Section 4
Health effects

Test Guideline No. 493

Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assays to Detect Chemicals with ER Binding Affinity

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) In Vitro Assays to Detect Chemicals with ER Binding Affinity

GENERAL INTRODUCTION

Performance-Based Test Guideline

1. This Performance-Based Test Guideline (PBTG) describes the methodology for human recombinant i*n vitro* assays to detect substances with estrogen receptor binding affinity (hrER binding assays). It comprises two mechanistically and functionally similar test methods for the identification of estrogen receptor (i.e. ERα) binders and should facilitate the development of new similar or modified test methods in accordance with the principles for validation set forth in the OECD Guidance Document (GD) on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (1). The fully validated reference test methods (Annex 2 and Annex 3) that provide the basis for this PBTG are:

- The Freyberger-Wilson (FW) *In Vitro* Estrogen Receptor (ER) Binding Assay Using a Full Length Human Recombinant ERα (2), and
- The Chemical Evaluation and Research Institute (CERI) *In Vitro* Estrogen Receptor Binding Assay Using a Human Recombinant Ligand Binding Domain Protein (2).

Performance standards (PS) (3) are available to facilitate the development and validation of similar test methods for the same hazard endpoint and allow for timely amendment of this PBTG so that new similar test methods can be added to an updated PBTG. However, similar test methods will only be added after review and agreement that performance standards are met. The test methods included in this Test Guideline can be used indiscriminately to address countries' requirements for test results on estrogen receptor binding while benefiting from the Mutual Acceptance of Data.

Background and principles of the test methods included in the PBTG

2. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new, Test Guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD conceptual framework (CF) for testing and assessment of potential endocrine disrupting chemicals was revised in 2012. The original and revised CFs are included as Annexes in the Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (4). The CF comprises five levels, each level corresponding to a different level of biological complexity. The ER binding assays described in this PBTG are level 2, which includes "*in vitro assays providing data about selected endocrine mechanism(s)/pathway(s).* This PBTG is for *in vitro* receptor binding test methods designed to identify ligands for the human estrogen receptor alpha (ER□).

3. The relevance of the *in vitro* ER binding assay to biological functions has been clearly demonstrated. ER binding assays are designed to identify chemicals that have the potential to disrupt the estrogen hormone pathway, and have been used extensively during the past two decades to characterise ER tissue distribution as well as to identify ER agonists/antagonists. These assays reflect the ligand-receptor interaction which is the initial step of the estrogen signaling pathway and essential for reproduction function in all vertebrates.

4. The interaction of estrogens with ERs can affect transcription of estrogen-controlled genes and induce non-genomic effects, which can lead to the induction or inhibition of cellular processes, including those necessary for cell proliferation, normal fetal development, and reproductive function (5) (6) (7). Perturbation of normal estrogenic systems may have the potential to trigger adverse effects on normal development (ontogenesis), reproductive health and the integrity of the reproductive system. Inappropriate ER signaling can lead to effects such as increased risk of hormone dependent cancer, impaired fertility, and alterations in fetal growth and development (8).

5. *In vitro* binding assays are based on a direct interaction of a substance with a specific receptor ligand binding site that regulates the gene transcription. The key component of the human recombinant estrogen receptor alpha (hrERα) binding assay measures the ability of a radiolabeled ligand ([^{3}H]17β-estradiol) to bind with the ER in the presence of increasing concentrations of a test chemical (i.e. competitor). Test chemicals that possess a high affinity for the ER compete with the radiolabeled ligand at a lower concentration as compared with those chemicals with lower affinity for the receptor. This assay consists of two major components: a saturation binding experiment to characterise receptor-ligand interaction parameters and document ER specificity, followed by a competitive binding experiment that characterises the competition between a test chemical and a radiolabeled ligand for binding to the ER.

6. Validation studies of the CERI and the FW binding assays have demonstrated their relevance and reliability for their intended purpose (2).

7. Definitions and abbreviations used in this Test Guideline are described in Annex 1.

Scope and limitations related to the receptor binding assays

8. These test methods are being proposed for screening and prioritisation purposes, but can also provide information for a molecular initiation event (MIE) that can be used in a weight of evidence approach. They address chemical binding to the ERα ligand binding domain in an *in vitro* system. Thus, results should not be directly extrapolated to the complex signaling and regulation of the intact endocrine system *in vivo*.

9. Binding of the natural ligand, 17β-estradiol, is the initial step of a series of molecular events that activates the transcription of target genes and ultimately, culminates with a physiological change (9). Thus binding to the ERα ligand binding domain is considered one of the key mechanisms of ER mediated endocrine disruption (ED), although there are other mechanisms through which ED can occur, including (i) interactions with sites of ERα other than the ligand binding pocket, (ii) interactions with other receptors relevant for estrogen signaling, ERβ and G-protein coupled estrogen receptor, other receptors and enzymatic systems within the endocrine system, (iii) hormone synthesis, (iv) metabolic activation and/or inactivation of hormones, (v) distribution of hormones to target tissues, and (vi) clearance of hormones from the body. None of the test methods under this PBTG address these modes of action.

10. This PBTG addresses the ability of substances to bind to human ERα and does not distinguish between ERα agonists or antagonists. This assay does not address either further downstream events such as gene transcription or physiological changes. Considering that only single substances were used during the validation, the applicability to test mixtures has not been addressed. The test method is nevertheless theoretically applicable to the testing of multi-constituent substances and mixtures. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

11. The cell free receptor systems have no intrinsic metabolic capability and they were not validated in combination with metabolic enzyme systems. However, it might be possible to incorporate metabolic activity in a study design but this would require further validation efforts.

12. Chemicals that may denature the protein (i.e. receptor protein), such as surfactant or chemicals that can change the pH of the assay buffer, may not be tested or may only be tested at concentrations devoid of

such interactions. Otherwise, the concentration range that can be tested in the assays for a test chemical is limited by its solubility in the assay buffer.

For informational purposes, Table 1 provides the test results for the 24 substances that were tested in both of the fully validated test methods described in this PBTG. Of these substances, 17 are classified as ER binders and 6 as non-binders based upon published reports, including in vitro assays for ER transcriptional activation and/or the uterotrophic assay (9) (10) (11) (12) (13) (14) (15). In reference to the data summarised in Table 1, there was almost 100% agreement between the two test methods on the classifications of all the substances up to 10^{-4}M, and each substance was correctly classified as an ER binder or non-binder. Supplementary information on this group of substances as well as additional substances tested in the ER binding test methods during the validation studies is provided in the Performance Standards for the hrER binding assay (3), Annex 2 (Tables 1, 2 and 3).

Table 1: Classification of substances as ER binders or non-binders when tested in the FW and CERI hrER Binding Assays with comparison with expected response

	Substance Name	CAS RN	Expected Response	FW Assay		CERI Assay		MESH Chemical Class	Product Class
				Concentration Range (M)	Classification	Concentration Range (M)	Classification		
1	17β-Estradiol	50-28-2	*Binder*	$1x10^{-11}$ – $1x10^{-6}$	Binder	$1x10^{-11}$ – $1x10^{-6}$	Binder	Steroid	Pharmaceutical, Veterinary Agent
2	Norethynodrel	68-23-5	*Binder*	$3x10^{-9}$ – $30x10^{-4}$	Binder	$3x10^{-9}$ – $30x10^{-4}$	Binder	Steroid	Pharmaceutical, Veterinary Agent
3	Norethindrone	68-22-4	*Binder*	$3x10^{-9}$ – $30x10^{-4}$	Binder	$3x10^{-9}$ – $30x10^{-4}$	Binder	Steroid	Pharmaceutical, Veterinary Agent
4	Di-*n*-butyl phthalate	84-74-2	*Non-binder**	$1x10^{-10}$ – $1x10^{-4}$	Non-Binder*†	$1x10^{-10}$ – $1x10^{-4}$	Non-Binder*†	Hydrocarbon (cyclic), Ester	Plasticizer, Chemical Intermediate
5	DES	56-53-1	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Hydrocarbon (Cyclic), Phenol	Pharmaceutical, Veterinary Agent
6	17α-ethynylestradiol	57-63-6	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Steroid	Pharmaceutical, Veterinary Agent
7	Meso-Hexestrol	84-16-2	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Hydrocarbon (Cyclic), Phenol	Pharmaceutical, Veterinary Agent
8	Genistein	446-72-0	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Hydrocarbon (heterocyclic), Flavonoid	Natural Product
9	Equol	531-95-3	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Phytoestrogen Metabolite	Natural Product
10	Butyl paraben (*n* butyl-4-hydroxybenzoate)	94-26-8	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Paraben	Preservative
11	Nonylphenol (mixture)	84852-15-3	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Alkylphenol	Intermediate Compound
12	*o,p'*-DDT	789-02-6	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Organochlorine	Insecticide
13	Corticosterone	50-22-6	*Non-binder**	$1x10^{-10}$ – $1x10^{-4}$	Non-binder	$1x10^{-10}$ – $1x10^{-4}$	Non-Binder	Steroid	Natural Product
14	Zearalenone	17924-92-4	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Hydrocarbon (heterocyclic), Lactone	Natural Product
15	Tamoxifen	10540-29-1	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Hydrocarbon, (Cyclic)	Pharmaceutical, Veterinary Agent
16	5α-dihydrotestosterone	521-18-6	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Steroid, Nonphenolic	Natural Product
17	Bisphenol A	80-05-7	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Phenol	Chemical Intermediate
18	4-*n*-heptylphenol	1987-50-4	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Equivocal [a]	$1x10^{-10}$ – $1x10^{-3}$	Binder	Alkylphenol	Intermediate

19	Kepone (Chlordecone)	143-50-0	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Hydrocarbon, (Halogenated)	Pesticide
20	Benz(a)anthracene	56-55-3	*Non-Binder*	$1x10^{-10}$ – $1x10^{-3}$	Non-Binder [b]	$1x10^{-10}$ – $1x10^{-3}$	Non-Binder [b]	Aromatic Hydrocarbon	Intermediate
21	Enterolactone	78473-71-9	*Binder*	$1x10^{-10}$ – $1x10^{-3}$	Binder	$1x10^{-10}$ – $1x10^{-3}$	Binder	Phytoestrogen	Natural Product
22	Progesterone	57-83-0	*Non-binder**	$1x10^{-10}$ – $1x10^{-4}$	Non-Binder	$1x10^{-10}$ – $1x10^{-4}$	Non-Binder	Steroid	Natural Product
23	Octyltriethoxysilane	2943-75-1	*Non-binder*	$1x10^{-10}$ – $1x10^{-3}$	Non-Binder	$1x10^{-10}$ – $1x10^{-3}$	Non-Binder	Silane	Surface Modifier
24	Atrazine	1912-24-9	*Non-binder**	$1x10^{-10}$ – $1x10^{-4}$	Non-Binder	$1x10^{-10}$ – $1x10^{-4}$	Non-Binder	Heterocyclic compound	Herbicide

*Limit of solubility < $1x\ 10^{-4}$M.

[*]The use and classification of di-n-butyl phthalate (DBP) as a non-binder was based on testing up to 10^{-4} M because the substance had been observed to be insoluble at 10^{-3}M (e.g. turbidity) in some laboratories during the pre-validation studies.

[†] During the validation study, di-*n*-butyl phthalate (DBP) was tested as a coded test substance at concentrations up to 10^{-3}M. Under these conditions, some laboratories observed either a decrease in radioligand binding at the highest concentration (10^{-3}M) and/or an ambiguous curve fit. For these runs, DBP was classified as 'equivocal' or 'binder' in 3/5 laboratories using the CERI assay and 5/6 laboratories using the FW assay (see Reference (2), Sections IV.B.3a,b and VI.A).

[a] Classification was not consistent with expected classification. Classification of 4-*n*-heptylphenol as 'equivocal' or 'non-binder' by 3/5 labs resulted in an average classification of equivocal. Closer inspection revealed that this was due to chemical solubility limitations that prevented the production of a full binding curve.

[b] During the validation study, benz(a)anthracene was reclassified as a non-binder (i.e. negative) based on published literature demonstrating that the *in vitro* estrogenic activity reported for this substance (16) is primarily dependent upon its metabolic activation (17) (18). Enzymatic metabolic activation of the substance would not be anticipated in the cell free hrER binding assays as used in this inter-validation study. Thus, the correct classification for this substance is a 'non-binder' when used under the experimental conditions for the FW and CERI assays.

hrER BINDING TEST METHOD COMPONENTS

Essential Test Method Components

13. This PBTG applies to methods using an ER receptor and a suitably strong ligand to the receptor that can be used as a marker/tracer for the assay and can be displaced with increasing concentrations of a test chemical. Binding assays contain the following two major components: 1) saturation binding and 2) competitive binding. The saturation binding assay is used to confirm the specificity and activity of the receptor preparations, while the competitive binding experiment is used to evaluate the ability of a test chemical to bind to hrER.

Control substances

14. The basis for the proposed concurrent reference estrogen and controls should be described. Concurrent controls (solvent (vehicle), positive (ER binder; strong and weak affinity), negative (non-binder)), as appropriate, serve as an indication that the test method is operative under the test conditions and provide a basis for experiment-to-experiment comparisons; they are usually part of the acceptability criteria for a given experiment (1). Full concentration curves for the reference estrogen and controls (i.e. weak binder and non-binder) should be used in one plate during each run. All other plates should contain: 1) a high- (approximately full displacement of radiolabeled ligand) and medium- (approximately the IC50) concentration each of E2 and weak binder in triplicate; 2) solvent control and non-specific binding, each in triplicate.

Standard Quality Control Procedures

15. Standard quality control procedures should be performed as described for each assay to ensure active receptors, the correct chemical concentrations, tolerance bounds remain stable through multiple replications, and retain the ability to provide the expected ER-binding responses over time.

Demonstration of Laboratory Proficiency

16. Prior to testing unknown chemicals with any of the test methods under this PBTG, each laboratory should demonstrate proficiency in using the test method by performing saturation assays to confirm specificity and activity of the ER preparation, and competitive binding assays with the reference estrogen and controls (weak binder and non-binder). A historical database with results for the reference estrogen and controls generated from 3-5 independent experiments conducted on different days should be established by the laboratory. These experiments will be the foundation for the reference estrogen and historical controls for the laboratory and will be used as a partial assessment of assay acceptability for future runs.

The responsiveness of the test system will also be confirmed by testing the proficiency substances listed in Table 2. The list of proficiency substances is a subset of the reference substances provided in the Performance Standards for the ER binding assays (3). These substances are commercially available, represent the classes of chemicals commonly associated with ER binding activity, exhibit a suitable range of potency expected for ER binding (i.e. strong to weak) and non-binders (i.e. negatives). For each proficiency substance, concentrations tested should cover the range provided in Table 2. At least three experiments should be performed for each substance and results should be in concordance with expected chemical activity. Each experiment should be conducted independently (i.e. with fresh dilutions of receptor, chemicals, and reagent), with three replicates for each concentration. Proficiency is demonstrated by correct classification (positive/negative) of each proficiency substance. Proficiency testing should be performed by each technician when learning the test methods.

Table 2: List of controls and proficiency substances for the hrER competitive binding assays.[1]

No.	Substance Name	CAS RN[2]	Expected Response[3,4]	Test concentration range (M)	MeSH chemical class[5]	Product class[6]
Controls (Reference estrogen, weak binder, non-binder)						
1	17β-estradiol	50-28-2	Binder	$1x10^{-11}$ – $1x10^{-6}$	Steroid	Pharmaceutical, Veterinary agent
2	Norethynodrel (or) Norethindrone	68-23-5 (or) 68-22-4	Binder	$3x10^{-9}$ – $30x10^{-6}$	Steroid	Pharmaceutical, Veterinary agent
3	Octyltriethoxysilane	2943-75-1	Non-binder	$1x10^{-10}$ – $1x10^{-3}$	Silane	Surface modifier
Proficiency substances[6]						
4	Diethylstilbestrol	56-53-1	Binder	$1x10^{-11}$ – $1x10^{-6}$	Hydrocarbon (cyclic), Phenol	Pharmaceutical, Veterinary agent
5	17α-ethynylestradiol	57-63-6	Binder	$1x10^{-11}$ – $1x10^{-6}$	Steroid	Pharmaceutical, Veterinary agent
6	*meso*-Hexestrol	84-16-2	Binder	$1x10^{-11}$ – $1x10^{-6}$	Hydrocarbon (cyclic), Phenol	Pharmaceutical, Veterinary agent
7	Tamoxifen	10540-29-1	Binder	$1x10^{-11}$ – $1x10^{-6}$	Hydrocarbon (cyclic)	Pharmaceutical, Veterinary agent
8	Genistein	446-72-0	Binder	$1x10^{-10}$ – $1x10^{-3}$	Heterocyclic compound, Flavonoid,	Natural product
9	Bisphenol A	80-05-7	Binder	$1x10^{-10}$ – $1x10^{-3}$	Phenol	Chemical intermediate
10	Zearalonone	17924-92-4	Binder	$1x10^{-11}$ – $1x10^{-3}$	Heterocyclic compound, Lactone	Natural Product
11	Butyl paraben	94-26-8	Binder	$1x10^{-11}$ – $1x10^{-3}$	Carboxylic acid, Phenol	Preservative
12	Atrazine	1912-24-9	Non-binder	$1x10^{-11}$ – $1x10^{-6}$	Heterocyclic compound	Herbicide
13	Di-n-butylphthalate (DBP)[7]	84-74-2	Non-binder[8]	$1x10^{-10}$ – $1x10^{-4}$	Hydrocarbon (cyclic), Ester	Plasticizer, Chemical intermediate
14	Corticosterone	50-22-6	Non-binder	$1x10^{-11}$ – $1x10^{-4}$	Steroid	Natural product

[1]If a proficiency substance is no longer commercially available, a substance with the same ER binding classification, comparable potency, and chemical class can be used.

