 Individual tubes

- Add 390 µl/tube of the master mixture above and keep on ice. Prepare the standard, weak standard, negative, and competitors 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, 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 50 µg protein/100 µl solution, or lower if required to keep ligand depletion acceptably low)
500	Total volume in each assay tube

Note: Make sure that that the temperature of the tubes and contents are at 4° 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.

9.4 Preparation of 60% HAP slurry

- Prepare HAP slurry the day before the step 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, R1881, and non-specific or blank 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 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).

- 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% 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 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.

9.5 Separation of bound [^{3}H]-17β-estradiol-ER 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. It is important to continue to keep the assay tubes cold.

- Following the vortexing step, add 2.0 ml of the cold (4° 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.

9.6 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 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).

9.7 Data analysis

9.7.1 Terminology

9.7.1.1 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.)

9.7.1.2 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).

9.7.1.3 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, even if, as recommended in Section 9.2.2, NSB tubes are included at both the top and the bottom of each run in order to compensate for any drift that may have occurred while preparing the other tubes in the run. There are two tubes per run (per replicate).

9.7.1.4 Specific binding

Total binding minus non-specific binding.

9.7.2 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 binder, a non-binder, 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 a binder or non-binder 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. 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.

Estimates of $\log(IC_{50})$ values should be determined using appropriate nonlinear curve fitting software to fit a 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.

Appendix C shows how to estimate $\log(IC_{50})$ and its standard error. Examples are given. It is important to characterize the variability of the $\log(IC_{50})$ using the method given.

In order to ensure that the assay has been performed with enough care to provide reliable results, EPA has placed limits on the variability of the data points at each concentration within a run for the reference chemical and for the positive and negative controls. Instructions on how to calculate the within-run standard deviation of data points is given in the file "lab $ run # chem @ YYMMDD SDw.xls" .

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% more radioligand binding (that is, 10% 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.

9.7.3 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 (e.g., the test chemical may be binding to multiple binding sites, the test chemical may not be soluble at higher concentrations in the assay buffer, or the test chemical is changing the pH of the reaction mixture). When the assay is correctly performed, inert estradiol exhibits typical one-site competitive binding behavior.
- The IC_{50} value for 17β-estradiol is approximately equal to the molar concentration of [^{3}H]-17β-estradiol plus the K_d determined from the saturation binding assay. The K_d determined from the competitive binding assay is less reliable and should not be used here.
- 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%. Ideally, it is less than 10%.
- The parameter values (top, bottom, and slope) for 17β-estradiol, the concurrent positive control (norethynodrel), and the concurrent negative control (R1881) are within the confidence limits for historical data. (See Table 9 for the acceptable ranges for the parameters.)
- The within-run variation for each run for 17β-estradiol, norethynodrel, and R1881 is no larger than the upper limit shown in Table 9. (See file "lab $ run # chem @ YYMMDD SDw.xls" for instructions on how to determine within-run variation.) Performance criteria for within-run variation across multiple runs, and for between-run variation, are not available even though these quantities must be calculated and reported.
- 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%.

- The negative control substance (R1881) does not displace more than 25% of the radioligand from the ER at any concentration.

Table 9. Upper and lower limits for parameters in Competitive Binding Assay curves for the standards (radioinert estradiol, norethynodrel, and R1881)

Parameter	Unit	Estradiol		Norethynodrel		R1881	
		Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit
$SD_{within\text{-}run}$ (within-run variation)	% binding	NA	5.0	NA	5.7	NA	10
Bottom plateau level	% binding	-5.0	1.0	-5.0	1.0	NA	NA
Top plateau level	% binding	90.0	110.0	90.0	110.0	NA	NA
(Hill) Slope	$\log_{10}(M)^{-1}$	-1.1	-0.7	-1.1	-0.7	NA	NA

9.7.4 Classification criteria

Classification of a test chemical is based on the results of three non-concurrent runs, each of which meet the performance criteria. Until further guidance is available from EPA, laboratories may average the log(IC_{50}) values obtained from the three runs to determine the appropriate classification.

A substance is classified as "**positive**" for binding to the ER if a log(IC_{50}) value can be obtained from an unconstrained curve fitted to the Hill equation that has a slope of approximately -1.0. There must be at least one data point at which 50% or more of the radioligand has been displaced; the log(IC_{50}) may not be extrapolated from data which show less than 50% displacement, even if an appropriate Hill curve can be fitted.

Due to solubility constraints (for example), some test substances might induce a significant reduction in binding of the radioligand to the ER but without reducing it below 50%. Until additional information becomes available about the significance of this category of dose response curves, substances with acceptable binding curves which reach 25% displacement but not 50% at the highest concentration with acceptable data should be classified as "**equivocal**[2]".

Test chemicals whose fitted curves have slopes that are unusually steep or unusually shallow should also be classified as "**equivocal**". Justification for classifying a chemical on this basis should cite the Hill slope obtained.

Substances for which acceptable binding curves can be fit, and which do not displace more than 25% of the radioligand from the receptor, are classified as "**negative**".

[2] In these cases, an additional K_i experiment may be useful to determine if the test chemical is truly a competitive inhibitor. An experimentally derived K_i requires adding increasing concentrations of radiolabeled ligand in the presence of fixed concentrations of test chemical and then plotting the data on a double reciprocal plot (Laws et al., 1996; Kelce et al, 1995; Segel, 1993). A pattern of lines that intersects on the ordinate axis is characteristic of competitive inhibition. Slopes obtained from each of the double reciprocal plots are then re-plotted as a function of the inhibitor concentration. The slope and intercept of the re-plot can be used to calculate a K_i value for the test chemical (Segel, 1993). Experimental K_i determination is not a requirement of the Endocrine Disruptor Screening Program.

Substances for which no binding curve can be fit and for which no data point at any concentration displaces more than 25% of the radioligand are also classified as "**negative**".

9.8 Test report

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

9.8.1 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)

9.8.2 Solvent/Vehicle

- Justification for choice of solvent/vehicle if other than ethanol.
- Maximum concentration of solvent in assay tubes. (Show calculations.)
- Information to demonstrate that the solvent/vehicle, if other than an established solvent, does not bind to, or otherwise affect, the ER.

9.8.3 Reference estrogen (viz., inert estradiol)

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

9.8.4 Estrogen receptor

- Type and 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.

9.8.5 Test conditions

- K_d of the reference estrogen. Report the K_d obtained from the Saturation Binding Assay for each batch of cytosol prepared, as well as the K_ds obtained from curve-fitting the Competitive Binding Assays of inert estradiol for each run.
- Concentration range of the reference estrogen tested.
- Composition of buffer(s) used.
- Concentration range and spacing of test substance, with justification if deviating from required range and spacing.
- Volume of vehicle used to dissolve test substance and volume of test substance added.
- Incubation time and temperature.
- Concentration range and spacing of positive, negative, and solvent/vehicle controls.
- 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 K_i and IC_{50} values (software used, formulas, etc.).
- Statistical methods used, if any.

9.8.6 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 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 of each data point, along with the unconstrained curve fitted to the Hill equation, which demonstrates the performance of the test chemical in repeat runs. 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. Data and curves shall be differentiated by run (i.e., the reference chemical, positive control, negative control, and test chemical data points and fitted curve from Run A should be differentiated from those of Run B and Run C, and Run B should be differentiated from Run C).

- K_i (calculated from the Cheng-Prusoff equation) and log(IC_{50}) values and confidence limits 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 shall be included.

9.8.7 Discussion

- K_d and log(IC_{50}) values for reference ligand, including ranges, means, and standard deviations.

- Reproducibility of the K_d and log(IC_{50}) values of the reference ligand.

- Positive control data with ranges, means, and standard deviations.

- Compare the K_ds obtained for 17β-estradiol to the historical range for this substance (0.05 to 0.5 nM).

- Compare the estimated IC_{50} values for 17β-estradiol to the value expected from the formula IC_{50} = [radioligand] + K_d .

9.8.8 Conclusion

- Classification of test substance with regard to in vitro ER-binding activity (positive, equivocal, or negative).

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

9.9 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.

10.0 References

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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 4° 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 4° 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(s)

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 examples 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. The title on this sheet, "Laboratory Code X", should be changed to reflect the Laboratory Code assigned by the coordinator if multiple laboratories are involved. The same code should be substituted for "X" on the Saturation and Competitive Binding Assay worksheets. Please do not forget to change the headers of the sheets as well. The coordinator shall provide the decoding sheet for the Laboratory Codes to EPA.
   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. There are three worksheets provided for Competitive Binding. They differ only in the number of test chemicals per run – three, four, or five.
b. One sheet should be submitted per run.
c. Sheets should be identified by type of assay (Saturation, "S", or Competitive, "C"), Lab Code and run ID on the tab. For example, C-A-1 would identify Competitive Binding Assay from Lab A, Run 1.
d. Column O (from row 34) is where the decay-per-minute (dpm) values for each tube are entered by the user.
e. 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".
f. 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.
g. 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.

h. 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.

i. 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}) and its standard error 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}) and its standard error 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[3]. 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.

[3] 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%):

$$50 = \text{Bottom} + (\text{Top}-\text{Bottom})/(1+10^{((\text{LogEC50}-X)*\text{HillSlope})})$$

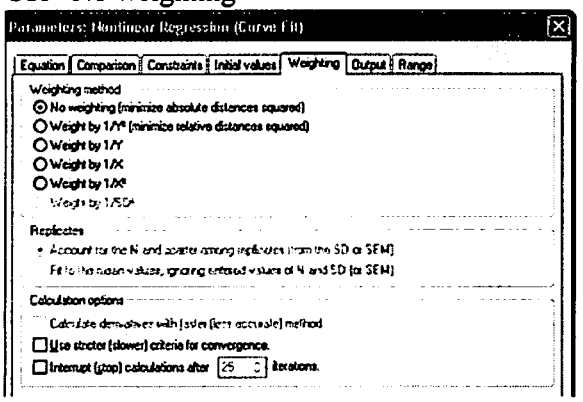
Prism can do this calculation. To set this up, we need to include a fake data point that has a "missing" X value in the data set. By including this fake data point and checking the "Unknown from standard curve" box, we can make Prism report the X value corresponding to Y = 50 % (which is the definition 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.