[2] Abbreviations: CAS RN = Chemical Abstracts Service Registry Number.

[3]Classification as an ERα Binder or Non-binder during the validation study for the CERI and FW hrER Binding Assays

(2).
[4]ER binding activity was based upon the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (9) as well as empirical data and other information obtained from referenced studies published and reviewed (10) (11) (12) (13) (14) (15).
[5] Substances were assigned into one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at: http://www.nlm.nih.gov/mesh).
[6] Substances were assigned into one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Database (available at:http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB)
[7] DPB can be used as an alternate control non-binder tested with maximum concentration of 10^{-4} M.
[8] Limit of solubility for this substance is 10^{-4} M. The use and classification of di-n-butyl phthalate (DBP) as a non-binder has been based on testing up to 10^{-4} M because the substance had been observed to be insoluble at 10^{-3}M (e.g. turbidity) in some laboratories during the pre-validation studies.

Solubility Testing and Concentration Range Finding for Test Chemicals

17. A preliminary test should be conducted to determine the limit of solubility for each test chemical and to identify the appropriate concentration range to use when conducting the test. The limit of solubility of each test chemical is to be initially determined in the solvent and further confirmed under assay conditions. The final concentration tested in the assay should not exceed 1 mM. Range finder testing consists of a solvent control along with eight, log serial dilutions, starting at the maximum acceptable concentration (e.g. 1 mM or lower, based upon the limit of solubility), and the presence of cloudiness or precipitate noted. Concentrations in the second and third experiments should be adjusted as appropriate to better characterise the concentration-response curve.

Test Run Acceptability Criteria

18. Acceptance or rejection of a test run is based on the evaluation of results obtained for the reference estrogen and control used for each experiment. First, for plate 1, the full concentration curves for the reference controls from each experiment should meet the measures of performance with curve-fit parameters (e.g. IC50 and Hillslope) based upon the results reported for the respective protocols for the CERI and FW assays (Annex 2 and 3), and the historical control data from the laboratory conducting the test. All controls (reference estrogen, weak binder, and non-binder) should be correctly classified for each experiment. Secondly, the controls on all subsequent plates need to be assessed for consistency with plate 1. A sufficient range of concentrations of the test chemical should be used to clearly define the top of the competitive binding curve. Variability among replicates at each concentration of the test chemical as well as among the three independent runs should be reasonable and scientifically defensible. The ability to consistently conduct the test method should be demonstrated by the development and maintenance of a historical database for the reference estrogen and controls. Standard deviations (SD) or coefficients of variation (CV) for the means of reference estrogen and control weak binder curves fitting parameters from multiple experiments may be used as a measure of within-laboratory reproducibility. Professional judgment should be applied when reviewing the plate control results from each run as well as for each test chemical.

In addition, the following principles regarding acceptability criteria should be met:

- Data should be sufficient for a quantitative assessment of ER binding
- The concentrations tested should remain within the solubility range of the test chemical.

Analysis of data

19. The defined data analysis procedure for saturation and competitive binding data should adhere to the key principles for characterising receptor-ligand interactions. Typically, saturation binding data are analyzed using a non-linear regression model that accounts for total and non-specific binding. A correction for ligand depletion (e.g. Swillens, 1995 (19)) may be needed when determining Bmax and Kd. Data from competitive binding assays are typically transformed (e.g. percent specific binding and concentration of test chemical (log M)). Estimates of log (IC_{50}) for each test chemical should be determined using an appropriate nonlinear curve fitting software to fit a four parameter Hill equation. Following an initial analysis, the curve fit parameters and a visual review of how well the binding data fit the generated competitive binding curve should be conducted. In some cases, additional analysis may be needed to obtain the best curve fit (e.g. constraining top and/or bottom of curve, use of 10% rule, see Annex 4 and Reference 2 (Section III.A.2).

20. Meeting the acceptability criteria (paragraph 20) indicates the assay system is operating properly, but it does not ensure that any particular test will produce accurate data. Replicating the correct results of the first test is the best indication that accurate data were produced.

General Data Interpretation Criteria

21. There is currently no universally agreed method for interpreting ER binding data. However, both qualitative (e.g. binder/non-binder) and/or quantitative (e.g. log IC_{50}, Relative Binding Affinity (RBA), etc.) assessments of hrER-mediated activity should be based on empirical data and sound scientific judgment.

Test Report

22. The test report should include the following information:

Test method:

- test method used;

Control/Reference/Test chemical

- source, lot number, limit date for use, if available
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known.
- measurement of pH, osmolality and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent/Vehicle:

- characterisation (nature, supplier and lot);
- justification for choice of solvent/vehicle;
- solubility and stability of the test chemical in solvent/vehicle, if known;

Receptors:

- source of receptors (supplier, catalog No., lot, species of receptor, active receptor concentration provided from supplier, certification from supplier)
- characterization of receptors (including saturation binding results): Kd, Bmax,
- storage of receptors
- radiolabeled ligand:
- supplier, catalog No., lot, specific activity

Test conditions:
- solubility limitations under assay conditions;
- composition of binding buffer;
- concentration of receptor;
- concentration of tracer (i.e. radiolabeled ligand);
- concentrations of test chemical;
- percent vehicle in final assay;
- incubation temperature and time;
- method of bound/free separation;
- positive and negative controls/reference substances;
- criteria for considering tests as positive, negative or equivocal;

Acceptability check:
- actual IC_{50} and Hillslope values for concurrent positive controls/reference substances;

Results:
- raw and bound/free data;
- denaturing confirmation check, if appropriate;
- if it exists, the lowest effective concentration (LEC);
- RBA and/or IC_{50} values, as appropriate;
- concentration-response relationship, where possible;
- statistical analyses, if any, together with a measure of error and confidence (e.g. SEM, SD, CV or 95% CI) and a description of how these values were obtained;

Discussion of the results:
- application of 10% rule

Conclusion

LITERATURE

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ANNEX 1: DEFINITIONS AND ABBREVIATIONS

10% Rule: Option to exclude from the analyses data points where the mean of the replicates for the percent [^{3}H]17β-estradiol specific bound is 10% or more above that observed for the mean value at a lower concentration (see annex 4).

Acceptability criteria: Minimum standards for the performance of experimental controls and reference standards. All acceptability criteria should be met for an experiment to be considered valid.

Accuracy (concordance): The closeness of agreement between test method results and an accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with "concordance" to mean the proportion of correct outcomes of a test method (1).

CF: The OECD Conceptual Framework for the Testing and Evaluation of Endocrine Disrupters.

CV: Coefficient of variation

E2: 17β-estradiol

ED: Endocrine disruption

hERα: Human estrogen receptor alpha

ER: Estrogen receptor

Estrogenic activity: The capability of a chemical to mimic 17β-estradiol in its ability to bind estrogen receptors. Binding to the hERα can be detected with this PBTG.

IC_{50}: The half maximal effective concentration of an inhibitory test chemical.

ICCVAM: The Interagency Coordinating Committee on the Validation of Alternative Methods.

Inter-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same substances, can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the prevalidation and validation processes, and indicates the extent to which a test method can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility (1).

Intra-laboratory reproducibility: A determination of the extent that qualified people within the same laboratory can successfully replicate results using a specific protocol at different times. Also referred to as "within-laboratory reproducibility" (1).

LEC: Lowest effective concentration is the lowest concentration of test chemical that produces a response (*i.e.* the lowest test chemical concentration at which the fold induction is statistically different from the concurrent vehicle control).

Me-too test: A colloquial expression for a test method that is structurally and functionally similar to a validated and accepted reference test method. Interchangeably used with similar test method

PBTG: Performance-Based Test Guideline

Performance standards: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are (1) essential test method components; (2) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (3) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of reference chemicals (1).

Proficiency substances: A subset of the Reference substances included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardized test method. Selection criteria for these substances typically include that they represent the range of responses, are commercially available, and have high quality reference data available.

Proficiency: The demonstrated ability to properly conduct a test method prior to testing unknown substances.

Reference estrogen: 17ß-estradiol (E2, CAS 50-28-2).

Reference test methods: The test methods upon which this PBTG is based.

RBA: Relative Binding Affinity. The RBA of a substance is calculated as a percent of the log (IC_{50}) for the substance relative to the log (IC_{50}) for 17β-estradiol

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (1).

Reliability: Measure of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility.

SD: Standard deviation.

Validated test method: A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose (1).

Validation: The process by which the reliability and relevance of a particular approach, method, process or assessment is established for a defined purpose (1).

ANNEX 2: THE FREYBERGER-WILSON *IN VITRO* ESTROGEN RECEPTOR (ERα) SATURATION AND COMPETITIVE BINDING ASSAYS USING FULL LENGTH RECOMBINANT ERα

INITIAL CONSIDERATIONS AND LIMITATIONS (See also GENERAL INTRODUCTION, page 1)

1. This *in vitro* Estrogen Receptor (ERα) saturation and competitive binding test method uses full length human receptor ERα (hrERα) that is produced in and isolated from baculovirus-infected insect cells. The protocol, developed by Freyberger and Wilson, underwent an international multi-laboratory validation study (2) which has demonstrated its relevance and reliability for the intended purpose of the test method.

2. This test method is a screening procedure for identifying substances that can bind to the full length hrERα. It is used to determine the ability of a test chemical to compete with 17β-estradiol for binding to hrERα. Quantitative assay results may include the IC_{50} (a measure of the concentration of test chemical needed to displace half of the [^{3}H]-17β-estradiol from the hrERα) and the relative binding affinities of test chemicals for the hrERα compared to 17β-estradiol. For chemical screening purposes, acceptable qualitative assay results may include classifications of test chemicals as either hrERα binders, non-binders, or equivocal based upon criteria described for the binding curves.

3. The test method uses a radioactive ligand that requires a radioactive materials license for the laboratory. All procedures with radioisotopes and hazardous chemicals should follow the regulations and procedures as described by national legislation.

4. The "**GENERAL INTRODUCTION**" and "**hrER BINDING TEST METHOD COMPONENTS"** (pages 1-14) should be read before using this test method for regulatory purposes. Definitions and abbreviations used in this TG are described in Annex 1.

PRINCIPLES OF THE TEST METHOD (See also GENERAL INTRODUCTION, page 1)

5. The hrERα binding assay measures the ability of a radiolabeled ligand ([^{3}H]17β-estradiol) to bind with the ER in the presence of increasing concentrations of a test chemical (i.e. competitor). Test chemicals that possess a high affinity for the ER compete with the radiolabeled ligand at a lower concentration as compared with those chemicals with lower affinity for the receptor.

6. This test method consists of two major components: a saturation binding experiment to characterise receptor-ligand interaction parameters, followed by a competitive binding experiment that characterises the competition between a test chemical and a radiolabeled ligand for binding to the ER.

7. The purpose of the saturation binding experiment is to characterise a particular batch of receptors for binding affinity and number in preparation for the competitive binding experiment. The saturation binding experiment measures, under equilibrium conditions, the affinity of a fixed concentration of the estrogen receptor for its natural ligand (represented by the dissociation constant, Kd), and the concentration of active receptor sites (Bmax).

8. The competitive binding experiment measures the affinity of a substance to compete with [^{3}H]17β-estradiol for binding to the ER. The affinity is quantified by the concentration of test chemical that, at equilibrium, inhibits 50% of the specific binding of the [^{3}H]17β-estradiol (termed the "inhibitory concentration 50%" or IC_{50}). This can also be evaluated using the relative binding affinity (RBA, relative to the IC_{50} of estradiol measured separately in the same run). The competitive binding experiment measures the binding of [^{3}H]17β-estradiol at a fixed concentration in the presence of a wide range (eight orders of magnitude) of test chemical concentrations. The data are then fit, where possible, to a

form of the Hill equation (Hill, 1910) that describes the displacement of the radioligand by a one-site competitive binder. The extent of displacement of the radiolabeled estradiol at equilibrium is used to characterise the test chemical as a binder, non-binder, or generating an equivocal response.

PROCEDURE

Demonstration of Acceptable hrERα Protein Performance

9. Prior to routinely conducting the saturation and competitive binding assays, each new batch of hrERα should be shown to be performing correctly in the laboratory in which it will be used. A two-step process should be used to demonstrate performance. These steps are the following:

- Conduct a saturation [^{3}H]-17β-estradiol binding assay to demonstrate hrERα specificity and saturation. Nonlinear regression analysis of these data (e.g. BioSoft; McPherson, 1985; Motulsky, 1995) and the subsequent Scatchard plot should document hrERα binding affinity of the [^{3}H]-17β-estradiol (Kd) and the number of receptors (Bmax) for each batch of hrERα.

- Conduct a competitive binding assay using the control substances (reference estrogen (17β-estradiol), a weak binder (e.g. norethynodrel or norethindrone), and a non-binder (octyltriethoxysilane, OTES). Each laboratory should establish an historical database to document the consistency of IC_{50} and other relevant values for the reference estrogen and weak binder among experiments and different batches of hrERα. The curve fit parameters of the competitive binding curves for the control substances should be within the range of the mean ±2SD (see Table 1) that were developed using data from laboratories that participated in the validation study for this test method (2).

Table 1. Performance criteria developed for the reference estrogen and weak binder, FW hrER Binding Assay.

Substance	Parameter	Mean[a]	Standard Deviation (n)	Range of performance criteria based on Mean±2SD [b]
17β-estradiol	Top (%)	100.44	10.84 (67)	78.76 ~ 122.12
	Bottom (%)	0.29	1.25 (67)	-2.21 ~ 2.79
	Hill Slope	-1.06	0.20 (67)	-1.46 ~ -0.66
	$LogIC_{50}$ (M)	-8.92[c]	0.18 (67)	-9.28 ~ -8.56
Norethynodrel	Top (%)	99.42	8.90 (68)	81.62 ~ 117.22
	Bottom (%)	2.02	3.42 (68)	-4.82 ~ 8.86
	Hill Slope	-1.01	0.38 (68)	-1.77 ~ -0.25
	LogIC50 (M)	-6.39	0.27 (68)	-6.93 ~ -5.85
Norethindrone[c]	Top (%)	96.14	8.44 (27)	79.26 ~ 113.02
	Bottom (%)	2.38	5.02 (27)	-7.66 ~ 12.42
	Hill Slope	-1.41	0.32 (27)	-2.05~-0.77
	$LogIC_{50}$(M)	-5.73	0.27 (27)	-6.27 ~ -5.19

[a] *Mean (n) ± Standard Deviation (SD) was calculated using curve fit parameter estimates (4-parameter Hill Equation) for control runs conducted in four laboratories during the validation study (see Reference 2, Annex N (pages 71-83, https://www.oecd.org/env/ehs/testing/35_ANN3.pdf).*

[b] *Ranges of Performance Criteria (based on the mean +/- 2SD for each parameter) are provided as a guide for assay acceptability.*

[c] *Testing of norethindrone was optional for Subtask 4 during the validation study (see Reference 2, Subtask 4, pages 79-85, https://www.oecd.org/env/ehs/testing/MONO(2015)35.pdf). Thus, the mean ± SD (n) for each parameter was calculated using curve fit estimates (4-parameter Hill equation) for control runs conducted in two laboratories.*

[d]*The range for the IC50 will be dependent upon the Kd of the receptor preparation and concentration of radiolabeled ligand used within each laboratory. Appropriate adjustment for the range of the IC50 based upon the conditions used to conduct the test method will be acceptable.*

Demonstration of laboratory proficiency

10. See paragraphs 17 and 18 and Table 2 in "**hrER BINDING TEST METHOD COMPONENTS**" of this Test Guideline. Each assay (saturation and competitive binding) should consist of three independent runs (i.e. with fresh dilutions of receptor, chemicals, and reagents) on different days, and each run should contain three replicates.