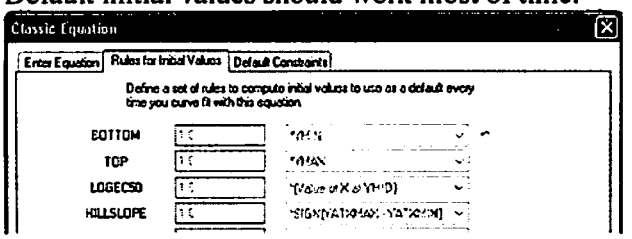
Details of options in Prism software

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)".

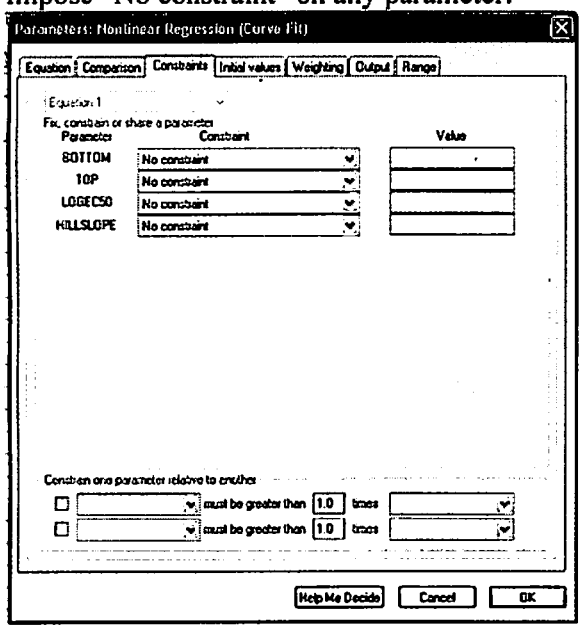
Use "No weighting"



Default initial values should work most of time.



Impose "No constraint" on any parameter.[4]



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

[4] 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)))

In our experience, the statistical software package Stata often is able to fit a model to a data set which Prism is unable to fit. The SAS statistical software is also likely to be able to fit a model where Prism cannot.

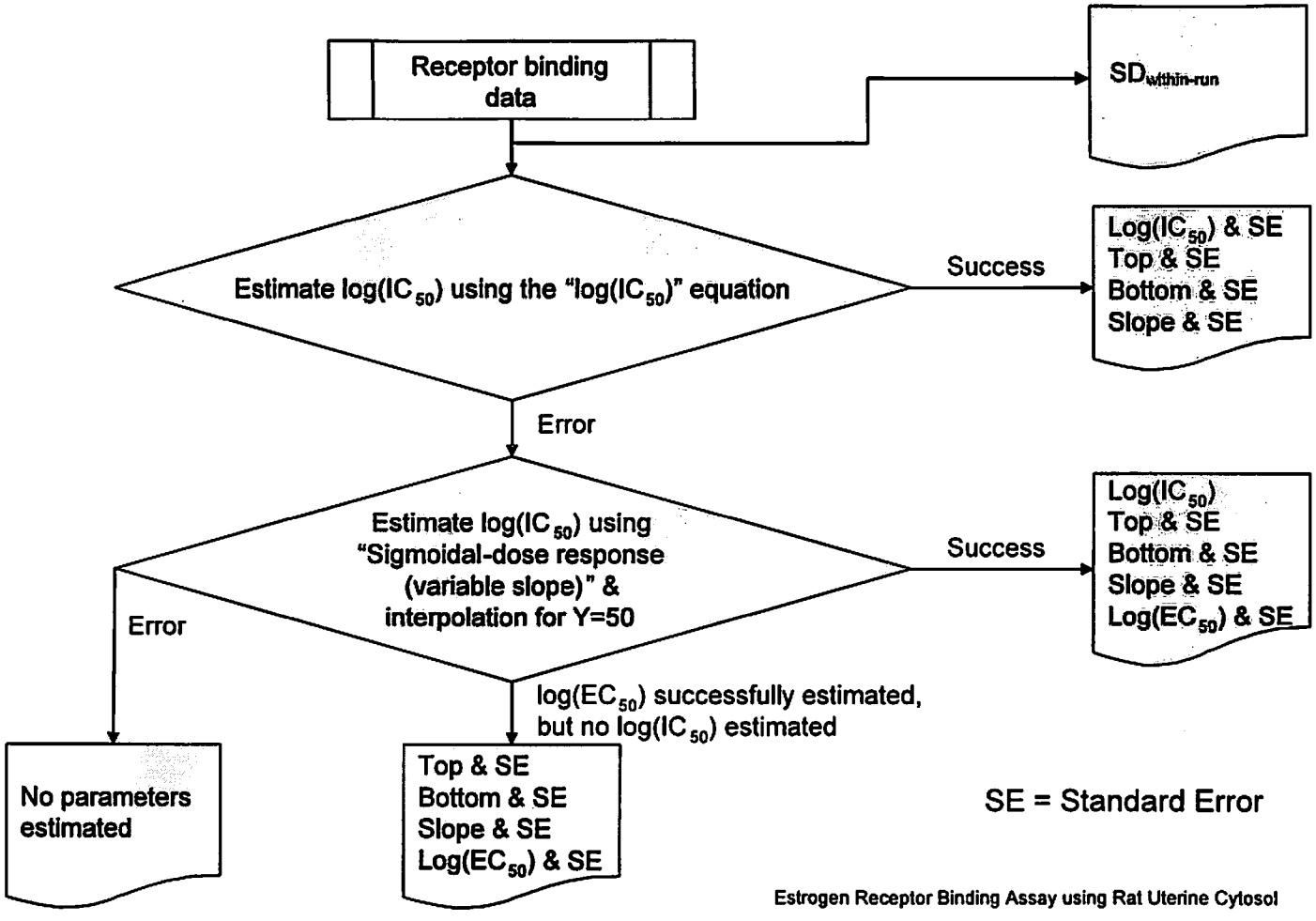
What to report

The attached flowchart shows the values that must be reported, depending on how successful the different Methods are.

It is important that log(IC_{50}) and log(EC_{50}) be clearly distinguished from each other when reporting. Different areas of the Data Entry and Analysis spreadsheet have been provided for log(IC_{50}) runs and log(EC_{50}) runs.

Note: If log(IC_{50}) is interpolated from the log(EC_{50}) model-fit, enter the Prism output in the log(EC_{50}) block but enter the interpolated log(IC_{50}) value in the log(IC_{50}) block. The rest of the log(IC_{50}) block will remain empty.

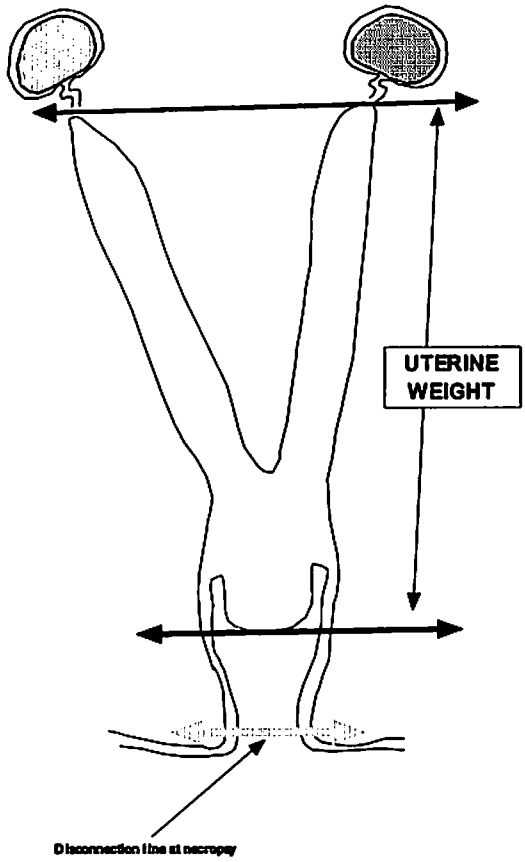
What to report for each run for each chemical



Estrogen Receptor Binding Assay using Rat Uterine Cytosol
USEPA Endocrine Disruptor Screening Program, July 2006

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.

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Appendix II

TC1-23 and Option

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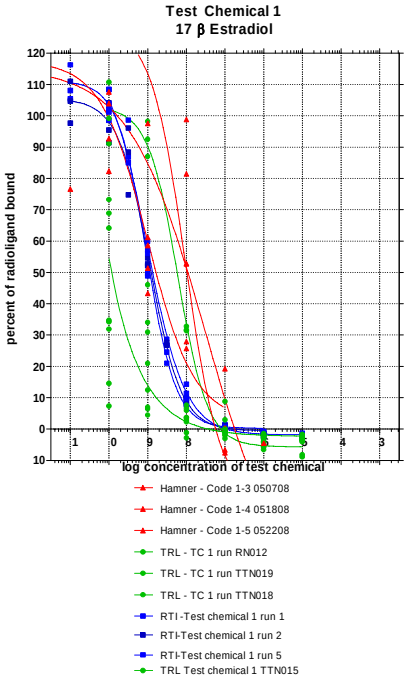
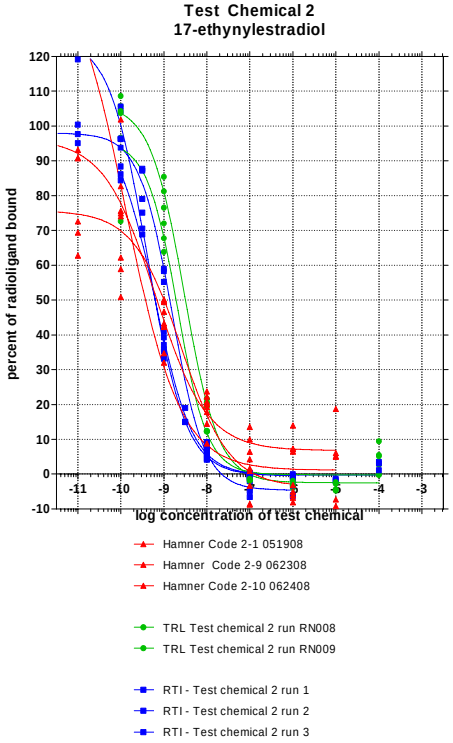
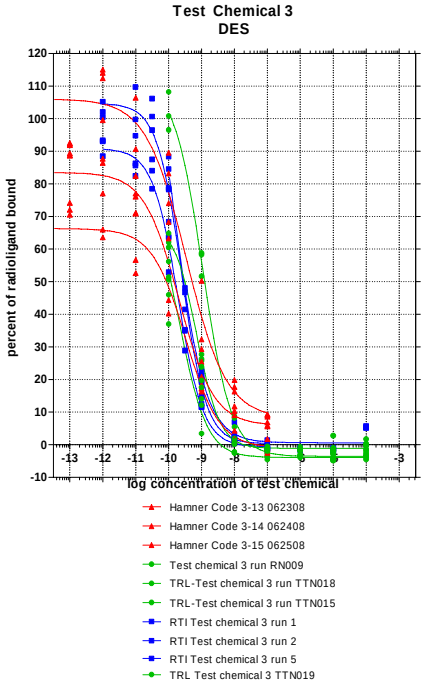
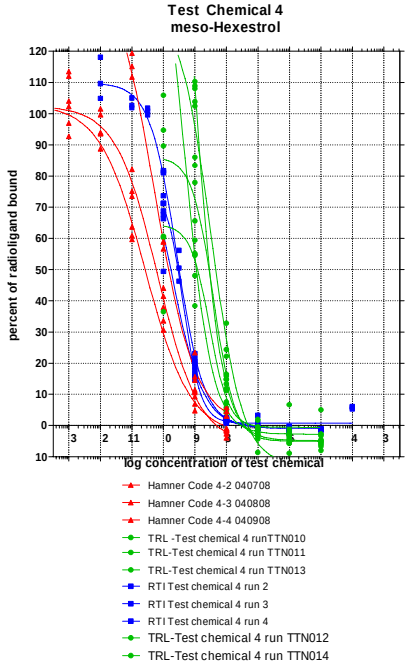
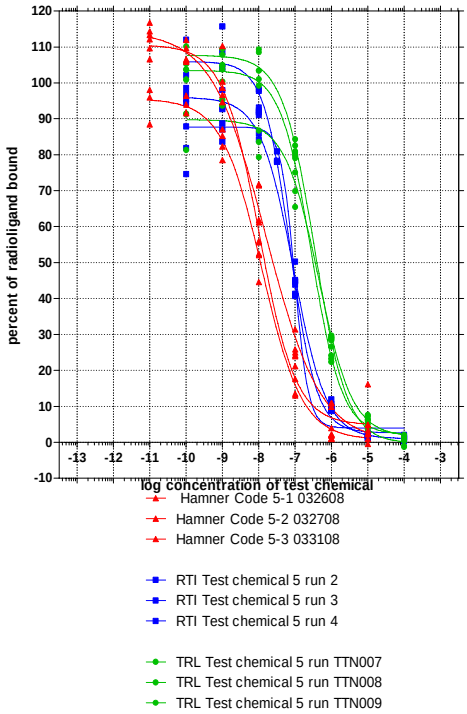

Test Chemical 1
17 β Estradiol
percent of radioligand bound
log concentration of test chemical
Hamner - Code 1-3 050708
Hamner - Code 1-4 051808
Hamner - Code 1-5 052208
TRL - TC 1 run RN012
TRL - TC 1 run TTN019
TRL - TC 1 run TTN018
RTI -Test chemical 1 run 1
RTI-Test chemical 1 run 2
RTI-Test chemical 1 run 5
TRL Test chemical 1 TTN015


Test Chemical 2
17-ethynylestradiol
percent of radioligand bound
log concentration of test chemical
Hamner Code 2-1 051908
Hamner Code 2-9 062308
Hamner Code 2-10 062408
TRL Test chemical 2 run RN008
TRL Test chemical 2 run RN009
RTI - Test chemical 2 run 1
RTI - Test chemical 2 run 2
RTI - Test chemical 2 run 3

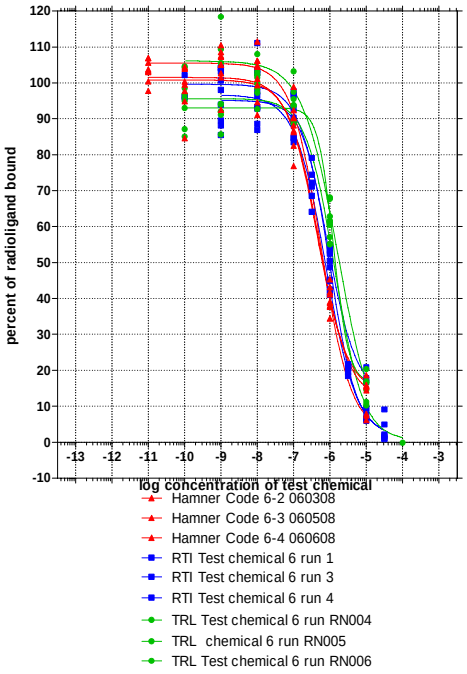

Test Chemical 3
DES
percent of radioligand bound
log concentration of test chemical
Hamner Code 3-13 062308
Hamner Code 3-14 062408
Hamner Code 3-15 062508
Test chemical 3 run RN009
TRL-Test chemical 3 run TTN018
TRL-Test chemical 3 run TTN015
RTI Test chemical 3 run 1
RTI Test chemical 3 run 2
RTI Test chemical 3 run 5
TRL Test chemical 3 TTN019


Test Chemical 4
meso-Hexestrol
percent of radioligand bound
log concentration of test chemical
Hamner Code 4-2 040708
Hamner Code 4-3 040808
Hamner Code 4-4 040908
TRL -Test chemical 4 runTTN010
TRL-Test chemical 4 run TTN011
TRL-Test chemical 4 run TTN013
RTI Test chemical 4 run 2
RTI Test chemical 4 run 3
RTI Test chemical 4 run 4
TRL-Test chemical 4 run TTN012
TRL-Test chemical 4 run TTN014