Determination of Receptor (hrERα) Concentration

11. The concentration of active receptor varies slightly by batch and storage conditions. For this reason, the concentration of active receptor as received from the supplier should be determined. This will yield the appropriate concentration of active receptor at the time of the run.

12. Under conditions corresponding to competitive binding (i.e. 1 nM [^{3}H]-estradiol), nominal concentrations of 0.25, 0.5, 0.75, and 1 nM receptor should be incubated in the absence (total binding) and presence (non-specific binding) of 1 µM unlabeled estradiol. Specific binding, calculated as the difference of total and non-specific binding, is plotted against the nominal receptor concentration. The concentration of receptor that gives specific binding values corresponding to 20% of added radiolabel is related to the corresponding nominal receptor concentration, and this receptor concentration should be used for saturation and competitive binding experiments. Frequently, a final hrER concentration of 0.5 nM will comply with this condition.

13. If the 20% criterion repeatedly cannot be met, the experimental set up should be checked for potential errors. Failure to achieve the 20% criterion may indicate that there is very little active receptor in the recombinant batch, and the use of another receptor batch should then be considered.

Saturation assay

14. Eight increasing concentrations of [^{3}H]17β-estradiol should be evaluated in triplicate, under the following three conditions (see Table 2):

a. In the absence of unlabelled 17β-estradiol and presence of ER. This is the determination of total binding by measure of the radioactivity in the wells that have only [^{3}H]17β-estradiol.
b. In the presence of a 1000- fold excess concentration of unlabelled 17β-estradiol over labelled 17β-estradiol and presence of ER. The intent of this condition is to saturate the active binding sites with unlabelled 17β-estradiol, and by measuring the radioactivity in the wells, determine the non-specific binding. Any remaining hot estradiol that can bind to the receptor is considered to be binding at a non-specific site as the cold estradiol should be at such a high concentration that it is bound to all of the available specific sites on the receptor.
c. In the absence of unlabelled 17β-estradiol and absence of ER (determination of total radioactivity)

Preparation of [^{3}H]-17β-estradiol and unlabelled 17β-estradiol solutions

15. Dilutions of [^{3}H]-17β-estradiol should be prepared by adding assay buffer to a 12 nM stock solution of [^{3}H]-17β-estradiol to obtain concentrations initially ranging from 0.12nM to 12 nM. By adding 40 µL of these solutions to the respective assay wells of a 96-well microtiter plate (in a final volume of 160 µL), the final assay concentrations, ranging from 0.03 to 3.0 nM, will be obtained. Preparation of assay buffer, [^{3}H]-17β-estradiol stock solution and dilutions and determination of the concentrations are described in depth in the FW protocol (2).

16. Dilutions of ethanolic 17β-estradiol solutions should be prepared by adding assay buffer to achieve eight increasing concentrations initially ranging from 0.06 µM to 6 µM. By adding 80 µL of these solutions to the respective assay wells of a 96-well microtiter plate (in a final volume of 160 µL), the final assay concentrations, ranging from 0.03µM to 3µM, will be obtained. The final concentration of unlabeled 17β-estradiol in the individual non-specific binding assay wells should be 1000-fold of the labeled [^{3}H]-17β-estradiol concentration. Preparation of unlabelled 17β-estradiol dilutions is described in depth in the FW protocol (2).

17. The nominal concentration of receptor that gives specific binding of 20±5% should be used (see paragraphs 12-13). The hrERα solution should be prepared immediately prior to use.

18. The 96-well microtiter plates are prepared as illustrated in Table 2, with 3 replicates per concentration. Example of plate concentration and volume assignment of [^{3}H]-17β-estradiol, unlabeled 17β-estradiol, buffer and receptor are provided in Appendix 2.

Table 2: Saturation Binding Assay Microtiter Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.03 nM [^{3}H] E_2 + ER			0.06 nM [^{3}H] E_2 + ER			0.08 nM [^{3}H] E_2 + ER			0.10 nM [^{3}H] E_2 + ER			Total Binding (Solvent)
B	0.30 nM [^{3}H] E_2 + ER			0.60 nM [^{3}H] E_2 + ER			1.0 nM [^{3}H] E_2 + ER			3.0 nM [^{3}H] E_2 + ER			
C													
D	0.03 nM [^{3}H] E_2 + ER + 0.03 µM E_2			0.06 nM [^{3}H] E_2 + ER + 0.06 µM E_2			0.08 nM [^{3}H] E_2 + ER + 0.08 µM E_2			0.10 nM [^{3}H] E_2 + ER + 0.10 µM E_2			Non-Specific Binding
E	0.30 nM [^{3}H] E_2 + ER + 0.30 µM E_2			0.60 nM [^{3}H] E_2 + ER + 0.60 µM E_2			1.0 nM [^{3}H] E_2 + ER			3.0 nM [^{3}H] E_2 + ER + 3.0 µM E_2			
F													
G													
H													

[^{3}H] E_2: [^{3}H]-17β-estradiol
ER: estrogen receptor
E_2: unlabelled 17β-estradiol

19. Assay microtiter plates should be incubated at 2° to 8°C for 16 to 20 hours and placed on a rotator during the incubation period.

Measurement of [3H]-17β-Estradiol bound to hrERα

20. [^{3}H]-17β-Estradiol bound to hrERα should be separated from free [^{3}H]-17β-Estradiol by adding 80 µL of cold DCC suspension to each well, shaking the microtiter plates for 10 minutes and centrifugating for 10 minutes at about 2500 RPM. To minimize dissociation of bound [^{3}H]-17β-estradiol from the hrERα during this process, it is extremely important that the buffers and assay wells be kept between 2 and 8°C and that each step be conducted quickly. A shaker for microtiter plates is necessary to process plates efficiently and quickly.

21. 50 µL of supernatant containing the hrERα-bound [^{3}H]-17β-estradiol should then be taken with extreme care, to avoid any contamination of the wells by touching DCC, and should be placed on a second microtiter plate.

22. 200 µL of scintillation fluid, capable of converting the kinetic energy of nuclear emissions into light energy, should then be added to each well (A1-B12 and D1 to E12). Wells G1-H12 (identified as total dpms) represent serial dilutions of the [^{3}H]-17β-estradiol (40 µL) that should be delivered directly into the scintillation fluid in the wells of the measurement plate as indicated in Table 3, i.e. these wells contain only 200 µL of scintillation fluid and the appropriate dilution of [3H]-17β-estradiol. These measures demonstrate how much [^{3}H]-17β-estradiol in dpms was added to each set of wells for the total binding and non-specific binding.

Table 3: Saturation Binding Assay Microtiter Plate Layout, Radioactivity Measurement

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.03 nM [^{3}H] E_2 + ER			0.06 nM [^{3}H] E_2 + ER			0.08 nM [^{3}H] E_2 + ER			0.10 nM [^{3} H] E_2 + ER			Total Binding (Solvent)
B	0.30 nM [^{3}H] E_2 + ER			0.60 nM [^{3}H] E_2 + ER			1.0 nM [^{3}H] E_2 + ER			3.0 nM [^{3}H] E_2 + ER			
C													
D	0.03 nM [^{3}H] E_2 + ER + 0.03 µM E_2			0.06 nM [^{3}H] E_2 + ER + 0.06 µM E_2			0.08 nM [^{3}H] E_2 + ER + 0.08 µM E_2			0.10 nM [^{3}H] E_2 + ER + 0.10 µM E_2			Non-Specific Binding
E	0.30 nM [^{3}H] E_2 + ER + 0.30 µM E_2			0.60 nM [^{3}H] E_2 + ER + 0.60 µM E_2			1.0 nM [^{3}H] E_2 + ER			3.0 nM [^{3}H] E_2 + ER + 3.0 µM E_2			
F													
G	0.03 nM [^{3}H] E_2 (total dpms)			0.06 nM [^{3}H] E_2			0.08 nM [^{3}H] E_2			0.10 nM [^{3}H] E_2			Total dpms*
H	0.30 nM [^{3}H] E_2			0.60 nM [^{3}H] E_2			1.0 nM [^{3}H] E_2			3.0 nM [^{3}H] E_2			

[^{3}H] E_2: [^{3}H]-17β-estradiol
ER: estrogen receptor
E_2: unlabelled 17β-estradiol
dpms: disintegrations per minute
*The hot serial dilutions of [^{3}H]-labeled estradiol here should be directly added into 200 µL of scintillation fluid in wells G1 – H12.

23. Measurement should start with a delay of at least 2 hours and counting time should be 40 minutes per well. A microtiter plate scintillation counter should be used for determination of dpm/well with quench correction. Alternatively, if a scintillation counter for a microtiter plate is not available, samples may be measured in a conventional counter. Under these conditions, a reduction of counting time may be considered.

Competitive binding assay

24. 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 chemical. Three concurrent replicates should be used at each concentration within one run. In addition, three non-concurrent runs should be performed for each chemical tested. The assay should be set up in one or more 96-well microtiter plates

Controls

25. When performing the assay, concurrent solvent and controls (i.e. reference estrogen, weak binder, and non-binder) should be included in each experiment. Full concentration curves for the reference estrogen and controls (i.e. weak binder and non-binder) should be used in one plate during each run. All other plates should contain (i) a high- (maximum displacement) and medium- (approximately the IC50) concentration each of E2 and weak binder in triplicate; (ii) solvent control and non-specific binding, each at least in triplicate. Procedures for the preparation of assay buffer, controls, [^{3}H]-17β-estradiol, hrERα and test chemical solutions are described in Reference 2 (Annex K, see FW Assay Protocol).

- Solvent control:

26. The solvent control indicates that the solvent does not interact with the test system and also measures total binding (TB). Ethanol is the preferred solvent. Alternatively, if the highest concentration of the test chemical is not soluble in ethanol, DMSO may be used. The concentration of ethanol or DMSO, if used, in the final assay wells is 1.5% and may not exceed 2%.

- Buffer control:

27. The buffer control (BC) should contain neither solvent nor test chemical, but all of the other components of the assay. The results of the buffer control are compared to the solvent control to verify that the solvent used does not affect the assay system.

- Strong binder (reference estrogen)

28. 17β-estradiol (CAS 50-28-2) is the endogenous ligand and binds with high affinity to the ER, alpha subtype. A standard curve using unlabeled 17β-estradiol should be prepared for each hrERα competitive binding assay, to allow for an assessment of variability when conducting the assay over time within the same laboratory. Eight solutions of unlabeled 17β-estradiol should be prepared in ethanol, with concentrations in the assay wells ranging from 100 nM – 10 pM (-7[logM] to -11[logM]), spaced as follows: (-7[logM], -8[logM], -8.5[logM], -9[logM], - 9.5[logM], -10[logM], -11[logM]). The highest concentration of unlabeled 17β-estradiol (1 µM) also serves as the non-specific binding indicator. This concentration is distinguished by the label “NSB” in Table 4 even though it is also part of the standard curve.

- Weak binder

29. A weak binder (norethynodrel (CAS68-23-5) or norethindrone (CAS 68-22-4)) should be included to demonstrate the sensitivity of each experiment and to allow an assessment of variability when conducting the assay over time. Eight solutions of the weak binder should be prepared in ethanol, with concentrations in the assay wells ranging from 3 nM to 30 µM (-8.5[logM] to -4.5[logM]), spaced as follows: -4.5[logM], -5[logM], -5.5[logM], -6[logM], -6.5[logM], -7[logM],-7.5[logM], -8.5[logM].

- Non binder

30. Octyltriethoxysilane (OTES, CAS 2943-75-1) should be used as the negative control (non-binder). It provides assurance that the assay as run, will detect when test chemicals do not bind to the hrERα. Eight solutions of the non-binder should be prepared in ethanol, with concentrations in the assay wells ranging from 0.1nM to 1000 µM (-10[logM] to -3[logM]), in log increments. Di-*n*-butyl phtalate (DBP) can be used as an alternate control non-binder. Its maximum solubility has been shown to be -4[logM].

hrERα concentration

31. The amount of receptor that gives specific binding of 20±5% of 1 nM radioligand should be used (see paragraphs 12-13 of Annex 2). The hrERα solution should be prepared immediately prior to use.

[^{3}H]-17β-estradiol

32. The concentration of [^{3}H]-17β-estradiol in the assay wells should be of 1.0 nM.

Test Chemicals

33. In the first instance, it is necessary to conduct a solubility test to determine the limit of solubility for each test chemical and to identify the appropriate concentration range to use when conducting the test protocol. The limit of solubility of each test chemical is to be initially determined in the solvent and further confirmed under assay conditions. The final concentration tested in the assay should not exceed 1 mM. Range finder testing consists of a solvent control along with 8 log serial dilutions, starting at the maximum acceptable concentration (e.g. 1 mM or lower, based upon the limit of solubility), and the presence of cloudiness or precipitate noted (see also paragraph 35). The test chemical should be tested using 8 log concentration spaced curves as defined by the preceding range finding test. Concentrations in the second and third experiments should be adjusted as appropriate to better characterise the concentration-response curve.

34. Dilutions of the test chemical should be prepared in the appropriate solvent (see paragraph 26 of Annex 2). If the highest concentration of the test chemical is not soluble in either ethanol or DMSO, and adding more solvent would cause the solvent concentration in the final tube to be greater than the acceptable limit, the highest concentration may be reduced to the next lower concentration. In this case, an additional concentration may be added at the low end of the concentration series. Other concentrations in the series should remain unchanged.

35. The test chemical solutions should be closely monitored when added to the assay well, as the test chemical may precipitate upon addition to the assay well. The data for all wells that contain precipitate should be excluded from curve-fitting, and the reason for exclusion of the data noted.

36. If there is prior existing information from other sources that provide a log(IC_{50}) of a test chemical, it may be appropriate to geometrically space the dilutions (i.e. 0.5 log units around the expected log(IC_{50}). The final result should reflect sufficient spread of concentrations on either side of the log(IC_{50}), including the “top” and “bottom”, such that the binding curve can be adequately characterised.

Assay plate organisation

37. Labeled microtiter plates should be prepared considering sextuple incubations with codes for the solvent control, the highest concentration of the reference estrogen which also serves as the non-specific binding (NSB) indicator, and the buffer control and considering triplicate incubations with codes for each of the eight concentrations of the non-binding control (octyltriethoxysilane), the 7 lower concentrations for the reference estrogen, the eight concentrations dose levels of the weak binder, and the 8 concentrations of each test chemical (TC). An example layout of the plate diagram for the full concentration curves for the reference estrogen and control is given below in Table 4. Additional microtiter plates are used for the test chemicals and should include plate controls (i.e. 1) a high- (maximum displacement) and medium- (approximately the IC50) concentration each of E2 and weak binder in triplicate; 2) solvent control and non-specific binding, each in sextuple (Table 5). An example of a competitive assay microtiter plate layout worksheet using three unknown test chemicals is provided in Appendix 3 of Annex 2. The concentrations indicated in Tables 4 and 5 are the final concentrations of the assay. The maximum concentration for E2 should be 1×10^{-7} M and for the weak binder, the highest concentration used for the weak binder on plate 1 should be used. The IC50 concentration has to be determined by the laboratory based on their historical control database. It is expected

that this value would be similar to that observed in the validation studies (see Table 1).