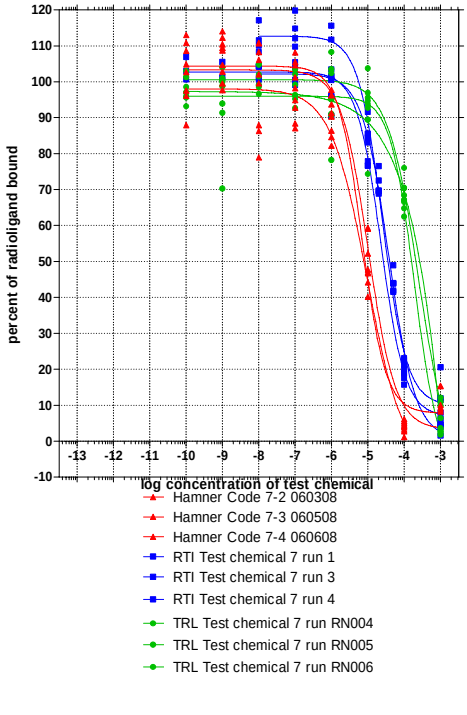
Test Chemical 5
Genistein



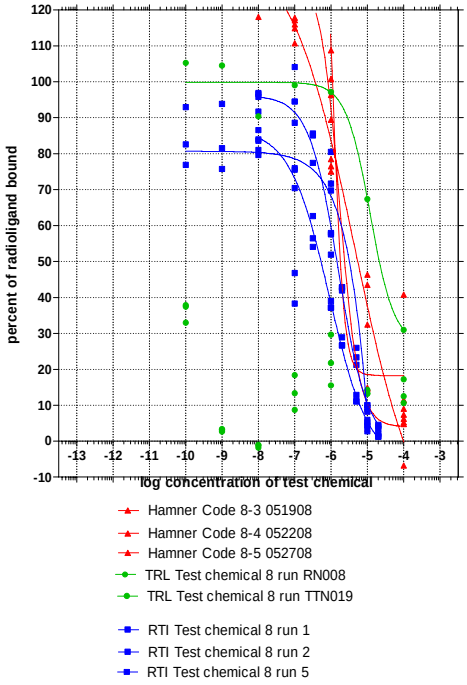
Test Chemical 6
Norethynodrel

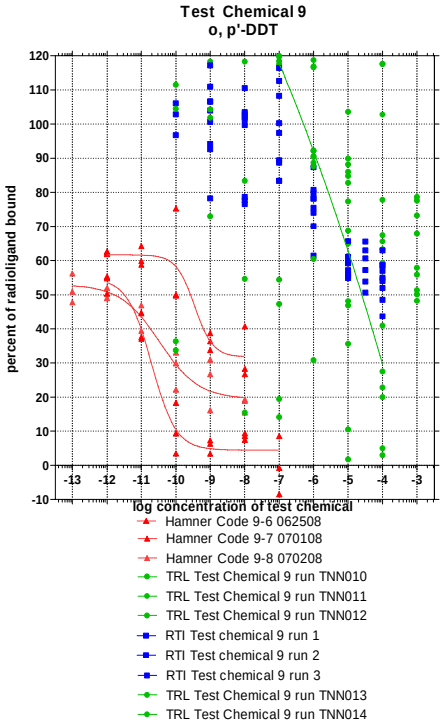


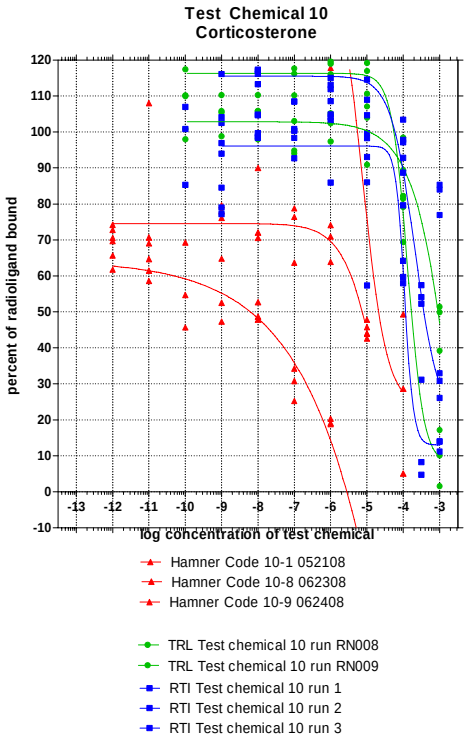
Test Chemical 7
Butyl paraben

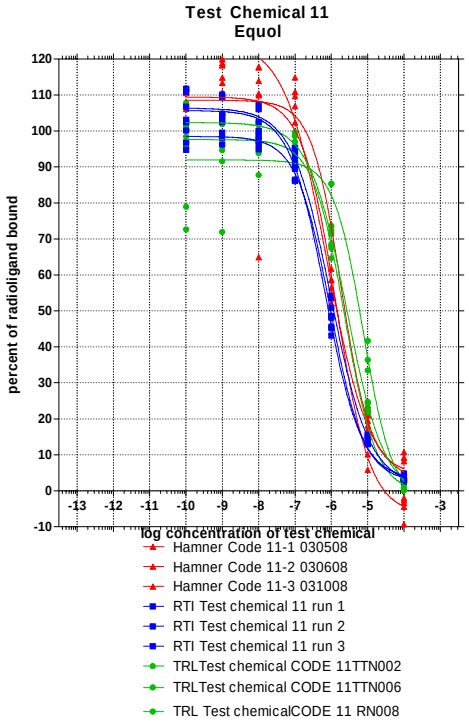


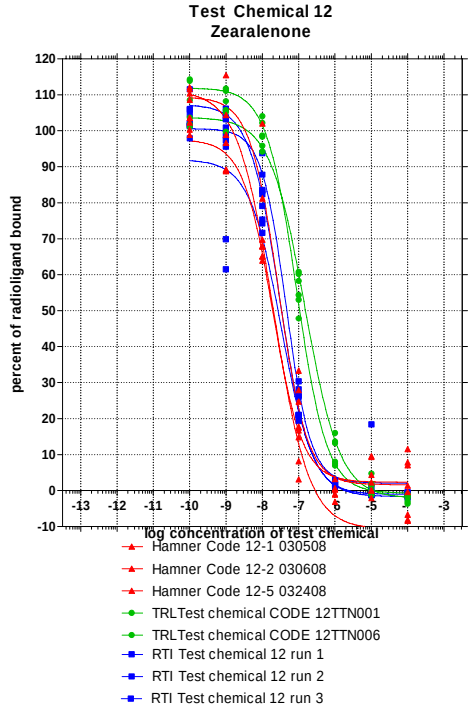
Test Chemical 8
Nonylphenol

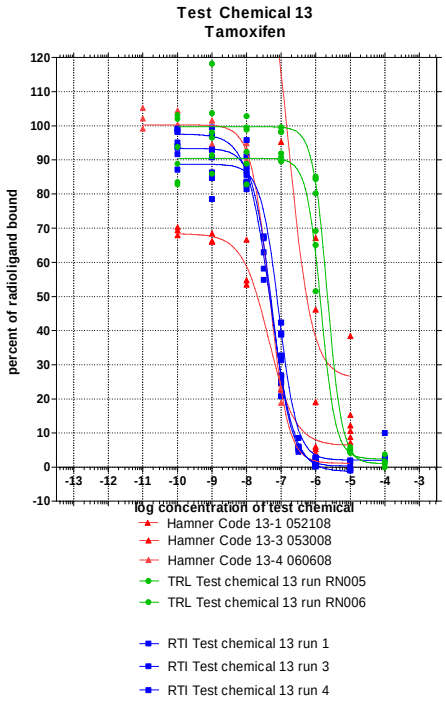


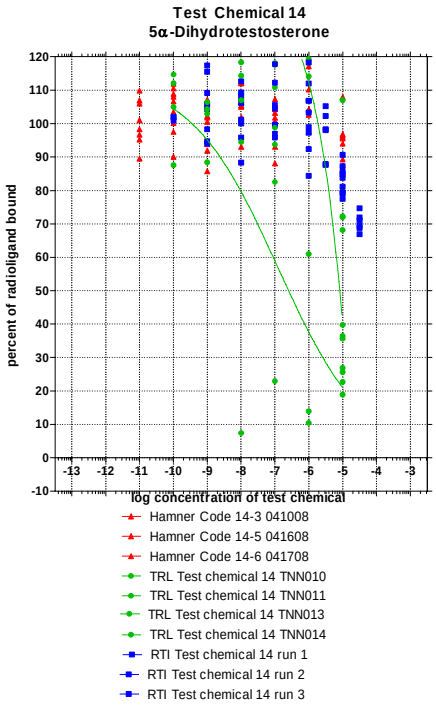

Test Chemical 9
o, p'-DDT
percent of radioligand bound
log concentration of test chemical
Hamner Code 9-6 062508
Hamner Code 9-7 070108
Hamner Code 9-8 070208
TRL Test Chemical 9 run TNN010
TRL Test Chemical 9 run TNN011
TRL Test Chemical 9 run TNN012
RTI Test chemical 9 run 1
RTI Test chemical 9 run 2
RTI Test chemical 9 run 3
TRL Test Chemical 9 run TNN013
TRL Test Chemical 9 run TNN014

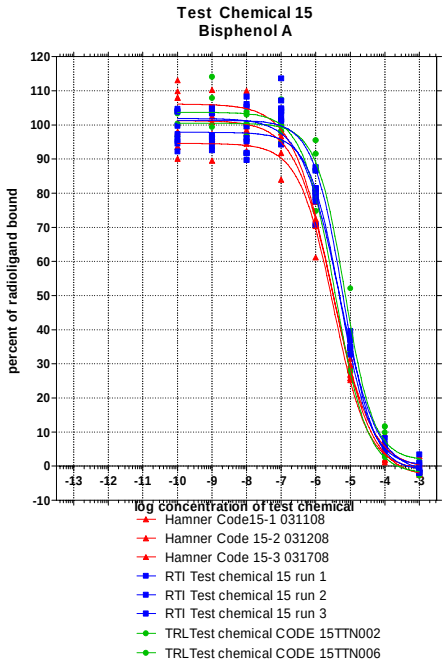

Test Chemical 10
Corticosterone
percent of radioligand bound
log concentration of test chemical
Hamner Code 10-1 052108
Hamner Code 10-8 062308
Hamner Code 10-9 062408
TRL Test chemical 10 run RN008
TRL Test chemical 10 run RN009
RTI Test chemical 10 run 1
RTI Test chemical 10 run 2
RTI Test chemical 10 run 3

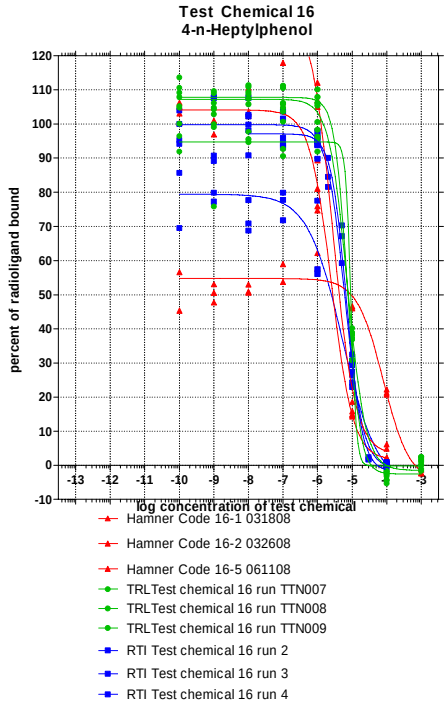

Test Chemical 11
Equol
percent of radioligand bound
log concentration of test chemical
Hamner Code 11-1 030508
Hamner Code 11-2 030608
Hamner Code 11-3 031008
RTI Test chemical 11 run 1
RTI Test chemical 11 run 2
RTI Test chemical 11 run 3
TRLTest chemical CODE 11TTN002
TRLTest chemical CODE 11TTN006
TRL Test chemicalCODE 11 RN008

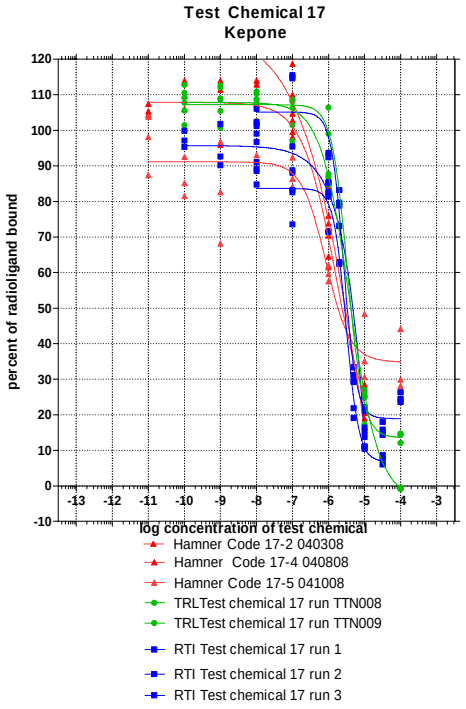

Test Chemical 12
Zearalenone
percent of radioligand bound
log concentration of test chemical
Hamner Code 12-1 030508
Hamner Code 12-2 030608
Hamner Code 12-5 032408
TRLTest chemical CODE 12TTN001
TRLTest chemical CODE 12TTN006
RTI Test chemical 12 run 1
RTI Test chemical 12 run 2
RTI Test chemical 12 run 3

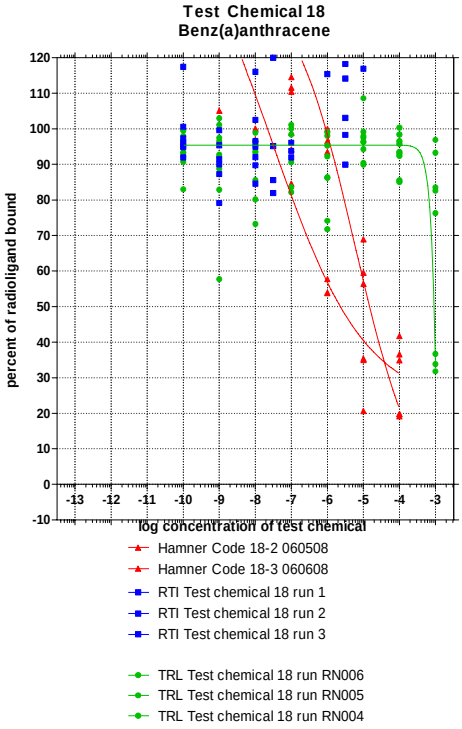

Test Chemical 13
Tamoxifen
percent of radioligand bound
log concentration of test chemical
Hamner Code 13-1 052108
Hamner Code 13-3 053008
Hamner Code 13-4 060608
TRL Test chemical 13 run RN005
TRL Test chemical 13 run RN006
RTI Test chemical 13 run 1
RTI Test chemical 13 run 3
RTI Test chemical 13 run 4

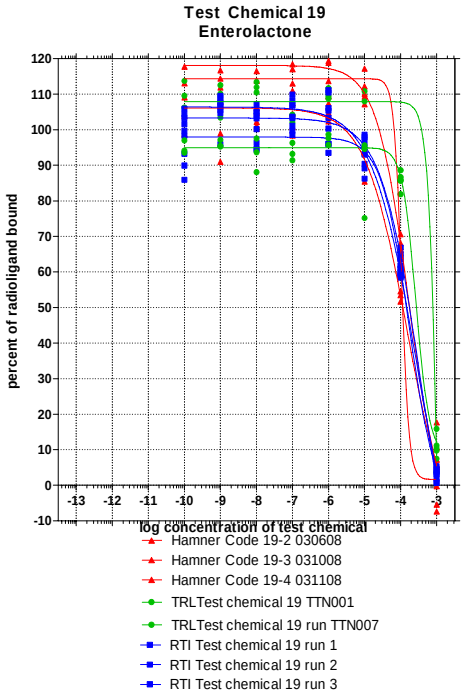

Test Chemical 14
5α-Dihydrotestosterone
percent of radioligand bound
log concentration of test chemical
Hamner Code 14-3 041008
Hamner Code 14-5 041608
Hamner Code 14-6 041708
TRL Test chemical 14 TNN010
TRL Test chemical 14 TNN011
TRL Test chemical 14 TNN013
TRL Test chemical 14 TNN014
RTI Test chemical 14 run 1
RTI Test chemical 14 run 2
RTI Test chemical 14 run 3

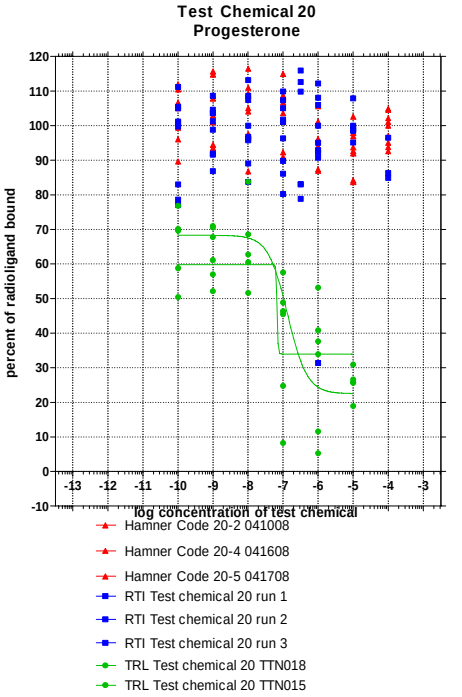

Test Chemical 15
Bisphenol A
percent of radioligand bound
log concentration of test chemical
Hamner Code15-1 031108
Hamner Code 15-2 031208
Hamner Code 15-3 031708
RTI Test chemical 15 run 1
RTI Test chemical 15 run 2
RTI Test chemical 15 run 3
TRLTest chemical CODE 15TTN002
TRLTest chemical CODE 15TTN006

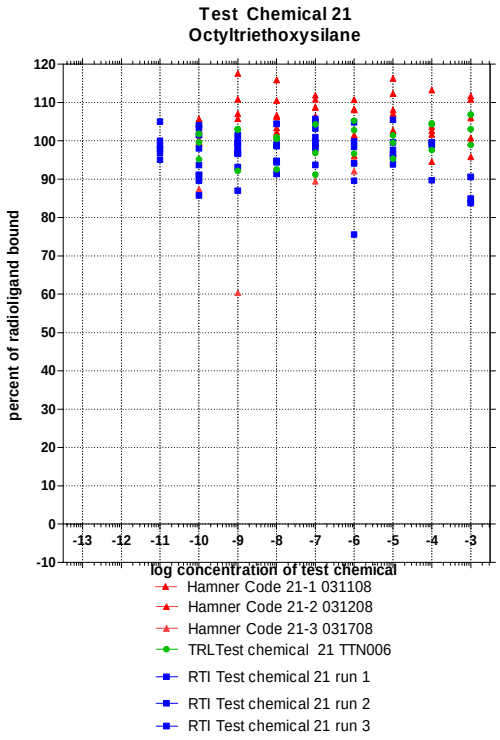

Test Chemical 16
4-n-Heptylphenol
percent of radioligand bound
log concentration of test chemical
Hamner Code 16-1 031808
Hamner Code 16-2 032608
Hamner Code 16-5 061108
TRLTest chemical 16 run TTN007
TRLTest chemical 16 run TTN008
TRLTest chemical 16 run TTN009
RTI Test chemical 16 run 2
RTI Test chemical 16 run 3
RTI Test chemical 16 run 4

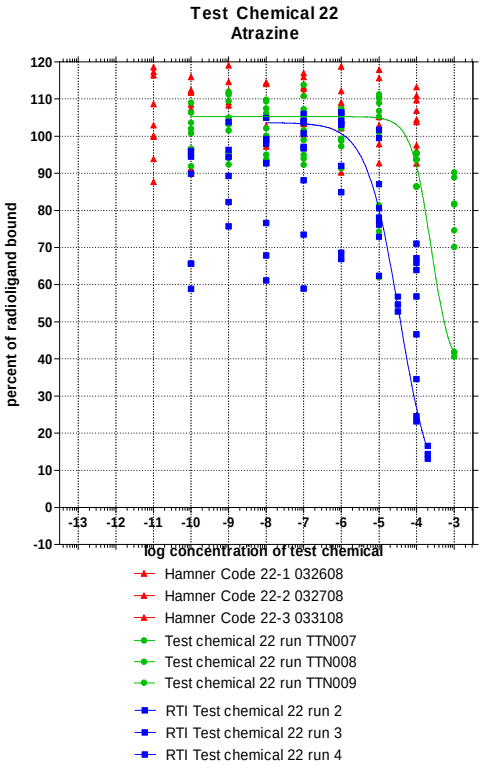

Test Chemical 17
Kepone
percent of radioligand bound
log concentration of test chemical
Hamner Code 17-2 040308
Hamner Code 17-4 040808
Hamner Code 17-5 041008
TRLTest chemical 17 run TTN008
TRLTest chemical 17 run TTN009
RTI Test chemical 17 run 1
RTI Test chemical 17 run 2
RTI Test chemical 17 run 3

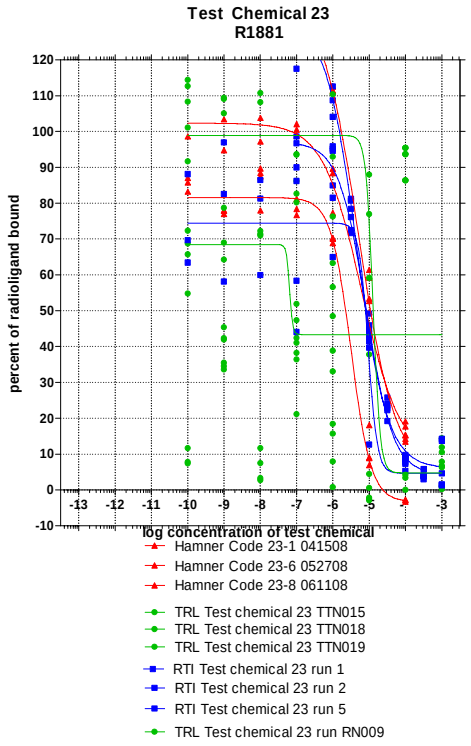

Test Chemical 18
Benz(a)anthracene
percent of radioligand bound
log concentration of test chemical
Hamner Code 18-2 060508
Hamner Code 18-3 060608
RTI Test chemical 18 run 1
RTI Test chemical 18 run 2
RTI Test chemical 18 run 3
TRL Test chemical 18 run RN006
TRL Test chemical 18 run RN005
TRL Test chemical 18 run RN004

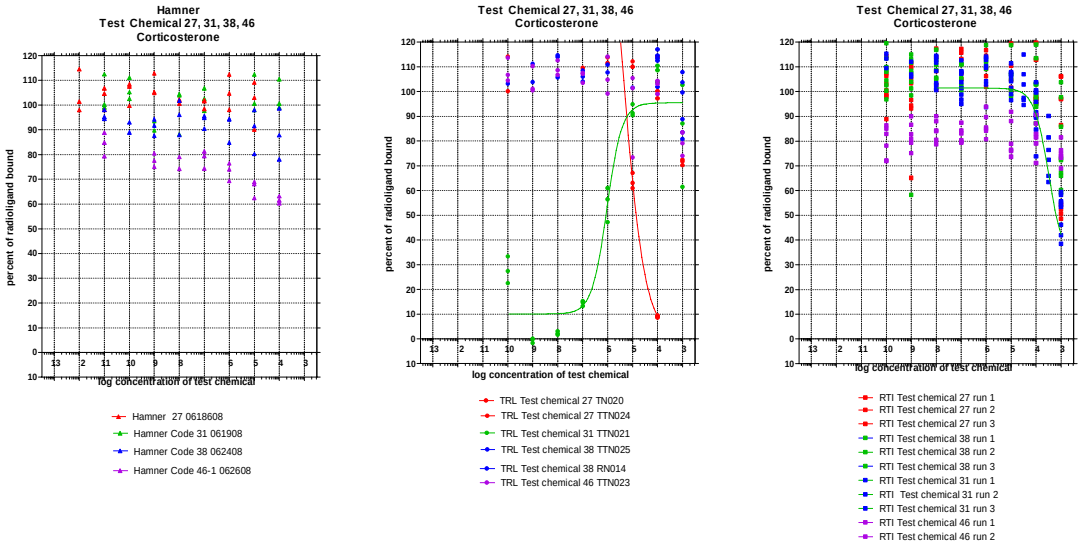

Test Chemical 19
Enterolactone
percent of radioligand bound
log concentration of test chemical
Hamner Code 19-2 030608
Hamner Code 19-3 031008
Hamner Code 19-4 031108
TRLTest chemical 19 TTN001
TRLTest chemical 19 run TTN007
RTI Test chemical 19 run 1
RTI Test chemical 19 run 2
RTI Test chemical 19 run 3