Table 4: Competitive Binding Assay Microtiter Plate Layout, Full Concentration Curves for Reference Estrogen and Controls (Plate 1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	TB (Solvent only)			TB (Solvent only)			NSB			NSB		
B	E_2 (1×10^{-7})			E_2 (1×10^{-8})			E_2 ($1\times10^{-8.5}$)			E_2 (1×10^{-9})		
C	E_2 ($1\times10^{-9.5}$)			E_2 (1×10^{-10})			E_2 (1×10^{-11})			Blank*		
D	NE ($1\times10^{-4.5}$)			NE (1×10^{-5})			NE ($1\times10^{-5.5}$)			NE (1×10^{-6})		
E	NE ($1\times10^{-6.5}$)			NE (1×10^{-7})			NE ($1\times10^{-7.5}$)			NE ($1\times10^{-8.5}$)		
F	OTES (1×10^{-3})			OTES (1×10^{-4})			OTES (1×10^{-5})			OTES (1×10^{-6})		
G	OTES (1×10^{-7})			OTES (1×10^{-8})			OTES (1×10^{-9})			OTES (1×10^{-10})		
H	Blank (for hot)**			Blank (for hot) **			Buffer control			Buffer control		

In this example, the weak binder is norethinodrel (NE)

* real blank, well not used

** blank not used during the incubation, but used to confirm the total radioactivity added.

Table 5: Competitive Binding Assay Microtiter Plate Layout, Full Concentration Curves for Test Chemicals and Plate Controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A	TB (Solvent only)			TB (Solvent only)			NSB			NSB		
B	TC1 (1×10^{-3})			TC1 (1×10^{-4})			TC1 (1×10^{-5})			TC1 (1×10^{-6})		
C	TC1 (1×10^{-7})			TC1 (1×10^{-8})			TC1 (1×10^{-9})			TC1 (1×10^{-10})		
D	TC2 (1×10^{-3})			TC2 (1×10^{-4})			TC2 (1×10^{-5})			TC2 (1×10^{-6})		
E	TC2 (1×10^{-7})			TC2 (1×10^{-8})			TC2 (1×10^{-9})			TC2 (1×10^{-10})		
F	TC3 (1×10^{-3})			TC3 (1×10^{-4})			TC3 (1×10^{-5})			TC3 (1×10^{-6})		
G	TC3 (1×10^{-7})			TC3 (1×10^{-8})			TC3 (1×10^{-9})			TC3 (1×10^{-10})		
H	NE (IC50)			NE ($1\times10^{-4.5}$)			E_2 (IC50)			E_2 (1×10^{-7})		

In this example, the weak binder is norethinodrel (NE)

Completion of competitive binding assay

38. As shown in Table 6, 80 µL of the solvent control, buffer control, reference estrogen, weak binder, non-binder, and test chemicals prepared in assay buffer should be added to the wells. Then, 40 µl of a 4 nM [3H]-17β-estradiol solution should be added to each well. After gentle rotation for 10 to 15 minutes between 2° to 8°C, 40 µl of hrERα solution should be added. Assay microtiter plates should be incubated at 2° to 8°C for 16 to 20 hours, and placed on a rotator during the incubation period.

Table 6: Volume of Assay Components for hrER Competitive Binding Assay, Microtiter Plates

Volume (µL)	*Constituent*
80	Unlabeled 17β-estradiol, norethynodrel, OTES, test chemicals, solvent or buffer
40	4 nM [^{3}H]-17β-estradiol solution
40	hrERα solution, concentration as determined
160	***Total volume in each assay well***

39. The quantification of [^{3}H]-17β-Estradiol bound to hrERα, following separation of [^{3}H]-17β-Estradiol bound to hrERα from free [^{3}H]-17β-Estradiol by adding 80 µL of cold DCC suspension to each well, should then be performed as described in paragraphs 20-23 for the saturation binding assay.

40. Wells H1-6 (identified as blank (for hot) in table 4) represent the dpms of the [^{3}H]-labeled-estradiol in 40 µL. The 40 µL aliquot should be delivered directly into the scintillation fluid in wells H1 – H6.

Acceptability criteria

Saturation binding assay

41. The specific binding curve should reach a plateau as increasing concentrations of [^{3}H]-17β-estradiol were used, indicating saturation of hrERα with ligand.

42. The specific binding at 1 nM of [3H]-17β-estradiol should be inside the acceptable range 15% to 25% of the average measured total radioactivity added across runs. Occasional slight excursions outside of this range are acceptable, but if runs are consistently outside this range or a particular run is significantly outside this range, the protein concentration should be adjusted and the saturation assay repeated.

43. The data should produce a linear Scatchard plot.

44. The non-specific binding should not be excessive. The value for non-specific binding should typically be <35% of the total binding. However, the ratio might occasionally exceed this limit when measuring very low dpm for the lowest concentration of radiolabeled 17β-Estradiol tested.

Competitive binding assay

45. Increasing concentrations of unlabeled 17β-estradiol should displace [^{3}H]-17β- estradiol from the receptor in a manner consistent with a one-site competitive binding.

46. The IC50 value for the reference estrogen (i.e. 17β-estradiol) should be approximately equal to the molar concentration of [^{3}H]-17β-estradiol plus the Kd determined from the saturation binding assay.

47. The total specific binding should be consistently within the acceptable range of 20 ± 5 % when the average measured concentration of total radioactivity added to each well was 1 nM across runs. Occasional slight excursions outside of this range are acceptable, but if runs are consistently outside this range or a particular run is significantly outside this range, the protein concentration should be adjusted.

48. The solvent should not alter the sensitivity or reproducibility of the assay. The results of the solvent control (TB wells) are compared to the buffer control to verify that the solvent used does not affect the assay system. The results of the TB and Buffer control should be comparable if there is no effect of the solvent on the assay.

49. The non-binder should not displace more than 25% of the [^{3}H]-17β-estradiol from the hrERα when tested up to10^{-3} M (OTES) or 10^{-4} M (DBP).

50. Performance criteria were developed for the reference estrogen and two weak binders (e.g. norethynodrel, norethindrone) using data from the validation study of the FW hrER Binding Assay (Reference 2, Annex N (pages 71-83, https://www.oecd.org/env/ehs/testing/35_ANN3.pdf)). Performance criteria were calculated using the mean +/- 2SD for the curve fit parameters (i.e. top, bottom, Hillslope, logIC_{50}) for the reference estrogen and weak binders. Table 1 provides ranges for the curve fit parameters that can be used as a guide for assay cceptability. In practice, the range of the IC_{50} may vary slightly based upon the Kd of receptor preparation and ligand concentration.

51. No performance criteria was developed for curve fit parameters for the test chemicals because of the wide array of existing potential test chemicals and variation in potential affinities and outcomes (e.g. Full curve, partial curve, no curve fit). However, professional judgment should be applied when reviewing results from each run for a test chemical. A sufficient range of concentrations of the test chemical should be used to clearly define the top (e.g. 90 - 100% of binding) of the competitive curve. Variability among replicates at each concentration of test chemical as well as among the 3 non-concurrent runs should be reasonable and scientifically defensible. Controls from each run for a test chemical should approach the measures of performance reported for this FW assay and be consistent historical control data from each respective

laboratory.

ANALYSIS OF DATA

Saturation binding assay

52. Both total and non-specific binding are measured. From these values, specific binding of increasing concentrations of [^{3}H]-17β-estradiol under equilibrium conditions is calculated by subtracting non-specific from total. A graph of specific binding versus [^{3}H]-17β-estradiol concentration should reach a plateau for maximum specific binding indicative of saturation of the hrERα with the [^{3}H]-17β-estradiol. In addition, analysis of the data should document the binding of the [^{3}H]-17β- estradiol to a single, high-affinity binding site. Non-specific, total, and specific binding should be displayed on a saturation binding curve. Further 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.

53. The data analysis should determine B_{max} and K_d from the total binding data alone, using the assumption that non- specific binding is linear, unless justification is given for using a different method. In addition, robust regression should be used when determining the best fit unless justification is given. The method chosen for robust regression should be stated. Correction for ligand depletion (e.g. using the method of Swillens 1995) should always be used when determining B_{max} and K_d from saturation binding data.

Competitive binding assay

54. The competitive binding curve is plotted as specific [^{3}H]-17β-estradiol binding versus the concentration (log10 units) of the competitor. The concentration of the test chemical that inhibits 50% of the maximum specific [^{3}H]-17β-estradiol binding is the IC_{50} value.

55. Estimates of log(IC_{50}) values for the positive controls (e.g. reference estrogen and weak binder) should be determined using an appropriate nonlinear curve fitting software to fit a four parameter Hill equation (e.g. BioSoft; McPherson, 1985; Motulsky, 1995). The top, bottom, slope, and log(IC_{50}) should generally be left unconstrained when fitting these curves. Robust regression should be used when determining the best fit unless justification is given. Correction for ligand depletion should not be used. Following the initial analysis, each binding curve should be reviewed to ensure appropriate fit to the model. The relative binding affinity (RBA) for the weak binder should be calculated as a percent of the log (IC_{50}) for the weak binder relative to the log (IC_{50}) for 17β-estradiol. Results from the positive controls and the non-binder control should be evaluated using the measures of the test method performance in paragraphs 45-50 in this Annex 2.

56. Data for all test chemicals should be analysed using a step-wise approach to ensure that data are appropriately analysed and that each competitive binding curve is properly classified. It is recommended that each run for a test chemical initially undergo a standardised data analysis that is identical to that used for the reference estrogen and weak binder controls (see paragraph 55 above). Once completed, a technical review of the curve fit parameters as well as a visual review of how well the data fit the generated competitive binding curve for each run should be conducted. During this technical review, the observations of a concentration dependent decrease in the percent [^{3}H]-17β-estradiol specifically bound, low variability among the technical replicates at each chemical concentration, and consistency in fit parameters among the three runs are a good indication that the assay and data analyses were conducted appropriately.

Data interpretation

57. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be a binder for the hrERα if a binding curve can be fit and the lowest point on the response curve within the range of the data is less than 50% (Figure 1).

58. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be a non-binder for the hrERα if:

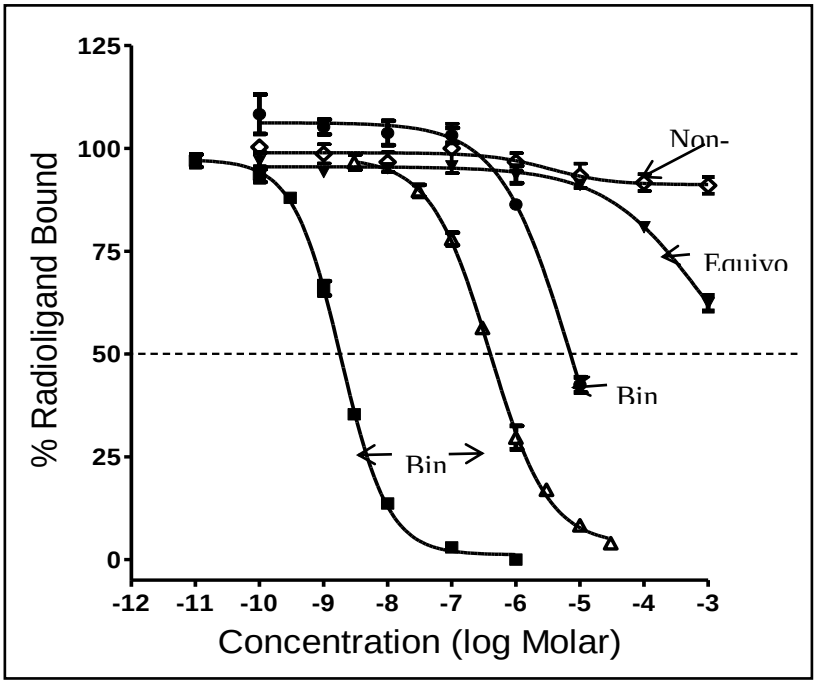
- A binding curve can be fit and the lowest point on the fitted response curve within the range of the data is above 75%, or
- A binding curve cannot be fit and the lowest unsmoothed average percent binding among the concentration groups in the data is above 75%.

59. Test chemicals are considered equivocal if none of the above conditions are met (e.g. the lowest point on the fitted response curve is between 76 – 51%).

Table7. Criteria for assigning classification based upon competitive binding curve for a test chemical.

Classification	Criteria
Binder[a]	A binding curve can be fit. • The **lowest point on the response curve within the range of the data is less than 50%.**
Non-binder[b]	If a binding curve can be fit, • the **lowest point on the fitted response curve within the range of the data is above 75%.** If a binding curve cannot be fit, • the **lowest unsmoothed average percent binding among the concentration groups in the data is above 75%.**
Equivocal[c]	Any testable run that is neither a binder nor a non-binder (*e.g.*, The lowest point on the fitted response curve is between 76 – 51%).

Figure 1. Examples of test chemical classification using competitive binding curve.



60. Multiple runs conducted within a laboratory for a test chemical are combined by assigning numeric values to each run and averaging across the runs as shown in Table 8. Results for the combined runs within each laboratory are compared with the expected classification for each test chemical.

Table 8. Method for classification of test chemical using multiple runs within a laboratory

To assign value to each run:	
Classification	**Numeric Value**
Binder	2
Equivocal	1
Non-binder	0
To classify average of numeric value across runs:	
Classification	**Numeric Value**
Binder	Average ≥ 1.5
Equivocal	0.5 ≤ Average < 1.5
Non-binder	Average < 0.5

TEST REPORT

61. See paragraph 24 of "**hrER BINDING TEST METHOD COMPONENTS**" of this Test Guideline.

Appendix 1: List of Terms

[^{3}H]E_2: 17β-Estradiol radiolabeled with tritium
DCC: Dextran-coated charcoal
E_2: Unlabeled 17β-estradiol (inert)
Assay buffer: 10 mM Tris, 10 mg Bovine Serum Albumin /mL, 2 mM DTT, 10% glycerol, 0.2 mM leupeptin, pH 7.5

hrERα: Human recombinant estrogen receptor alpha (ligand binding domain)
Replicate: One of multiple wells that contain the same contents at the same concentrations and are assayed concurrently within a single run. In this protocol, each concentration of test chemical is tested in triplicate; that is, there are three replicates that are assayed simultaneously at each concentration of test chemical.
Run: A complete set of concurrently-run microtiter plate assay wells that provides all the information necessary to characterize binding of a test chemical to the hrERα (viz., total [^{3}H]-17β-estradiol added to the assay well, maximum binding of [^{3}H]-17β-estradiol to the hrERα, nonspecific binding, and total binding at various concentrations of test chemical). A run could consist of as few as one assay well (i.e. replicate) per concentration, but since this protocol requires assaying in triplicate, one run consists of three assay wells per concentration. In addition, this protocol requires three independent (i.e. non-concurrent) runs per chemical.

Appendix 2: Typical [^{3}H]-17β-Estradiol Saturation Assay with Three Replicate Wells

Typical [^{3}H]-17β-Estradiol Saturation Assay with Three Replicate Wells											
Position	Replicate	Well Type Code	Hot E2 Initial Concentration (nM)	Hot E2 Volume (uL)	Hot E2 Final Concentration (nM)	Cold E2 Initial Concentration (uM)	Cold E2 Volume (uL)	Cold E2 Final Concentration (uM)	Buffer Volume (uL)	Receptor Volume (uL)	Total volume in wells
A1	1	H	0.12	40	0.03	—	—	—	80	40	160
A2	2	H	0.12	40	0.03	—	—	—	80	40	160
A3	3	H	0.12	40	0.03	—	—	—	80	40	160
A4	1	H	0.24	40	0.06	—	—	—	80	40	160
A5	2	H	0.24	40	0.06	—	—	—	80	40	160
A6	3	H	0.24	40	0.06	—	—	—	80	40	160
A7	1	H	0.32	40	0.08	—	—	—	80	40	160
A8	2	H	0.32	40	0.08	—	—	—	80	40	160
A9	3	H	0.32	40	0.08	—	—	—	80	40	160
A10	1	H	0.40	40	0.10	—	—	—	80	40	160
A11	2	H	0.40	40	0.10	—	—	—	80	40	160
A12	3	H	0.40	40	0.10	—	—	—	80	40	160
B1	1	H	1.20	40	0.30	—	—	—	80	40	160
B2	2	H	1.20	40	0.30	—	—	—	80	40	160
B3	3	H	1.20	40	0.30	—	—	—	80	40	160
B4	1	H	2.40	40	0.60	—	—	—	80	40	160
B5	2	H	2.40	40	0.60	—	—	—	80	40	160
B6	3	H	2.40	40	0.60	—	—	—	80	40	160
B7	1	H	4.00	40	1.00	—	—	—	80	40	160
B8	2	H	4.00	40	1.00	—	—	—	80	40	160
B9	3	H	4.00	40	1.00	—	—	—	80	40	160
B10	1	H	12.00	40	3.00	—	—	—	80	40	160
B11	2	H	12.00	40	3.00	—	—	—	80	40	160
B12	3	H	12.00	40	3.00	—	—	—	80	40	160
D1	1	HC	0.12	40	0.03	0.06	80	0.03	—	40	160
D2	2	HC	0.12	40	0.03	0.06	80	0.03	—	40	160
D3	3	HC	0.12	40	0.03	0.06	80	0.03	—	40	160
D4	1	HC	0.24	40	0.06	0.12	80	0.06	—	40	160
D5	2	HC	0.24	40	0.06	0.12	80	0.06	—	40	160
D6	3	HC	0.24	40	0.06	0.12	80	0.06	—	40	160
D7	1	HC	0.32	40	0.08	0.16	80	0.08	—	40	160
D8	2	HC	0.32	40	0.08	0.16	80	0.08	—	40	160
D9	3	HC	0.32	40	0.08	0.16	80	0.08	—	40	160
D10	1	HC	0.40	40	0.10	0.2	80	0.1	—	40	160
D11	2	HC	0.40	40	0.10	0.2	80	0.1	—	40	160
D12	3	HC	0.40	40	0.10	0.2	80	0.1	—	40	160