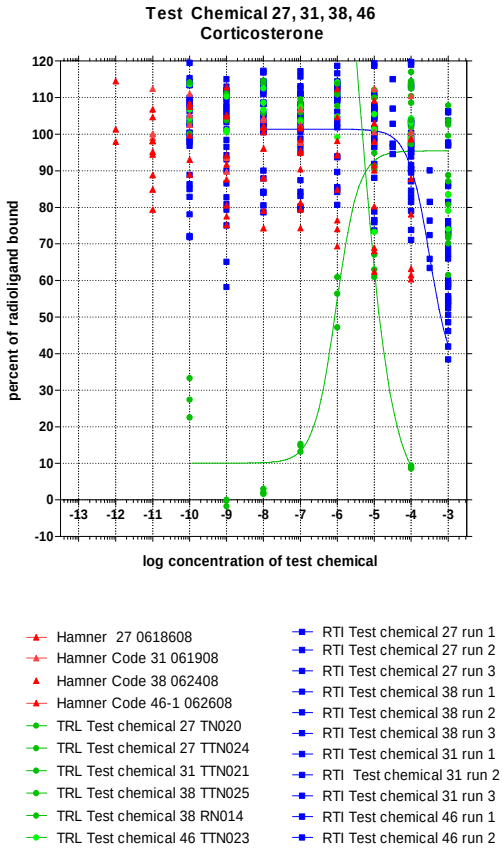

Test Chemical 20
Progesterone
percent of radioligand bound
log concentration of test chemical
Hamner Code 20-2 041008
Hamner Code 20-4 041608
Hamner Code 20-5 041708
RTI Test chemical 20 run 1
RTI Test chemical 20 run 2
RTI Test chemical 20 run 3
TRL Test chemical 20 TTN018
TRL Test chemical 20 TTN015

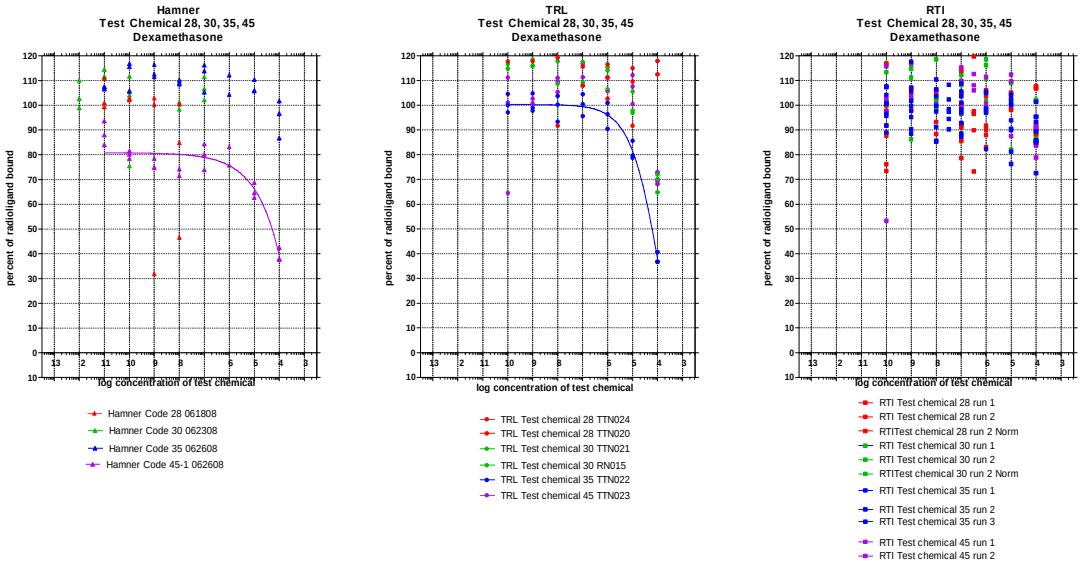

Test Chemical 21
Octyltriethoxysilane
percent of radioligand bound
log concentration of test chemical
Hamner Code 21-1 031108
Hamner Code 21-2 031208
Hamner Code 21-3 031708
TRLTest chemical 21 TTN006
RTI Test chemical 21 run 1
RTI Test chemical 21 run 2
RTI Test chemical 21 run 3

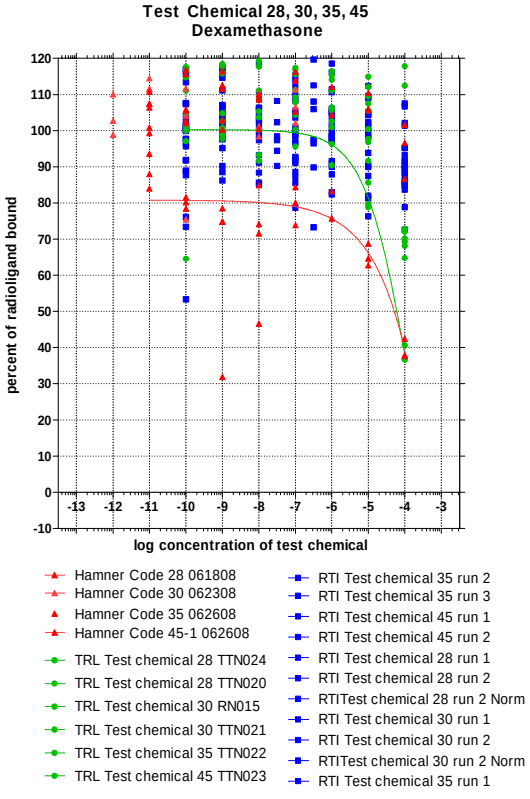

Test Chemical 22
Atrazine
percent of radioligand bound
log concentration of test chemical
Hamner Code 22-1 032608
Hamner Code 22-2 032708
Hamner Code 22-3 033108
Test chemical 22 run TTN007
Test chemical 22 run TTN008
Test chemical 22 run TTN009
RTI Test chemical 22 run 2
RTI Test chemical 22 run 3
RTI Test chemical 22 run 4

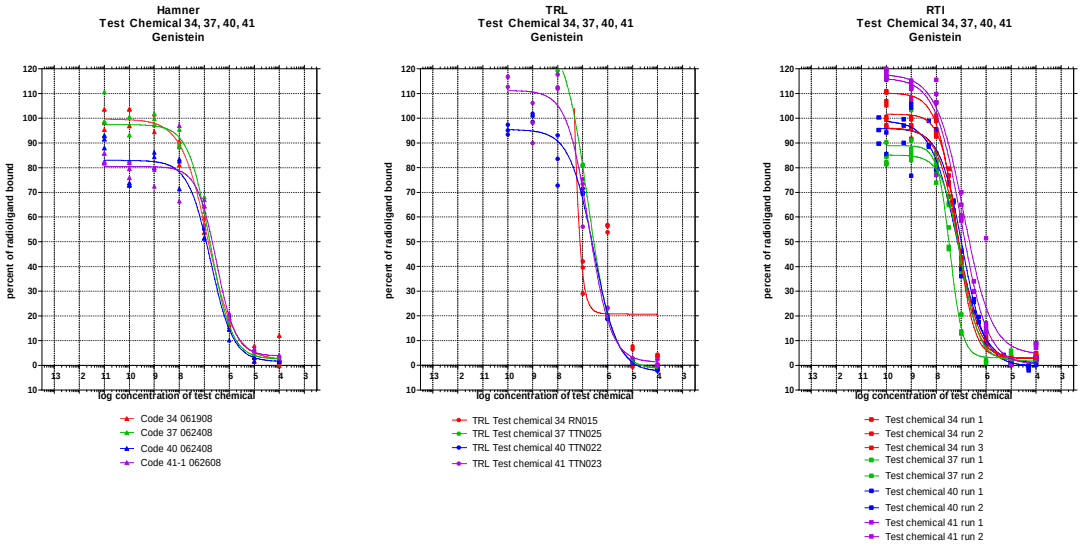

Test Chemical 23
R1881
percent of radioligand bound
log concentration of test chemical
Hamner Code 23-1 041508
Hamner Code 23-6 052708
Hamner Code 23-8 061108
TRL Test chemical 23 TTN015
TRL Test chemical 23 TTN018
TRL Test chemical 23 TTN019
RTI Test chemical 23 run 1
RTI Test chemical 23 run 2
RTI Test chemical 23 run 5
TRL Test chemical 23 run RN009


Hamner
Test Chemical 27, 31, 38, 46
Corticosterone
percent of radioligand bound
log concentration of test chemical
Hamner 27 0618608
Hamner Code 31 061908
Hamner Code 38 062408
Hamner Code 46-1 062608
Test Chemical 27, 31, 38, 46
Corticosterone
percent of radioligand bound
log concentration of test chemical
TRL Test chemical 27 TN020
TRL Test chemical 27 TTN024
TRL Test chemical 31 TTN021
TRL Test chemical 38 TTN025
TRL Test chemical 38 RN014
TRL Test chemical 46 TTN023
Test Chemical 27, 31, 38, 46
Corticosterone
percent of radioligand bound
log concentration of test chemical
RTI Test chemical 27 run 1
RTI Test chemical 27 run 2
RTI Test chemical 27 run 3
RTI Test chemical 38 run 1
RTI Test chemical 38 run 2
RTI Test chemical 38 run 3
RTI Test chemical 31 run 1
RTI Test chemical 31 run 2
RTI Test chemical 31 run 3
RTI Test chemical 46 run 1
RTI Test chemical 46 run 2