Typical [^{3}H]-17β-Estradiol Saturation Assay with Three Replicate Wells											
Position	Replicate	Well Type Code	Hot E2 Initial Concentration (nM)	Hot E2 Volume (uL)	Hot E2 Final Concentration (nM)	Cold E2 Initial Concentration (uM)	Cold E2 Volume (uL)	Cold E2 Final Concentration (uM)	Buffer Volume (uL)	Receptor Volume (uL)	Total volume in wells
E1	1	HC	1.20	40	0.30	0.6	80	0.3	—	40	160
E2	2	HC	1.20	40	0.30	0.6	80	0.3	—	40	160
E3	3	HC	1.20	40	0.30	0.6	80	0.3	—	40	160
E4	1	HC	2.40	40	0.60	1.2	80	0.6	—	40	160
E5	2	HC	2.40	40	0.60	1.2	80	0.6	—	40	160
E6	3	HC	2.40	40	0.60	1.2	80	0.6	—	40	160
E7	1	HC	4.00	40	1.00	2	80	1	—	40	160
E8	2	HC	4.00	40	1.00	2	80	1	—	40	160
E9	3	HC	4.00	40	1.00	2	80	1	—	40	160
E10	1	HC	12.00	40	3.00	6	80	3	—	40	160
E11	2	HC	12.00	40	3.00	6	80	3	—	40	160
E12	3	HC	12.00	40	3.00	6	80	3	—	40	160
G1	1	Hot	0.12	40	0.03	—	—	—	—	—	40
G2	2	Hot	0.12	40	0.03	—	—	—	—	—	40
G3	3	Hot	0.12	40	0.03	—	—	—	—	—	40
G4	1	Hot	0.24	40	0.06	—	—	—	—	—	40
G5	2	Hot	0.24	40	0.06	—	—	—	—	—	40
G6	3	Hot	0.24	40	0.06	—	—	—	—	—	40
G7	1	Hot	0.32	40	0.08	—	—	—	—	—	40
G8	2	Hot	0.32	40	0.08	—	—	—	—	—	40
G9	3	Hot	0.32	40	0.08	—	—	—	—	—	40
G10	1	Hot	0.40	40	0.10	—	—	—	—	—	40
G11	2	Hot	0.40	40	0.10	—	—	—	—	—	40
G12	3	Hot	0.40	40	0.10	—	—	—	—	—	40
H1	1	Hot	1.20	40	0.30	—	—	—	—	—	40
H2	2	Hot	1.20	40	0.30	—	—	—	—	—	40
H3	3	Hot	1.20	40	0.30	—	—	—	—	—	40
H4	1	Hot	2.40	40	0.60	—	—	—	—	—	40
H5	2	Hot	2.40	40	0.60	—	—	—	—	—	40
H6	3	Hot	2.40	40	0.60	—	—	—	—	—	40
H7	1	Hot	4.00	40	1.00	—	—	—	—	—	40
H8	2	Hot	4.00	40	1.00	—	—	—	—	—	40
H9	3	Hot	4.00	40	1.00	—	—	—	—	—	40
H10	1	Hot	12.00	40	3.00	—	—	—	—	—	40
H11	2	Hot	12.00	40	3.00	—	—	—	—	—	40
H12	3	Hot	12.00	40	3.00	—	—	—	—	—	40

Note that the "hot" wells are empty during incubation. The 40 µl are added only for scintillation counting.

Appendix 3 :Competitive Binding Assay Well Layout

Plate	Position	Replicat	Well type	Well Code	Concentration	Competitor Initial Concentration	hrER stock (uL)	Buffer Volume (uL)	Tracer (Hot E2) Volume	Volume from dilution plate(uL)	Final Volume (ul)	Competitor Final Concentration
S	A1	1	total binding	TB	TB1	-	40	-	40	80	160	-
S	A2	2	total binding	TB	TB2	-	40	-	40	80	160	-
S	A3	3	total binding	TB	TB3	-	40	-	40	80	160	-
S	A4	1	total binding	TB	TB4	-	40	-	40	80	160	-
S	A5	2	total binding	TB	TB5	-	40	-	40	80	160	-
S	A6	3	total binding	TB	TB6	-	40	-	40	80	160	-
S	A7	1	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	A8	2	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	A9	3	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	A10	1	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	A11	2	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	A12	3	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	B1	1	cold E2	S	S1	2.00E-07	40	-	40	80	160	1.0E-07
S	B2	2	cold E2	S	S1	2.00E-07	40	-	40	80	160	1.0E-07
S	B3	3	cold E2	S	S1	2.00E-07	40	-	40	80	160	1.0E-07
S	B4	1	cold E2	S	S2	2.00E-08	40	-	40	80	160	1.0E-08
S	B5	2	cold E2	S	S2	2.00E-08	40	-	40	80	160	1.0E-08
S	B6	3	cold E2	S	S2	2.00E-08	40	-	40	80	160	1.0E-08
S	B7	1	cold E2	S	S3	6.00E-09	40	-	40	80	160	3.0E-09
S	B8	2	cold E2	S	S3	6.00E-09	40	-	40	80	160	3.0E-09
S	B9	3	cold E2	S	S3	6.00E-09	40	-	40	80	160	3.0E-09
S	B10	1	cold E2	S	S4	2.00E-09	40	-	40	80	160	1.0E-09
S	B11	2	cold E2	S	S4	2.00E-09	40	-	40	80	160	1.0E-09
S	B12	3	cold E2	S	S4	2.00E-09	40	-	40	80	160	1.0E-09
S	C1	1	cold E2	S	S5	6.00E-10	40	-	40	80	160	3.0E-10
S	C2	2	cold E2	S	S5	6.00E-10	40	-	40	80	160	3.0E-10
S	C3	3	cold E2	S	S5	6.00E-10	40	-	40	80	160	3.0E-10
S	C4	1	cold E2	S	S6	2.00E-10	40	-	40	80	160	1.0E-10
S	C5	2	cold E2	S	S6	2.00E-10	40	-	40	80	160	1.0E-10
S	C6	3	cold E2	S	S6	2.00E-10	40	-	40	80	160	1.0E-10
S	C7	1	cold E2	S	S7	2.00E-11	40	-	40	80	160	1.0E-11
S	C8	2	cold E2	S	S7	2.00E-11	40	-	40	80	160	1.0E-11
S	C9	3	cold E2	S	S7	2.00E-11	40	-	40	80	160	1.0E-11
S	C10	1	blank	blank	B1	-	-	160	-	-	160	-
S	C11	2	blank	blank	B2	-	-	160	-	-	160	-
S	C12	3	blank	blank	B3	-	-	160	-	-	160	-
S	D1	1	norethynodrel	NE	WP1	6.00E-05	40	-	40	80	160	3.0E-05
S	D2	1	norethynodrel	NE	WP1	6.00E-05	40	-	40	80	160	3.0E-05
S	D3	1	norethynodrel	NE	WP1	6.00E-05	40	-	40	80	160	3.0E-05
S	D4	1	norethynodrel	NE	WP2	2.00E-05	40	-	40	80	160	1.0E-05
S	D5	1	norethynodrel	NE	WP2	2.00E-05	40	-	40	80	160	1.0E-05
S	D6	1	norethynodrel	NE	WP2	2.00E-05	40	-	40	80	160	1.0E-05
S	D7	1	norethynodrel	NE	WP3	6.00E-06	40	-	40	80	160	3.0E-06
S	D8	1	norethynodrel	NE	WP3	6.00E-06	40	-	40	80	160	3.0E-06
S	D9	1	norethynodrel	NE	WP3	6.00E-06	40	-	40	80	160	3.0E-06
S	D10	1	norethynodrel	NE	WP4	2.00E-06	40	-	40	80	160	1.0E-06
S	D11	1	norethynodrel	NE	WP4	2.00E-06	40	-	40	80	160	1.0E-06
S	D12	1	norethynodrel	NE	WP4	2.00E-06	40	-	40	80	160	1.0E-06

Competitive Binding Assay Well Layout

Plate	Position	Replicate	Well type	Well Code	Concentration Code	Competitor Initial Concentration (M)	hrER stock (uL)	Buffer Volume (uL)	Tracer (Hot E2) Volume (uL)	Volume from dilution plate(uL)	Final Volume (ul)	Competitor Final Concentration (M)
S	E1	1	norethynodrel	NE	WP5	6.00E-07	40	-	40	80	160	3.0E-07
S	E2	2	norethynodrel	NE	WP5	6.00E-07	40	-	40	80	160	3.0E-07
S	E3	3	norethynodrel	NE	WP5	6.00E-07	40	-	40	80	160	3.0E-07
S	E4	1	norethynodrel	NE	WP6	2.00E-07	40	-	40	80	160	1.0E-07
S	E5	2	norethynodrel	NE	WP6	2.00E-07	40	-	40	80	160	1.0E-07
S	E6	3	norethynodrel	NE	WP6	2.00E-07	40	-	40	80	160	1.0E-07
S	D7	1	norethynodrel	NE	WP7	6.00E-08	40	-	40	80	160	3.0E-08
S	E8	2	norethynodrel	NE	WP7	6.00E-08	40	-	40	80	160	3.0E-08
S	E9	3	norethynodrel	NE	WP7	6.00E-08	40	-	40	80	160	3.0E-08
S	E10	1	norethynodrel	NE	WP8	6.00E-09	40	-	40	80	160	3.0E-09
S	E11	2	norethynodrel	NE	WP8	6.00E-09	40	-	40	80	160	3.0E-09
S	E12	3	norethynodrel	NE	WP8	6.00E-09	40	-	40	80	160	3.0E-09
S	F1	1	OTES	N	OTES1	2.00E-03	40	-	40	80	160	1.0E-03
S	F2	2	OTES	N	OTES1	2.00E-03	40	-	40	80	160	1.0E-03
S	F3	3	OTES	N	OTES1	2.00E-03	40	-	40	80	160	1.0E-03
S	F4	1	OTES	N	OTES2	2.00E-04	40	-	40	80	160	1.0E-04
S	F5	2	OTES	N	OTES2	2.00E-04	40	-	40	80	160	1.0E-04
S	F6	3	OTES	N	OTES2	2.00E-04	40	-	40	80	160	1.0E-04
S	F7	1	OTES	N	OTES3	2.00E-05	40	-	40	80	160	1.0E-05
S	F8	2	OTES	N	OTES3	2.00E-05	40	-	40	80	160	1.0E-05
S	F9	3	OTES	N	OTES3	2.00E-05	40	-	40	80	160	1.0E-05
S	F10	1	OTES	N	OTES4	2.00E-06	40	-	40	80	160	1.0E-06
S	F11	2	OTES	N	OTES4	2.00E-06	40	-	40	80	160	1.0E-06
S	F12	3	OTES	N	OTES4	2.00E-06	40	-	40	80	160	1.0E-06
S	G1	1	OTES	N	OTES5	2.00E-07	40	-	40	80	160	1.0E-07
S	G2	2	OTES	N	OTES5	2.00E-07	40	-	40	80	160	1.0E-07
S	G3	3	OTES	N	OTES5	2.00E-07	40	-	40	80	160	1.0E-07

Note that the "hot" wells are empty during incubation. The 40 µl are added only for scintillation counting.

Competitive Binding Assay Well Layout

Plate	Position	Replicat	Well type	Well Code	Concentration	Competitor Initial Concentration	hrER stock (uL)	Buffer Volume (uL)	Tracer (Hot E2) Volume	Volume from dilution plate(uL)	Final Volume (ul)	Competitor Final Concentration
P1	A1	1	total binding	TB	T	-	40	-	40	80	160	-
P1	A2	2	total binding	TB	T	-	40	-	40	80	160	-
P1	A3	3	total binding	TB	T	-	40	-	40	80	160	-
P1	A4	1	total binding	TB	T	-	40	-	40	80	160	-
P1	A5	2	total binding	TB	T	-	40	-	40	80	160	-
P1	A6	3	total binding	TB	T	-	40	-	40	80	160	-
P1	A7	1	cold E2 (high)	NSB	S	2.00E-06	40	-	40	80	160	1.0E-06
P1	A8	2	cold E2 (high)	NSB	S	2.00E-06	40	-	40	80	160	1.0E-06
P1	A9	3	cold E2 (high)	NSB	S	2.00E-06	40	-	40	80	160	1.0E-06
P1	A10	1	cold E2 (high)	NSB	S	2.00E-06	40	-	40	80	160	1.0E-06
P1	A11	2	cold E2 (high)	NSB	S	2.00E-06	40	-	40	80	160	1.0E-06
P1	A12	3	cold E2 (high)	NSB	S	2.00E-06	40	-	40	80	160	1.0E-06
P1	B1	1	Test Chemical 1	TC1	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	B2	2	Test Chemical 1	TC1	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	B3	3	Test Chemical 1	TC1	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	B4	1	Test Chemical 1	TC1	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	B5	2	Test Chemical 1	TC1	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	B6	3	Test Chemical 1	TC1	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	B7	1	Test Chemical 1	TC1	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	B8	2	Test Chemical 1	TC1	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	B9	3	Test Chemical 1	TC1	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	B10	1	Test Chemical 1	TC1	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	B11	2	Test Chemical 1	TC1	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	B12	3	Test Chemical 1	TC1	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	C1	1	Test Chemical 1	TC1	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	C2	2	Test Chemical 1	TC1	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	C3	3	Test Chemical 1	TC1	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	C4	1	Test Chemical 1	TC1	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	C5	2	Test Chemical 1	TC1	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	C6	3	Test Chemical 1	TC1	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	C7	1	Test Chemical 1	TC1	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	C8	2	Test Chemical 1	TC1	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	C9	3	Test Chemical 1	TC1	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	C10	1	Test Chemical 1	TC1	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	C11	2	Test Chemical 1	TC1	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	C12	3	Test Chemical 1	TC1	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	D1	1	Test Chemical 2	TC2	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	D2	2	Test Chemical 2	TC2	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	D3	3	Test Chemical 2	TC2	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	D4	1	Test Chemical 2	TC2	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	D5	2	Test Chemical 2	TC2	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	D6	3	Test Chemical 2	TC2	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	D7	1	Test Chemical 2	TC2	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	D8	2	Test Chemical 2	TC2	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	D9	3	Test Chemical 2	TC2	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	D10	1	Test Chemical 2	TC2	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	D11	2	Test Chemical 2	TC2	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	D12	3	Test Chemical 2	TC2	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	E1	1	Test Chemical 2	TC2	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	E2	2	Test Chemical 2	TC2	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	E3	3	Test Chemical 2	TC2	5	2.00E-07	40	0	40	80	160	1.0E-07

Competitive Binding Assay Well Layout

Plate	Position	Replicate	Well type	Well Code	Concentration Code	Competitor Initial Concentration (M)	hrER stock (uL)	Buffer Volume (uL)	Tracer (Hot E2) Volume (uL)	Volume from dilution plate(uL)	Final Volume (ul)	Competitor Final
P1	E4	1	Test Chemical 2	TC2	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	E5	2	Test Chemical 2	TC2	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	E6	3	Test Chemical 2	TC2	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	E7	1	Test Chemical 2	TC2	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	E8	2	Test Chemical 2	TC2	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	E9	3	Test Chemical 2	TC2	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	E10	1	Test Chemical 2	TC2	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	E11	2	Test Chemical 2	TC2	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	E12	3	Test Chemical 2	TC2	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	F1	1	Test Chemical 3	TC3	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	F2	2	Test Chemical 3	TC3	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	F3	3	Test Chemical 3	TC3	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	F4	1	Test Chemical 3	TC3	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	F5	2	Test Chemical 3	TC3	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	F6	3	Test Chemical 3	TC3	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	F7	1	Test Chemical 3	TC3	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	F8	2	Test Chemical 3	TC3	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	F9	3	Test Chemical 3	TC3	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	F10	1	Test Chemical 3	TC3	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	F11	2	Test Chemical 3	TC3	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	F12	3	Test Chemical 3	TC3	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	G1	1	Test Chemical 3	TC3	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	G2	2	Test Chemical 3	TC3	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	G3	3	Test Chemical 3	TC3	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	G4	1	Test Chemical 3	TC3	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	G5	2	Test Chemical 3	TC3	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	G6	3	Test Chemical 3	TC3	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	G7	1	Test Chemical 3	TC3	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	G8	2	Test Chemical 3	TC3	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	G9	3	Test Chemical 3	TC3	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	G10	1	Test Chemical 3	TC3	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	G11	2	Test Chemical 3	TC3	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	G12	3	Test Chemical 3	TC3	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	H1	1	norethynodrel	NE	IC50		40	0	40	80	160	
P1	H2	2	norethynodrel	NE	IC50		40	0	40	80	160	
P1	H3	3	norethynodrel	NE	IC50		40	0	40	80	160	
P1	H4	1	norethynodrel	NE	1.00E-4.5		40	0	40	80	160	
P1	H5	2	norethynodrel	NE	1.00E-4.5		40	0	40	80	160	
P1	H6	3	norethynodrel	NE	1.00E-4.5		40	0	40	80	160	
P1	H7	1	cold E2	S	IC50		40	0	40	80	160	
P1	H8	2	cold E2	S	IC50		40	0	40	80	160	
P1	H9	3	cold E2	S	IC50		40	0	40	80	160	
P1	H10	1	cold E2	S	1.00E-7.		40	0	40	80	160	
P1	H11	2	cold E2	S	1.00E-7		40	0	40	80	160	
P1	H12	3	cold E2	S	1.00E-7		40	0	40	80	160	

ANNEX 3: THE CHEMICAL EVALUATION AND RESEARCH INSTITUTE (CERI) *IN VITRO* ESTROGEN RECEPTOR BINDING ASSAY USING A HUMAN RECOMBINANT ERA LIGAND BINDING DOMAIN PROTEIN

INITIAL CONSIDERATIONS AND LIMITATIONS (See also GENERAL INTRODUCTION, page 1)

1. This *in vitro* Estrogen Receptor (ERα) saturation and competitive binding test method uses a ligand binding domain (LBD) of the human ERα (hrERα). This protein construct was produced by the Chemicals Evaluation Research Institute (CERI), Japan, and exists as a glutathione-S-transferase (GST) fusion protein, and is expressed in *E. coli.* The CERI protocol underwent an international multi-laboratory validation study (2) which has demonstrated its relevance and reliability for the intended purpose of the test method.

2. This test method is a screening procedure for identifying substances that can bind to the hrERα. It is used to determine the ability of a test chemical to compete with 17β-estradiol for binding to hrERα-LBD. Quantitative assay results may include the IC_{50} (a measure of the concentration of test chemical needed to displace half of the [^{3}H]-17β-estradiol from the hrERα) and the relative binding affinities of test chemicals for the hrERα compared to 17β-estradiol. For chemical screening purposes, acceptable qualitative assay results may include classifications of test chemicals as either hrERα binders, non-binders, or equivocal based upon criteria described for the binding curves.

3. The test method uses a radioactive ligand that requires a radioactive materials license for the laboratory. All procedures with radioisotopes and hazardous chemicals should follow the regulations and procedures as described by national legislation.

4. The "**GENERAL INTRODUCTION**" and "**hrER BINDING TEST METHOD COMPONENTS"** (pages 1-14) should be read before using this test method for regulatory purposes. Definitions and abbreviations used in this TG are described in Annex 1.

PRINCIPLES OF THE TEST METHOD (See also GENERAL INTRODUCTION, page 1)

5. The hrERα binding assay measures the ability of a radiolabeled ligand ([^{3}H]17β-estradiol) to bind with the ER in the presence of increasing concentrations of a test chemical (i.e. competitor). Test chemicals that possess a high affinity for the ER compete with the radiolabeled ligand at a lower concentration as compared with those chemicals with lower affinity for the receptor.

6. This test method consists of two major components: a saturation binding experiment to characterise receptor-ligand interaction parameters, followed by a competitive binding experiment that characterises the competition between a test chemical and a radiolabeled ligand for binding to the ER.

7. The purpose of the saturation binding experiment is to characterise a particular batch of receptors for binding affinity and number in preparation for the competitive binding experiment. The saturation binding experiment measures, under equilibrium conditions, the affinity of a fixed concentration of the estrogen receptor for its natural ligand (represented by the dissociation constant, Kd), and the concentration of active receptor sites (Bmax).

8. The competitive binding experiment measures the affinity of a substance to compete with [^{3}H]17β-estradiol for binding to the ER. The affinity is quantified by the concentration of test chemical that, at equilibrium, inhibits 50% of the specific binding of the [^{3}H]17β-estradiol (termed the "inhibitory concentration 50%" or IC50). This can also be evaluated using the relative binding affinity (RBA, relative to the IC50 of estradiol measured separately in the same run). The competitive binding experiment measures the binding of [^{3}H]17β-estradiol at a fixed concentration in the presence of a wide range (eight orders of magnitude) of

test chemical concentrations. The data are then fit, where possible, to a form of the Hill equation (Hill, 1910) that describes the displacement of the radioligand by a one-site competitive binder. The extent of displacement of the radiolabeled estradiol at equilibrium is used to characterise the test chemical as a binder, non-binder, or generating an equivocal response.

PROCEDURE

Demonstration of Acceptable hrERα Protein Performance

9. Prior to routinely conducting the saturation and competitive binding assays, each new batch of hrERα should be shown to be performing correctly in the laboratory in which it will be used. A two-step process should be used to demonstrate performance. These steps are the following:

- Conduct a saturation [^{3}H]-17β-estradiol binding assay to demonstrate hrERα specificity and saturation. Nonlinear regression analysis of these data (e.g. BioSoft; McPherson, 1985; Motulsky, 1995) and the subsequent Scatchard plot should document hrERα binding affinity of the [^{3}H]-17β-estradiol (Kd) and the number of receptors (Bmax) for a particular batch of hrERα.

- Conduct a competitive binding assay using the control substances (reference estrogen (17β-estradiol), a weak binder (e.g. norethynodrel or norethindrone), and a non-binder (octyltriethoxysilane, OTES). Each laboratory should establish an historical database to document the consistency of IC_{50} and the relevant values for the reference estrogen and weak binder among experiments and different batches of hrERα. In addition, the curve fit parameters of the competitive binding curves for the control substances should be within the range of the mean +/- 2SD (see Table 1) that were developed using data from laboratories that participated in the validation study for this test method (2).

Table 1. Performance criteria developed for the reference estrogen and weak binder, CERI hrER Binding Assay.

Substance	Parameter	Mean[a]	Standard Deviation(n)	Range of performance criteria based on Mean±2SD [b]
17β-estradiol	Top	104.74	13.12 (70)	78.5 ~ 130.98
	Bottom	0.85	2.41 (70)	-3.97 ~ 5.67
	HillSlope	-1.22	0.20 (70)	-1.62 ~ -0.82
	$LogIC_{50}$	-8.93	0.23 (70)	-9.39 ~ -8.47
Norethynodrel	Top	101.31	10.55 (68)	80.21 ~ 122.41
	Bottom	2.39	5.01 (68)	-7.63 ~ 12.41
	HillSlope	-1.04	0.21 (68)	-1.46 ~ -0.62
	$LogIC_{50}$	-6.19	0.40 (68)	-6.99 ~ -5.39
Norethindrone[C]	Top	92.27	7.79 (23)	76.69 ~ 107.85
	Bottom	16.52	10.59 (23)	-4.66 ~ 37.7
	Hill Slope	-1.18	0.32 (23)	-1.82 ~ -0.54
	$LogIC_{50}$	-6.01	0.54 (23)	-7.09 ~ -4.93

[a] *Mean ± Standard Deviation (SD) with (sample size (n) was calculated using curve fit estimates (4-parameter Hill equation) for control runs conducted in four laboratories during the validation study (see Reference 2, Annex N (pages 58-70, https://www.oecd.org/env/ehs/testing/35_ANN3.pdf)).*

[b] *Ranges of Performance Criteria (based on the mean +/- 2SD for each parameter) are provided as a guide for assay acceptability.*

[c] *Testing of norethindrone was optional for Subtask 4 during validation study (see Reference 2, Subtask 4, pages 79-85, https://www.oecd.org/env/ehs/testing/MONO(2015)35.pdf). Thus, the mean ± SD (n) for each parameter was calculated using curve fit estimates (4-parameter Hill equation) for control runs conducted in two laboratories.*

[d] *The range for the IC50 will be dependent upon the Kd of the receptor preparation and concentration of radiolabeled ligand used within each laboratory. Appropriate adjustment for the range of the IC50 based upon the conditions used to conduct the test method will be acceptable.*

Demonstration of laboratory proficiency

10. See paragraphs 17 and 18 and Table 2 in "**hrER BINDING TEST METHOD COMPONENTS**" of this Test Guideline. Each assay (saturation and competitive binding) should consist of three independent runs (i.e. with fresh dilutions of receptor, chemicals, and reagents) on different days, and each run should contain three replicates.

Determination of Receptor (hrERα) Concentration

11. The concentration of active receptor varies slightly by batch and storage conditions. For this reason, the concentration of active receptor as received from the supplier should be determined. This will yield the appropriate concentration of active receptor at the time of the run.

12. Under conditions corresponding to competitive binding (i.e. 0.5 nM [^{3}H]-estradiol), nominal concentrations of 0.1, 0.2, 0.4 and 0.6 nM receptor should be incubated in the absence (total binding) and presence (non-specific binding) of 1 µM unlabeled estradiol. Specific binding, calculated as the difference of total and non-specific binding, is plotted against the nominal receptor concentration. The concentration of receptor that gives specific binding values corresponding to 40% of added radiolabel is related to the corresponding receptor concentration, and this receptor concentration should be used for saturation and

competitive binding experiments. Frequently, a final hrER concentration of 0.2 nM will comply with this condition.

13. If the 40% criterion repeatedly cannot be met, the experimental set up should be checked for potential errors. Failure to achieve the 40% criterion may indicate that there is very little active receptor in the recombinant batch, and the use of another receptor batch should then be considered.

Saturation assay

14. Eight increasing concentrations of [^{3}H]17β-estradiol should be evaluated in triplicate, under the following three conditions (see Table 2):

a. In the absence of unlabelled 17β-estradiol and presence of ER. This is the determination of total binding by measure of the radioactivity in the wells that have only [^{3}H]17β-estradiol.
b. In the presence of a 2000- fold excess concentration of unlabelled 17β-estradiol over labelled 17β-estradiol and presence of ER. The intent of this condition is to saturate the active binding sites with unlabelled 17β-estradiol, and by measuring the radioactivity in the wells, determine the non-specific binding. Any remaining hot estradiol that can bind to the receptor is considered to be binding at a non-specific site as the cold estradiol should be at such a high concentration that it is bound to all of the available specific sites on the receptor.
c. In the absence of unlabelled 17β-estradiol and absence of ER (determination of total radioactivity)

*Preparation of [^{3}H]-17β-estradiol, unlabelled 17β-estradiol solutions and hrER*α

A 40 nM solution of [^{3}H]-17β-estradiol should be prepared from a 1 µM stock solution of [^{3}H]-17β-estradiol in DMSO, by adding DMSO (to prepare 200 nM) and assay buffer at room temperature (to prepare 40 nM). Using this 40 nM solution, the series of [^{3}H]-17β-estradiol dilutions prepared, ranging from 0.313 nM to 40 nM with assay buffer at room temperature (as represented in lane 12 of Table 2).

15. The final assay concentrations, ranging from 0.0313 to 4.0 nM, will be obtained by adding 10 µL of these solutions to the respective assay wells of a 96-well microtiter plate (see Tables 2 and 3). Preparation of assay buffer, calculation of the original [^{3}H]-17β-estradiol stock solution based on its specific activity, preparation of dilutions and determination of the concentrations are described in depth in the CERI protocol (2).

16. Dilutions of unlabeled 17β-estradiol solutions should be prepared from a 1 nM 17β-estradiol stock solution by adding assay buffer to achieve eight increasing concentrations initially ranging from 0.625 µM to 80 µM. The final assay concentrations, ranging from 0.0625 to 8 µM, will be obtained by adding 10 µL of these solutions to the respective assay wells of a 96-well microtiter plate dedicated to the measurement of non-specific binding (see Tables 2 and 3). Preparation of unlabelled 17β-estradiol dilutions is described in depth in the CERI protocol (2).

17. The concentration of receptor that gives 40±10% specific binding should be used (see paragraphs 12-13). The hrERα solution should be prepared with ice-cold assay buffer immediately prior to use, i.e. after all wells for total binding, non-specific binding and hot ligand alone have been prepared.

18. The 96-well microtiter plates are prepared as illustrated in Table 2, with 3 replicates per [^{3}H]-17β-estradiol concentration. Volume assignment of [^{3}H]-17β-estradiol, unlabeled 17β-estradiol, buffer and receptor are provided in Table 3.

Table 2: Saturation Binding Assay Microtiter Plate Layout

	*1**	*2**	*3**	*4**	*5**	*6**	*7**	*8**	*9**	*10*	*11***	*12***
	For measurement of TB			For measurement of NSB			For determination of hot ligand alone				unlabeled E_2 dilutions for plate column 4-6	$[^3H]E_2$ dilutions for plate column 1-9
A	0.0313 nM [^3H] E_2 + ER			0.0313 nM [^3H] E_2 + 0.0625 µM E_2 + ER			0.0313 nM				0.625 µM	0.313 nM
B	0.0625 nM [^3H] E_2 + ER			0.0625 nM [^3H] E_2 + 0.125 µM E_2 + ER			0.0625 nM				1.25 µM	0.625 nM
C	30.125 nM [^3H] E_2 + ER			0.125 nM [^3H] E_2 + 0.25 µM E_2 + ER			0.125 nM				2.5 µM	1.25 nM
D	0.250 nM [^3H] E_2 + ER			0.250 nM [^3H] E_2 + 0.5 µM E_2 + ER			0.250 nM				5 µM	2.5 nM
E	0.50 nM [H] E_2 + ER			0.50 nM [^3H] E_2 + 1 µM E_2 + ER			0.50 nM				10 µM	5 nM
F	1.00 nM [^3H] E_2 + ER			1.00 nM [^3H] E_2 + 2 µM E_2 + ER			1.00 nM				20 µM	10 nM
G	2.00 nM [^3H] E_2 + ER			2.00 nM [^3H] E_2 + 4 µM E_2 + ER			2.00 nM				40 µM	20 nM
H	4.00 nM [^3H] E_2 + ER			4.00 nM [^3H] E_2 + 8 µM E_2 + ER			4.00 nM				80 µM	40 nM

TB: total binding,
NSB: non-specific binding
[^3H] E_2: [^3H]17β-estradiol
E_2: unlabelled 17β-estradiol
**The indicated concentrations here are the final concentrations in each well.*
***The dilutions of unlabeled E_2 and [^3H]E_2 can be prepared in a different plate.*

Table 3. Reagent Volumes for Saturation Microtiter Plate

Lane Number		***1***	***2***	***3***	***4***	***5***	***6***	***7****	***8****	***9****
Preparation Steps		***TB Wells***			***NSB Wells***			***Hot Ligand Alone***		
Volume of components for reaction wells above and order to add	Buffer	60 µL			50 µL			90 µL		
	unlabeled E_2 from lane 11 in Table2	-			10 µL			-		
	[^{3}H]E_2 from lane12 in Table2	10 µL			10 µL			10 µL		
	hrERα	30 µL			30 µL			-		
Total reaction volume		100 µL			100 µL			100 µL		
Incubation		**FOLLOWING 2 HOUR INCUBATION REACTION**						Quantification of the radioactivity just after the preparation. No incubation		
Treatment with 0.4% DCC		**Yes**			**Yes**			**No**		
Volume of 0.4% DCC		100 µL			100 µL			-		
Filtration		Yes			Yes			No		
MEASURING THE DPMS										
Quantification volume added to scintillation cocktail		100 µL**			100 µL**			50 µL		

** If an LSC for microplates is used for measuring dpms, the preparation of hot ligand alone in the same assay plate of TB and NSB wells is not appropriate. The hot ligand alone should be prepared in a different plate.*

*** If centrifugation is used to separate DCC, the 50 µL of supernatant should be measured by LSC in order to avoid contamination of DCC.*

19. Assay microtiter plates for the determination of total binding and non-specific binding should be incubated at room temperature (22°C to 28°C) for two hours.