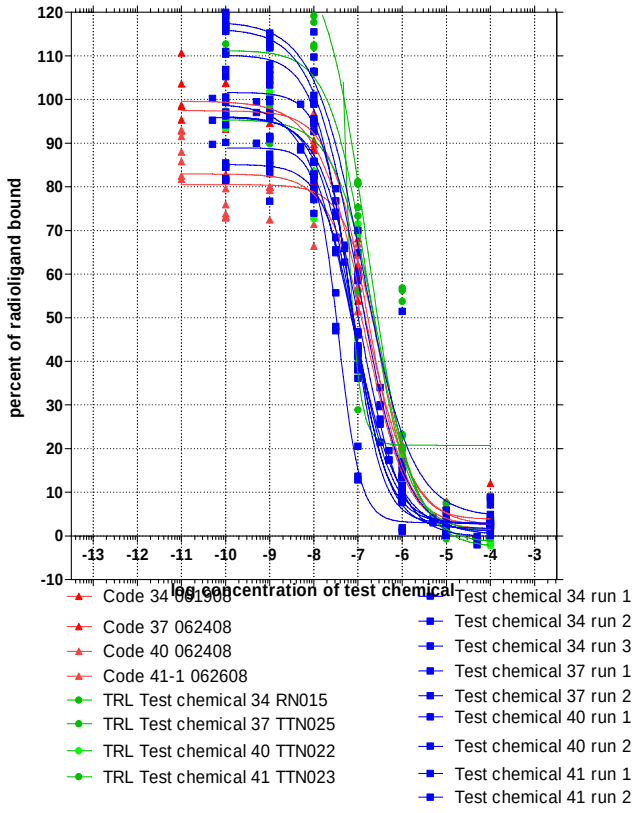

Test Chemical 27, 31, 38, 46
Corticosterone
percent of radioligand bound
log concentration of test chemical
Hamner 27 0618608
Hamner Code 31 061908
Hamner Code 38 062408
Hamner Code 46-1 062608
TRL Test chemical 27 TN020
TRL Test chemical 27 TTN024
TRL Test chemical 31 TTN021
TRL Test chemical 38 TTN025
TRL Test chemical 38 RN014
TRL Test chemical 46 TTN023
RTI Test chemical 27 run 1
RTI Test chemical 27 run 2
RTI Test chemical 27 run 3
RTI Test chemical 38 run 1
RTI Test chemical 38 run 2
RTI Test chemical 38 run 3
RTI Test chemical 31 run 1
RTI Test chemical 31 run 2
RTI Test chemical 31 run 3
RTI Test chemical 46 run 1
RTI Test chemical 46 run 2

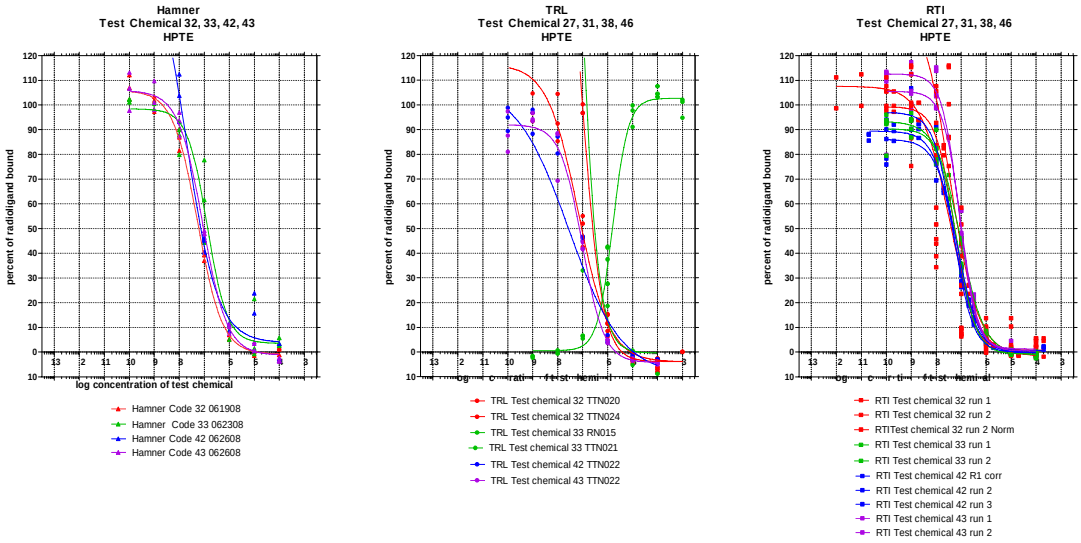

Hamner
Test Chemical 28, 30, 35, 45
Dexamethasone
percent of radioligand bound
log concentration of test chemical
Hamner Code 28 061808
Hamner Code 30 062308
Hamner Code 35 062608
Hamner Code 45-1 062608
TRL
Test Chemical 28, 30, 35, 45
Dexamethasone
percent of radioligand bound
log concentration of test chemical
TRL Test chemical 28 TTN024
TRL Test chemical 28 TTN020
TRL Test chemical 30 TTN021
TRL Test chemical 30 RN015
TRL Test chemical 35 TTN022
TRL Test chemical 45 TTN023
RTI
Test Chemical 28, 30, 35, 45
Dexamethasone
percent of radioligand bound
log concentration of test chemical
RTI Test chemical 28 run 1
RTI Test chemical 28 run 2
RTITest chemical 28 run 2 Norm
RTI Test chemical 30 run 1
RTI Test chemical 30 run 2
RTITest chemical 30 run 2 Norm
RTI Test chemical 35 run 1
RTI Test chemical 35 run 2
RTI Test chemical 35 run 3
RTI Test chemical 45 run 1
RTI Test chemical 45 run 2

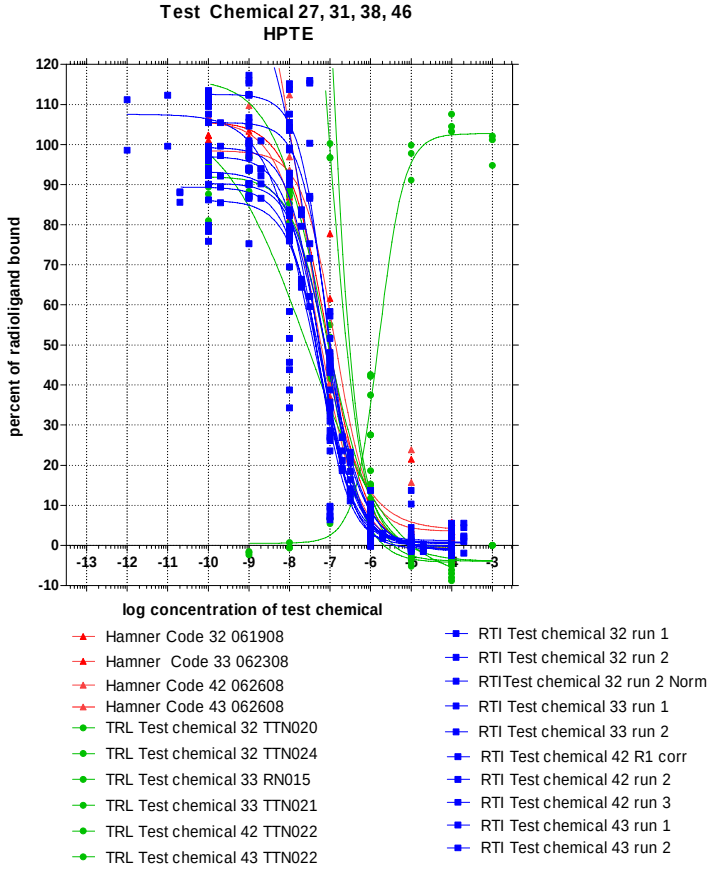

Test Chemical 28, 30, 35, 45
Dexamethasone
percent of radioligand bound
log concentration of test chemical
Hamner Code 28 061808
Hamner Code 30 062308
Hamner Code 35 062608
Hamner Code 45-1 062608
TRL Test chemical 28 TTN024
TRL Test chemical 28 TTN020
TRL Test chemical 30 RN015
TRL Test chemical 30 TTN021
TRL Test chemical 35 TTN022
TRL Test chemical 45 TTN023
RTI Test chemical 35 run 2
RTI Test chemical 35 run 3
RTI Test chemical 45 run 1
RTI Test chemical 45 run 2
RTI Test chemical 28 run 1
RTI Test chemical 28 run 2
RTITest chemical 28 run 2 Norm
RTI Test chemical 30 run 1
RTI Test chemical 30 run 2
RTITest chemical 30 run 2 Norm
RTI Test chemical 35 run 1