Measurement of [^{3}H]-17β-Estradiol bound to hrERα

20. Following the two hour incubation period, [^{3}H]-17β-Estradiol bound to hrERα should be separated from free [^{3}H]-17β-Estradiol by adding 100µL an ice cold 0.4% DCC suspension to the wells. The plates should then be placed on ice for 10 minutes and the reaction mixture and DCC suspension should be filtered, by transfer to a mictotiter plate filter, to remove DCC. A 100 µL of the filtrate should then be added to scintillation fluid in LSC vials for determination of disintegration per minute (dpms) per vial by liquid scintillation counting.

21. Alternatively, if a microplate filter is not available, removal of DCC can be obtained by centrifugation. A 50 µL of supernatant containing the hrERα-bound [^{3}H]-17β-estradiol should then be taken with extreme care, to avoid any contamination of the wells by touching DCC, and should be used for scintillation counting.

22. The hot ligand alone condition is used for determining the disintegration per minute (dpm) of [^{3}H]-17β-estradiol added to the assay wells. The radioactivity should be quantified just after preparation. These wells should not be incubated and should not be treated with DCC suspension but their content should be delivered directly into the scintillation fluid. These measures demonstrate how much [^{3}H]-17β-estradiol in dpms was added to each set of wells for the total binding and non-specific binding.

Competitive binding assay

23. 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 chemical. Three concurrent replicates should be used at each concentration within one run. In addition, three non-concurrent runs should be performed for each chemical tested. The assay should be set up in one or more 96-well microtiter plates.

Controls

24. When performing the assay, concurrent solvent and controls (i.e. reference estrogen, weak binder, and non-binder) should be included in each experiment. Full concentration curves for the reference estrogen and controls (i.e. weak binder and non-binder) should be used in one plate during each run. All other plates should contain (i) a high- (maximum displacement i.e. approximately full displacement of radiolabeled ligand) and medium- (approximately, the IC50) concentration of E2 and weak binder in triplicate; (ii) solvent control and non-specific binding, each in triplicate. Procedures for the preparation of assay buffer, [^{3}H]-17β-estradiol, hrERα and test chemical solutions are described in depth in the CERI protocol (2).

- Solvent control:

25. The solvent control indicates that the solvent does not interact with the test system and also measures total binding (TB). DMSO is the preferred solvent. Alternatively, if the highest concentration of the test chemical is not soluble in DMSO, ethanol may be used. The concentration of DMSO in the final assay wells should be 2.05% and could be increased up to 2.5% in case of lack of solubility of the test chemical. Concentrations of DMSO above 2.5% should not be used because of interference of higher solvent concentrations with the assay. For test chemicals that are not soluble in DMSO, but are soluble in ethanol, a maximum of 2% ethanol may be used in the assay without interference.

- Buffer control:

26. The buffer control (BC) should contain neither solvent nor test chemical, but all of the other components of the assay. The results of the buffer control are compared to the solvent control to verify that the solvent used does not affect the assay system.

- Strong binder (reference estrogen)

27. 17β-estradiol (CAS 50-28-2) is the endogenous ligand and binds with high affinity to the ER, alpha subtype. A standard curve using unlabeled 17β-estradiol should be prepared for each hrERα competitive binding assay, to allow for an assessment of variability when conducting the assay over time within the same laboratory. Eight solutions of unlabeled 17β-estradiol should be prepared in DMSO and assay buffer, with final concentrations in the assay wells to be used for the standard curve spaced as follows: 10^{-6}, 10^{-7}, 10^{-8}, $10^{-8.5}$, 10^{-9}, $10^{-9.5}$, 10^{-10}, 10^{-11} M. The highest concentration of unlabeled 17β-estradiol (1 µM) should serve as the non-specific binding indicator. This concentration is distinguished by the label “NSB” in Table 4 even though it is also part of the standard curve.

- Weak binder

28. A weak binder (norethynodrel (CAS68-23-5), or alternate, norethindrone (CAS 68-22-4)) should be included to demonstrate the sensitivity of each experiment and to allow an assessment of variability when conducting the assay over time. Eight solutions of the weak binder should be prepared in DMSO and assay buffer, with final concentrations in the assay wells as follows: $10^{-4.5}$, $10^{-5.5}$, 10^{-6}, $10^{-6.5}$, 10^{-7}, $10^{-7.5}$, 10^{-8} and 10^{-9} M.

- Non binder

29. Octytriethoxysilane (OTES, CAS 2943-75-1) should be used as the negative control (non-binder). It provides assurance that the assay as run, will detect test chemicals that do not bind to the hrERα. Eight solutions of the non-binder should be prepared in DMSO and assay buffer, with final concentrations in the

assay wells as follows: 10^{-3},10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M. Di-n-butyl phthalate (DBP, CAS 84-72-2) can be used as an alternative non-binder, but only tested up to 10^{-4}M. The maximum solubility of DBP in the assay has been demonstrated to be 10^{-4}M.

hrERα concentration

30. The amount of receptor that gives specific binding of 40±10% should be used (see paragraphs 12-13 of Annex 3). The hrERα solution should be prepared by dilution of the functional hrERα into ice cold assay buffer, immediately prior to use.

[^{3}H]-17β-estradiol

31. The final concentration of [^{3}H]-17β-estradiol in the assay wells should be of 0.5 nM.

Test Chemicals

32. In the first instance, it is necessary to conduct a solubility test to determine the limit of solubility for each test chemical and to identify the appropriate concentration range to use when conducting the test protocol. The limit of solubility of each test chemical is to be initially determined in the solvent and then further confirmed under assay conditions. The final concentration tested in the assay should not exceed 1mM. Range finder testing includes a solvent control along with at least 8 log serial dilutions, starting at maximum acceptable concentration (e.g. 1 mM or lower, based upon the limit of solubility), and the presence of cloudiness or precipitate noted (see also paragraph 35 of Annex 3). Once the concentration range for testing has been determine, a test chemical should be tested using 8 log concentrations spaced appropriately as defined in the preceding range finding test. Concentrations tested in the second and third experiments should be further adjusted as appropriate to better characterise the concentration response curve, if necessary.

33. Dilutions of the test chemical should be prepared in the appropriate solvent (see paragraph 25 of Annex 3). If the highest concentration of the test chemical is not soluble in either DMSO or ethanol, and adding more solvent would cause the solvent concentration in the final tube to be greater than the acceptable limit, the highest concentration may be reduced to the next lower concentration. In this case, an additional concentration may be added at the low end of the concentration series. Other concentrations in the series should remain unchanged.

34. The test chemical solutions should be closely monitored when added to the assay well, as the test chemical may precipitate upon addition to the assay well. The data for all wells that contain precipitate should be excluded from curve-fitting, and the reason for exclusion of the data noted.

35. If there is prior existing information from other sources that provide a log(IC_{50}) of a test chemical, it may be appropriate to geometrically space the dilutions more closely around the expected log(IC_{50}) (i.e. 0.5 log units). The final results should show enough sufficient spread of concentrations on either side of the log(IC_{50}), including the “top” and “bottom”, such that the binding curve can be adequately characterised.

Assay plate organisation

36. Labeled microtiter plates should be prepared using sextuple incubations for the solvent control, the highest concentration the reference estrogen (E2) which also serves as the non-specific binding (NSB) indicator, the buffer control, the eight concentrations of the non-binding control (octyltriethoxysilane), the seven lower concentrations for the reference estrogen (E2), the eight concentrations of the weak binder (norethynodrel or norethindrone), and the eight concentrations of each test chemical (TC). An example layout of the plate layout diagram for the full concentration curves for the reference estrogen and controls is give below in Table 4. Additional microtiter plates are used for the test chemical and should contain plate controls (i.e. (i) a high- (maximum displacement) and medium- (approximately, the IC_{50}) concentration of E2 and weak binder in triplicate; (ii) solvent control (as total binding) and non-specific binding, each in sextuple (Table 5). An example of a competitive assay microtiter plate layout worksheet using three unknown test chemicals is provided in Appendix 3 of Annex 3. The concentrations indicated in the worksheet as well as in Tables 4 and

5 refer to the final concentrations used in each assay well. The maximum concentration for E2 should be 1×10^{-7} M and for the weak binder, the highest concentration used for the weak binder on plate 1 should be used. The IC50 concentration has to be determined by the laboratory based on their historical control database. The expectation is that this value would be similar to that observed in the validation studies (see table 1).

Table 4: Competitive Binding Assay Microtiter Plate Layout[1,2], Full Concentration Curves for Reference Estrogen and Controls (Plate 1)

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
	Buffer Control and Positive Control (E2)			Weak Positive (Norethynodrel)			Negative Control (OTES)			TB and NSB		
A	Blank*			1×10^{-9} M			1×10^{-10} M			TB (solvent control) (2.05% DMSO)		
B	E2 (1×10^{-11} M)			1×10^{-8} M			1×10^{-9} M					
C	E2 (1×10^{-10} M)			$1\times10^{-7.5}$ M			1×10^{-8} M			NSB (10^{-6} M E_2)		
D	E2 ($1\times10^{-9.5}$ M)			1×10^{-7} M			1×10^{-7} M					
E	E2 (1×10^{-9} M)			$1\times10^{-6.5}$ M			1×10^{-6} M			Buffer control		
F	E2 ($1\times10^{-8.5}$ M)			1×10^{-6} M			1×10^{-5} M					
G	E2 (1×10^{-8} M)			$1\times10^{-5.5}$ M			1×10^{-4} M			Blank (for hot)**		
H	E2 (1×10^{-7} M)			$1\times10^{-4.5}$ M			1×10^{-3} M					

[1] Sample set up for the standards microtiter plate to be run with each experiment.
[2] Note that this microtiter plate is made using the dilutions made in the dilution plate described for the standards in the previous sections.
In this example, the weak binder is norethinodrel (NE)
** real blank, well not used*
*** blank, not used during the incubation, but used to confirm the total radioactivity added.*

Table 5: Competitive Binding Assay Microtiter Plate Layout, Additional Plates for Test Chemicals (TC) and Plate Controls.

	1	2	3	4	5	6	7	8	9	10	11	12
	Test Chemical-1 (TC-1)			**Test Chemical-2 (TC-2)**			**Test Chemical-3 (TC-3)**			**Controls**		
A	TC-1 (1×10^{-10} M)			TC-2 (1×10^{-10} M)			TC-3 (1×10^{-10} M)			E_2 (1×10^{-7}M)		
B	TC-1 (1×10^{-9} M)			TC-2 (1×10^{-9} M)			TC-3 (1×10^{-9} M)			E_2 (IC_{50})		
C	TC-1 (1×10^{-8} M)			TC-2 (1×10^{-8} M)			TC-3 (1×10^{-8} M)			NE ($1\times10^{-4.5}$M)		
D	TC-1 (1×10^{-7} M)			TC-2 (1×10^{-7} M)			TC-3 (1×10^{-7} M)			NE (IC_{50})		
E	TC-1 (1×10^{-6} M)			TC-2 (1×10^{-6} M)			TC-3 (1×10^{-6} M)			NSB (10^{-6} M E_2)		
F	TC-1 (1×10^{-5} M)			TC-2 (1×10^{-5} M)			TC-3 (1×10^{-5} M)					
G	TC-1 (1×10^{-4} M)			TC-2 (1×10^{-4} M)			TC-3 (1×10^{-4} M)			TB (Solvent control)		
H	TC-1 (1×10^{-3} M)			TC-2 (1×10^{-3} M)			TC-3 (1×10^{-3} M)					

In this example, the weak binder is norethinodrel (NE)

Completion of competitive binding assay

37. Excepting wells for total binding and blanks (for hot), as shown in Table 6, 50 µL of the assay buffer should be placed in each well, and should be mixed with 10 µL of the solvent control, reference estrogen (E2), weak binder, non-binder, and test chemicals, respectively, 10 µL of a 5 nM [3H]-17β-estradiol solution. Then, 30µL of ice cold receptor solution was added to each plate and mixed gently. The hrERα solution should be the last reagent to be added. Assay microtiter plates should be incubated at room temperature (22° to 28°C) for 2 hours.

Table 6: Volume of Assay Components for hrER Competitive Binding Assay, Microtiter Plates

Lane Number Preparation Steps		Other than TB wells	TB wells	Blank (for hot)
Volume of components for reaction wells above and order to add	Room Temperature assay Buffer	50 µL	60 µL	90 µL
	Unlabeled E2, weak binder, non-binder, solvent and test chemicals*	10 µL	-	-
	[^{3}H]-17β-estradiol to yield final concentration of 0.5 nM (i.e. 5 nM)	10 µL	10 µL	10 µL
	rERα concentration as determined (see paragraphs 12-13)	30 µL	30 µL	-
Total volume in each assay well		100 µL	100 µL	100 µL

*properly prepared to obtain final concentration within the acceptable solvent concentration

38. The quantification of [^{3}H]-17β-Estradiol bound to hrERα, following separation of [^{3}H]-17β-Estradiol bound to hrERα from free [^{3}H]-17β-Estradiol by adding 100 µL of ice-cold DCC suspension to each well, should then be performed as described in paragraphs 21-23 of Annex 3 for the saturation binding assay.

39. Wells G10-12 and H10-12 (identified as blank (for hot) in Table 4) represent the dpms of the [^{3}H]-labeled-estradiol in 10 µL. The 10 µL aliquot should be delivered directly into the scintillation fluid.

Acceptability criteria

Saturation binding assay

40. The specific binding curve should reach a plateau as increasing concentrations of [^{3}H]-17β-estradiol were used, indicating saturation of hrERα with ligand.

41. The specific binding at 0.5 nM of [^{3}H]-17β-estradiol should be inside the acceptable range 30% to 50% of the average measured total radioactivity added across runs. Occasional slight excursions outside of this range are acceptable, but if runs are consistently outside this range or a particular run is significantly outside this range, the protein concentration should be adjusted and the saturation assay repeated.

42. The data should produce a linear Scatchard plot.

43. The non-specific binding should not be excessive. The value for non-specific binding should typically be <35% of the total binding. However, the ratio might occasionally exceed this limit when measuring very low dpm for the lowest concentration of radiolabeled 17β-estradiol tested.

Competitive binding assay

44. Increasing concentrations of unlabeled 17β-estradiol should displace [^{3}H]-17β-estradiol from the receptor in a manner consistent with a one-site competitive binding.

45. The IC_{50} value for the reference estrogen (i.e. 17β-estradiol) should be approximately equal to the molar concentration of [^{3}H]-17β-estradiol plus the Kd determined from the saturation binding assay.

46. The total specific binding should be consistently within the acceptable range of 40 ± 10 % when the average measured concentration of total radioactivity added to each well was 0.5 nM across runs. Occasional slight excursions outside of this range are acceptable, but if runs are consistently outside this range or a particular run is significantly outside this range, the protein concentration should be adjusted.

47. The solvent should not alter the sensitivity or reproducibility of the assay. The results of the solvent control (TB wells) are compared to the buffer control to verify that the solvent used does not affect the assay system. The results of the TB and Buffer control should be comparable if there is no effect of the solvent on the assay.

48. The non-binder should not displace more than 25% of the [^{3}H]-17β-estradiol from the hrERα when tested up to 10^{-3} M (OTES) or 10^{-4} M (DBP).

49. Performance criteria were developed for the reference estrogen and two weak binders (e.g. norethynodrel, norethindrone) using data from the validation study for the CERI hrER Binding Assay (see Reference 2, Annex N (pages 58-70, https://www.oecd.org/env/ehs/testing/35_ANN3.pdf of reference 2)). Performance criteria were calculated using the mean +/- 2SD for the curve fit parameters (i.e. top, bottom, Hillslope and Log IC_{50}) for the reference estrogen and weak binders. Table 1 provides expected ranges for the curve fit parameters that can be used as a guide for assay acceptability. In practice, the range of the IC_{50} may vary slightly based upon the experimentally derived Kd of the receptor preparation and ligand concentration used for the test method.

50. No performance criteria were developed for curve fit parameters for the test chemicals because of the wide array of existing potential test chemicals and variation in potential affinities and outcomes (e.g. Full curve, partial curve, no curve fit). However, professional judgment should be applied when reviewing results from each run for a test chemical. A sufficient range of concentrations of the test chemical should be used to clearly define the top (e.g. 90 - 100% of binding) of the competitive curve. Variability among replicates at each concentration of test chemical as well as among the 3 non-concurrent runs should be reasonable and scientifically defensible. Controls from each run for a test chemical should approach the measures of performance reported for this CERI test method and be consistent historical control data from each respective laboratory.