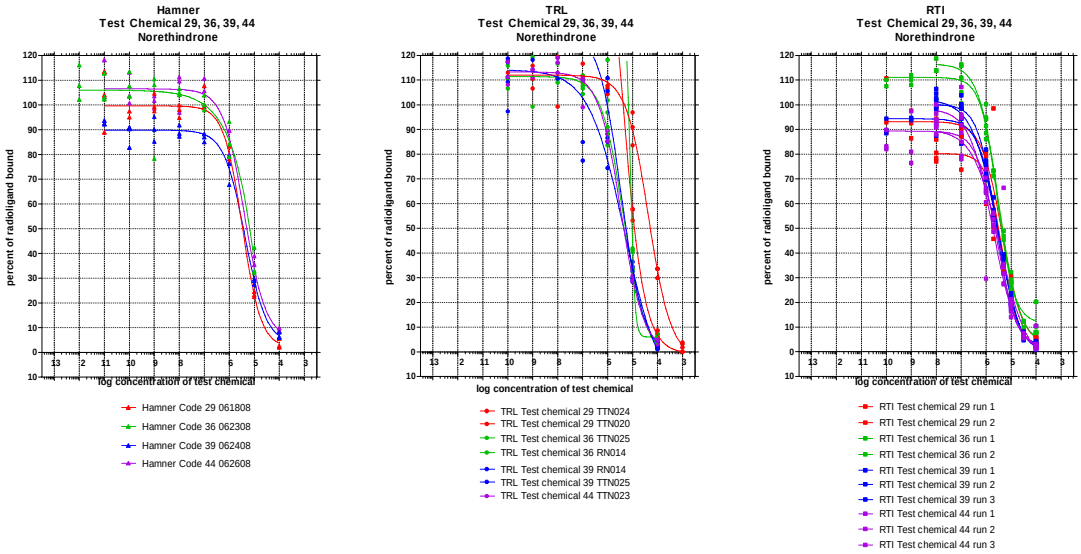


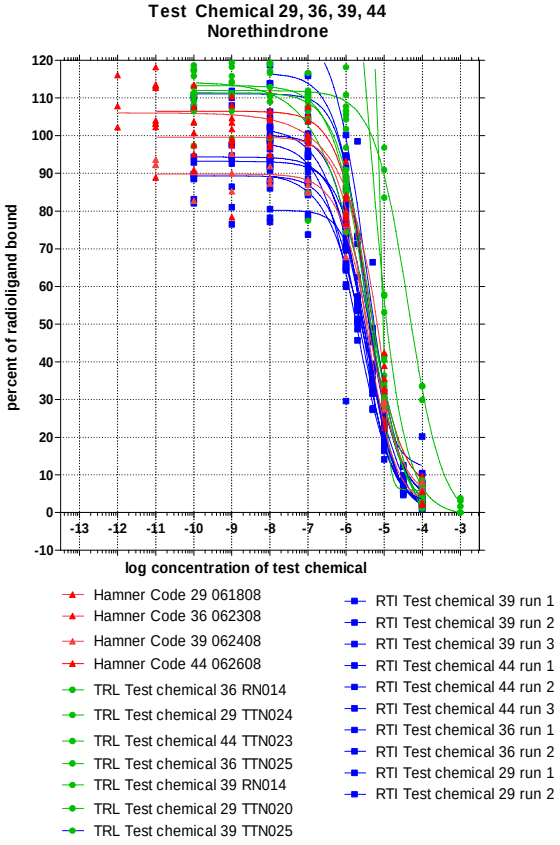
Test Chemical 34, 37, 40, 41
Genistein




Hamner
Test Chemical 32, 33, 42, 43
HPTE
TRL
Test Chemical 27, 31, 38, 46
HPTE
RTI
Test Chemical 27, 31, 38, 46
HPTE
percent of radioligand bound
log concentration of test chemical
Hamner Code 32 061908
Hamner Code 33 062308
Hamner Code 42 062608
Hamner Code 43 062608
TRL Test chemical 32 TTN020
TRL Test chemical 32 TTN024
TRL Test chemical 33 RN015
TRL Test chemical 33 TTN021
TRL Test chemical 42 TTN022
TRL Test chemical 43 TTN022
RTI Test chemical 32 run 1
RTI Test chemical 32 run 2
RTITest chemical 32 run 2 Norm
RTI Test chemical 33 run 1
RTI Test chemical 33 run 2
RTI Test chemical 42 R1 corr
RTI Test chemical 42 run 2
RTI Test chemical 42 run 3
RTI Test chemical 43 run 1
RTI Test chemical 43 run 2


Test Chemical 27, 31, 38, 46
HPTE
percent of radioligand bound
log concentration of test chemical
Hamner Code 32 061908
Hamner Code 33 062308
Hamner Code 42 062608
Hamner Code 43 062608
TRL Test chemical 32 TTN020
TRL Test chemical 32 TTN024
TRL Test chemical 33 RN015
TRL Test chemical 33 TTN021
TRL Test chemical 42 TTN022
TRL Test chemical 43 TTN022
RTI Test chemical 32 run 1
RTI Test chemical 32 run 2
RTITest chemical 32 run 2 Norm
RTI Test chemical 33 run 1
RTI Test chemical 33 run 2
RTI Test chemical 42 R1 corr
RTI Test chemical 42 run 2
RTI Test chemical 42 run 3
RTI Test chemical 43 run 1
RTI Test chemical 43 run 2


Hamner
Test Chemical 29, 36, 39, 44
Norethindrone
percent of radioligand bound
log concentration of test chemical
Hamner Code 29 061808
Hamner Code 36 062308
Hamner Code 39 062408
Hamner Code 44 062608
TRL
Test Chemical 29, 36, 39, 44
Norethindrone
percent of radioligand bound
log concentration of test chemical
TRL Test chemical 29 TTN024
TRL Test chemical 29 TTN020
TRL Test chemical 36 TTN025
TRL Test chemical 36 RN014
TRL Test chemical 39 RN014
TRL Test chemical 39 TTN025
TRL Test chemical 44 TTN023
RTI
Test Chemical 29, 36, 39, 44
Norethindrone
percent of radioligand bound
log concentration of test chemical
RTI Test chemical 29 run 1
RTI Test chemical 29 run 2
RTI Test chemical 36 run 1
RTI Test chemical 36 run 2
RTI Test chemical 39 run 1
RTI Test chemical 39 run 2
RTI Test chemical 39 run 3
RTI Test chemical 44 run 1
RTI Test chemical 44 run 2
RTI Test chemical 44 run 3


Test Chemical 29, 36, 39, 44
Norethindrone
percent of radioligand bound
log concentration of test chemical
Hamner Code 29 061808
Hamner Code 36 062308
Hamner Code 39 062408
Hamner Code 44 062608
TRL Test chemical 36 RN014
TRL Test chemical 29 TTN024
TRL Test chemical 44 TTN023
TRL Test chemical 36 TTN025
TRL Test chemical 39 RN014
TRL Test chemical 29 TTN020
TRL Test chemical 39 TTN025
RTI Test chemical 39 run 1
RTI Test chemical 39 run 2
RTI Test chemical 39 run 3
RTI Test chemical 44 run 1
RTI Test chemical 44 run 2
RTI Test chemical 44 run 3
RTI Test chemical 36 run 1
RTI Test chemical 36 run 2
RTI Test chemical 29 run 1
RTI Test chemical 29 run 2

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Appendix III

Assay Worksheets

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Protein Assay using BioRad Protein Assay Kit

Date: _____

Buffer: _____

Check when done

Standard: BioRad Protein Assay Standard II — Add 20 mL ddH_2O and mix to reconsitute
Bovine serum albumin — Standard may be aliquoted and frozen at -20 for future use.
Control #: _____
Exp Date _____

Reagents: BioRad Protein Assay — Dye Reagent Concentrate Control # _____
Exp date: _____

Dilute 1:5 in ddH_2O. _____ mL dye _____ mL ddH_2O
Filter to remove particulates

Standard Curve: Prepare the following dilutions of the Standard

mg protein/ mL	µL Standard Soln.	µL Buffer
0.89	300	200
0.74	250	250
0.59	400	600
0.44	150	350
0.3	200	800
0.22	75	425
0	0	500

Assay:
1) Add 100 µL of each standard and sample to triplicate tubes.
2) Add 5 mL diluted dye reagent to each standard and sample tube. Vortex
3) Let sit at room temperature at least 5 min, but not more than 1 h.
4) Transfer each standard and sample to a disposable semimicro cuvette
5) Read absorbance at 595 nm on the spectrophotometer

Standards

mg protein/ mL	Abs	Abs	Abs
0.89			
0.74			
0.59			
0.44			
0.3			
0.22			
0			

Samples

Sample ID	Abs	Abs	Abs

Signature _____ Date _____

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Appendix IV

QAPP Deviations

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QAPP Deviations Identified During EDSP Task Order 6

Deviation	Location of Requirement	Comment	Impact
1. No RTI guidance document or reporting template was provided for final reports.	TO 6 QAPP, Sections 6.3 and 9.3	The guidance document was not thought to be necessary by task management.	No significant impact.
2. No technical systems audit (TSA) was performed at RTI or TRL/UIC during Task 6 of TO 6.	TO 6 QAPP, Section 20.1	The failure to perform a TSA at RTI was due to a QA oversight. The failure at TRL/UIC was due to their QA not receiving the QAPP until after the work on Task 6 was completed.	Not significant. While the loss of quality oversight is unfortunate, it is not the primary determinant of the quality of the laboratory work and resulting data. The data and final report were assessed by the QA groups from the respective laboratories.
3. Closed out audit and inspection reports were not provided by TRL/UIC or Hamner	TO6 QAPP, Sections 4.3, 9.3.2, and 20.2; also QMP Section 10.2	Both subcontractor labs have internal company policies against the distribution of QA reports outside of their facilities. They have agreed to provide a list of deviations to RTI.	Not significant. The QA unit at each laboratory has documented any problems identified during inspections and audits that they have performed. The individual QAUs have assured that any issues identified have been resolved prior to issuing their final reports.
4. RTI audit reports did not contain the RTI	TO6 QAPP, Section 20.3	Due to the need for independence of the study conduct	No significant impact.

Task Order Leader (TOL) name and were not circulated to the RTI TOL.		from overall project management, (the RTI DMPK laboratory was acting like a subcontractor to RTI LST), audit reports were circulated to the study director and test facility management.	
5. The QAPP was not amended to add the general schedule as stated in Section 6.3, p. 20.	TO6 QAPP, Section 6.3.	The schedule was made, but never formally added to the QAPP.	No significant impact.
6. No final QA summary describing outstanding issues was submitted with the Task 6 final report.	TO6 QAPP, Section 6.3	The final reports for TO6, Task 6, were never finalized, therefore this report was not prepared.	No significant impact.

Appendix V

QA Statement

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RTI INTERNATIONAL

Quality Assurance Statement

Study Title: Second Inter-laboratory Validation of the Estrogen Receptor Binding Assay (Rat Uterine Cytosol)

Report Title: Task 7 – Test Coded Chemicals

Sponsor: U.S. Environmental Protection Agency

Sponsor Contract Number: EPA EP-W-06-026, Task Order 6

Project Number: 0210114.006.007.001

This study was audited by the Sciences and Engineering – Quality Assurance Unit and the results of the inspections and audits were reported to the Study Director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Study Director and Management
Report Audit	July 21-22, 2008	July 22, 2008

Prepared by:

Michelle Oh
Quality Assurance Specialist

7/22/08
Date

Reviewed by:

Celia Keller
Quality Assurance Specialist

07/22/08
Date

Appendix V

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