ANALYSIS OF DATA

Saturation binding assay

51. Both total and non-specific binding are measured. From these values, specific binding of increasing concentrations of [^{3}H]-17β-estradiol under equilibrium conditions is calculated by subtracting non-specific from total. A graph of specific binding versus [^{3}H]-17β-estradiol concentration should reach a plateau for maximum specific binding indicative of saturation of the hrERα with the [^{3}H]-17β-estradiol. In addition, analysis of the data should document the binding of the [^{3}H]-17β- estradiol to a single, high-affinity binding site. Non-specific, total, and specific binding should be displayed on a saturation binding curve. Further 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.

52. The data analysis should determine B_{max} and K_d from the total binding data alone, using the assumption that non-specific binding is linear, unless justification is given for using a different method. In addition, robust regression should be used when determining the best fit unless justification is given. The method chosen for robust regression should be stated. Correction for ligand depletion (e.g. using the method of Swillens 1995) should always be used when determining B_{max} and K_d from saturation binding data.

Competitive binding assay

53. The competitive binding curve is plotted as specific [^{3}H]-17β- estradiol binding versus the concentration (log10 units) of the competitor. The concentration of the test chemical that inhibits 50% of the maximum specific [^{3}H]-17β-estradiol binding is the IC50 value.

54. Estimates of log(IC_{50}) values for the positive controls (e.g. reference estrogen and weak binder) should be determined using an appropriate nonlinear curve fitting software to fit a four parameter Hill equation (e.g. BioSoft; McPherson, 1985; Motulsky, 1995). The top, bottom, slope, and log(IC_{50}) should generally be left unconstrained when fitting these curves. Robust regression should be used when determining the best fit unless justification is given. Correction for ligand depletion should not be used. Following the initial analysis, each binding curve should be reviewed to ensure appropriate fit to the model. The relative binding affinity (RBA) for the weak binder should be calculated as a percent of the log (IC_{50}) for the weak binder relative to the log (IC_{50}) for 17β-estradiol. Results from the positive controls and the non-binder control should be evaluated using the measures of the test method performance in paragraphs 44-49 of this Annex 3.

55. Data for all test chemicals should be analyzed using a step-wise approach to ensure that data are appropriately analyzed and that each competitive binding curve is properly classified. It is recommended that each run for a test chemical initially undergo a standardized data analysis that is identical to that used for the reference estrogen and weak binder controls (see paragraph 54 of this Annex 3). Once completed, a technical review of the curve fit parameters as well as a visual review of how well the data fit the generated competitive binding curve for each run should be conducted. During this technical review, the observations of a concentration dependent decrease in the percent [^{3}H]-17β-estradiol specifically bound, low variability among the technical replicates at each test chemical concentration, and consistency in fit parameters among the three runs are a good indication that the assay and data analyses were conducted appropriately.

Data interpretation

56. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be a binder for the hrERα if a binding curve can be fit and the lowest point on the response curve within the range of the data is less than 50% (Figure 1).

57. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be a non-binder for the hrERα if:

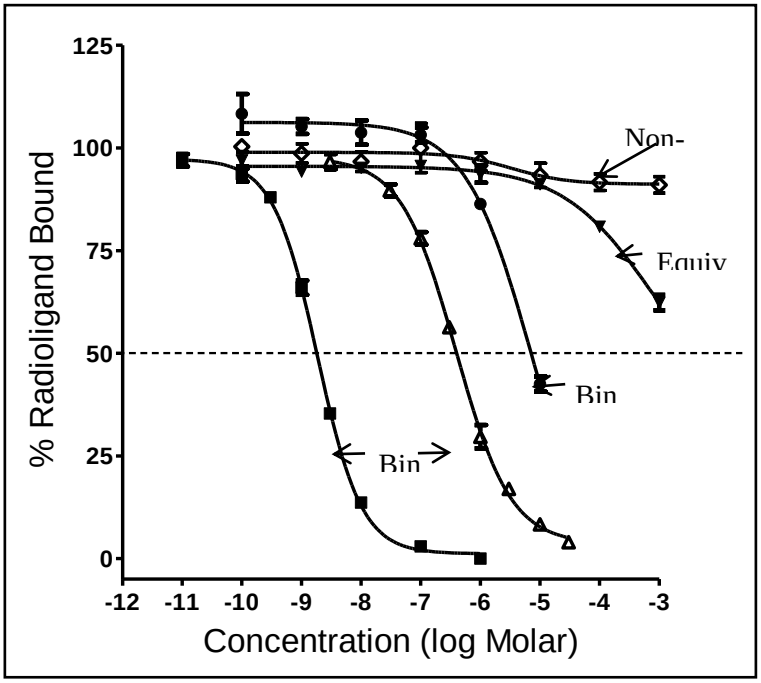
- A binding curve can be fit and the lowest point on the fitted response curve within the range of the data is above 75%, or
- A binding curve cannot be fit and the lowest unsmoothed average percent binding among the concentration groups in the data is above 75%.

58. Test chemicals are considered equivocal if none of the above conditions are met (e.g. the lowest point on the fitted response curve is between 76 – 51%).

Table 7. Criteria for assigning classification based upon competitive binding curve for a test chemical.

Classification	Criteria
Binder[a]	A binding curve can be fit. • The **lowest point on the response curve within the range of the data is less than 50%.**
Non-binder[b]	If a binding curve can be fit, • the **lowest point on the fitted response curve within the range of the data is above 75%.** If a binding curve cannot be fit, • the **lowest unsmoothed average percent binding among the concentration groups in the data is above 75%.**
Equivocal[c]	Any testable run that is neither a binder nor a non-binder (*e.g.* The lowest point on the fitted response curve is between 76 – 51%).

Figure 1. Examples of test chemical classification using competitive binding curve.



59. Multiple runs conducted within a laboratory for a test chemical are combined by assigning numeric values to each run and averaging across the runs as shown in Table 8. Results for the combined runs within each laboratory are compared with the expected classification for each test chemical.

Table 8. Method for classification of test chemical using multiple runs within a laboratory

To assign value to each run:	
Classification	**Numeric Value**
Binder	2
Equivocal	1
Non-binder	0
To classify average of numeric value across runs:	
Classification	**Numeric Value**
Binder	Average ≥ 1.5
Equivocal	0.5 ≤ Average < 1.5
Non-binder	Average < 0.5

TEST REPORT

60. See paragraph 24 of "**hrER BINDING TEST METHOD COMPONENTS**" of this Test Guideline.

APPENDIX 1: LIST OF TERMS

[^{3}H]E_2: 17β-Estradiol radiolabeled with tritium
DCC: Dextran-coated charcoal
E_2: Unlabeled 17β-estradiol (inert)
Assay buffer: 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1mM EGTA, 1 mM $NaVO_3$, 10 % Glycerol, 0.2 mM Leupeptin, 1 mM Dithiothreitol and 10 mg/mL Bovine Serum Albumin

hrERα: Human recombinant estrogen receptor alpha (ligand binding domain)
Replicate: One of multiple wells that contain the same contents at the same concentrations and are assayed concurrently within a single run. In this protocol, each concentration of test chemical is tested in triplicate; that is, there are three replicates that are assayed simultaneously at each concentration of test chemical.
Run: A complete set of concurrently-run microtiter plate assay wells that provides all the information necessary to characterize binding of a test chemical to the hrERα (viz., total [^{3}H]-17β-estradiol added to the assay well, maximum binding of [^{3}H]-17β-estradiol to the hrERα, nonspecific binding, and total binding at various concentrations of test chemical). A run could consist of as few as one assay well (i.e. replicate) per concentration, but since this protocol requires assaying in triplicate, one run consists of three assay wells per concentration. In addition, this protocol requires three independent (i.e. non-concurrent) runs per chemical.

APPENDIX 2: COMPETITIVE BINDING ASSAY WELL LAYOUT

Plate	Position	Replicat	Well type	Well Code	Concentration	Competitor Initial Concentration	hrER stock (uL)	Buffer Volume (uL)	Tracer (Hot E2) Volume	Volume from dilution plate(uL)	Final Volume (ul)	Competitor Final Concentration
S	A1	1	Blank	BK	BK1	—	—	—	—	—	—	—
S	A2	2	Blank	BK	BK2	—	—	—	—	—	—	—
S	A3	3	Blank	BK	BK3	—	—	—	—	—	—	—
S	B1	1	cold E2	S	S1	1.00E-10	30	50	10	10	100	1.0E-11
S	B2	2	cold E2	S	S1	1.00E-10	30	50	10	10	100	1.0E-11
S	B3	3	cold E2	S	S1	1.00E-10	30	50	10	10	100	1.0E-11
S	C1	1	cold E2	S	S2	1.00E-09	30	50	10	10	100	1.0E-10
S	C2	2	cold E2	S	S2	1.00E-09	30	50	10	10	100	1.0E-10
S	C3	3	cold E2	S	S2	1.00E-09	30	50	10	10	100	1.0E-10
S	D1	1	cold E2	S	S3	3.16E-09	30	50	10	10	100	3.2E-10
S	D2	2	cold E2	S	S3	3.16E-09	30	50	10	10	100	3.2E-10
S	D3	3	cold E2	S	S3	3.16E-09	30	50	10	10	100	3.2E-10
S	E1	1	cold E2	S	S4	1.00E-08	30	50	10	10	100	1.0E-09
S	E2	2	cold E2	S	S4	1.00E-08	30	50	10	10	100	1.0E-09
S	E3	3	cold E2	S	S4	1.00E-08	30	50	10	10	100	1.0E-09
S	F1	1	cold E2	S	S5	3.16E-08	30	50	10	10	100	3.2E-09
S	F2	2	cold E2	S	S5	3.16E-08	30	50	10	10	100	3.2E-09
S	F3	3	cold E2	S	S5	3.16E-08	30	50	10	10	100	3.2E-09
S	G1	1	cold E2	S	S6	1.00E-07	30	50	10	10	100	1.0E-08
S	G2	2	cold E2	S	S6	1.00E-07	30	50	10	10	100	1.0E-08
S	G3	3	cold E2	S	S6	1.00E-07	30	50	10	10	100	1.0E-08
S	H1	1	cold E2	S	S7	1.00E-06	30	50	10	10	100	1.0E-07
S	H2	2	cold E2	S	S7	1.00E-06	30	50	10	10	100	1.0E-07
S	H3	3	cold E2	S	S7	1.00E-06	30	50	10	10	100	1.0E-07
S	A4	1	norethynodrel	NE	WP1	1.00E-08	30	50	10	10	100	1.0E-09
S	A5	2	norethynodrel	NE	WP1	1.00E-08	30	50	10	10	100	1.0E-09
S	A6	3	norethynodrel	NE	WP1	1.00E-08	30	50	10	10	100	1.0E-09
S	B4	1	norethynodrel	NE	WP2	1.00E-07	30	50	10	10	100	1.0E-08
S	B5	2	norethynodrel	NE	WP2	1.00E-07	30	50	10	10	100	1.0E-08
S	B6	3	norethynodrel	NE	WP2	1.00E-07	30	50	10	10	100	1.0E-08
S	C4	1	norethynodrel	NE	WP3	3.16E-07	30	50	10	10	100	3.2E-08
S	C5	2	norethynodrel	NE	WP3	3.16E-07	30	50	10	10	100	3.2E-08
S	C6	3	norethynodrel	NE	WP3	3.16E-07	30	50	10	10	100	3.2E-08
S	D4	1	norethynodrel	NE	WP4	1.00E-06	30	50	10	10	100	1.0E-07
S	D5	2	norethynodrel	NE	WP4	1.00E-06	30	50	10	10	100	1.0E-07
S	D6	3	norethynodrel	NE	WP4	1.00E-06	30	50	10	10	100	1.0E-07
S	E4	1	norethynodrel	NE	WP5	3.16E-06	30	50	10	10	100	3.2E-07
S	E5	2	norethynodrel	NE	WP5	3.16E-06	30	50	10	10	100	3.2E-07
S	E6	3	norethynodrel	NE	WP5	3.16E-06	30	50	10	10	100	3.2E-07
S	F4	1	norethynodrel	NE	WP6	1.00E-05	30	50	10	10	100	1.0E-06
S	F5	2	norethynodrel	NE	WP6	1.00E-05	30	50	10	10	100	1.0E-06
S	F6	3	norethynodrel	NE	WP6	1.00E-05	30	50	10	10	100	1.0E-06
S	G4	1	norethynodrel	NE	WP7	3.16E-05	30	50	10	10	100	3.2E-06
S	G5	2	norethynodrel	NE	WP7	3.16E-05	30	50	10	10	100	3.2E-06
S	G6	3	norethynodrel	NE	WP7	3.16E-05	30	50	10	10	100	3.2E-06

Competitive Binding Assay Well Layout

Plate	Position	Replicat	Well type	Well Code	Concentration Code	Competitor Initial Concentration	hrER stock (uL)	Buffer Volume (uL)	Tracer (Hot E2) Volume	Volume from Dilution plate(uL)	Final Volume (ul)	Competitor Final Concentration
S	H4	1	norethynodrel	NE	WP8	3.16E-04	30	50	10	10	100	3.2E-05
S	H5	2	norethynodrel	NE	WP8	3.16E-04	30	50	10	10	100	3.2E-05
S	H6	3	norethynodrel	NE	WP8	3.16E-04	30	50	10	10	100	3.2E-05
S	A7	1	OTES	N	OTES1	1.00E-09	30	50	10	10	100	1.0E-10
S	A8	2	OTES	N	OTES1	1.00E-09	30	50	10	10	100	1.0E-10
S	A9	3	OTES	N	OTES1	1.00E-09	30	50	10	10	100	1.0E-10
S	B7	1	OTES	N	OTES2	1.00E-08	30	50	10	10	100	1.0E-09
S	B8	2	OTES	N	OTES2	1.00E-08	30	50	10	10	100	1.0E-09
S	B9	3	OTES	N	OTES2	1.00E-08	30	50	10	10	100	1.0E-09
S	C7	1	OTES	N	OTES3	1.00E-07	30	50	10	10	100	1.0E-08
S	C8	2	OTES	N	OTES3	1.00E-07	30	50	10	10	100	1.0E-08
S	C9	3	OTES	N	OTES3	1.00E-07	30	50	10	10	100	1.0E-08
S	D7	1	OTES	N	OTES4	1.00E-06	30	50	10	10	100	1.0E-07
S	D8	2	OTES	N	OTES4	1.00E-06	30	50	10	10	100	1.0E-07
S	D9	3	OTES	N	OTES4	1.00E-06	30	50	10	10	100	1.0E-07
S	E7	1	OTES	N	OTES5	1.00E-05	30	50	10	10	100	1.0E-06
S	E8	2	OTES	N	OTES5	1.00E-05	30	50	10	10	100	1.0E-06
S	E9	3	OTES	N	OTES5	1.00E-05	30	50	10	10	100	1.0E-06
S	F7	1	OTES	N	OTES6	1.00E-04	30	50	10	10	100	1.0E-05
S	F8	2	OTES	N	OTES6	1.00E-04	30	50	10	10	100	1.0E-05
S	F9	3	OTES	N	OTES6	1.00E-04	30	50	10	10	100	1.0E-05
S	G7	1	OTES	N	OTES7	1.00E-03	30	50	10	10	100	1.0E-04
S	G8	2	OTES	N	OTES7	1.00E-03	30	50	10	10	100	1.0E-04
S	G9	3	OTES	N	OTES7	1.00E-03	30	50	10	10	100	1.0E-04
S	H7	1	OTES	N	OTES8DBP7	1.00E-02	30	50	10	10	100	1.0E-03
S	H8	2	OTES	N	OTES88	1.00E-02	30	50	10	10	100	1.0E-03
S	H9	3	OTES	N	OTES8	1.00E-02	30	50	10	10	100	1.0E-03
S	A10	1	total binding	TB	TB1	-	30	60	10	-	100	-
S	A11	2	total binding	TB	TB2	-	30	60	10	-	100	-
S	A12	3	total binding	TB	TB3	-	30	60	10	-	100	-
S	B10	4	total binding	TB	TB4	-	30	60	10	-	100	-
S	B11	5	total binding	TB	TB5	-	30	60	10	-	100	-
S	B12	6	total binding	TB	TB6	-	30	60	10	-	100	-
S	C10	1	cold E2 (high)	NSB	S1	1.00E-05	30	50	10	10	100	1.0E-06
S	C11	2	cold E2 (high)	NSB	S2	1.00E-05	30	50	10	10	100	1.0E-06
S	C12	3	cold E2 (high)	NSB	S3	1.00E-05	30	50	10	10	100	1.0E-06
S	D10	4	cold E2 (high)	NSB	S4	1.00E-05	30	50	10	10	100	1.0E-06
S	D11	5	cold E2 (high)	NSB	S5	1.00E-05	30	50	10	10	100	1.0E-06
S	D12	6	cold E2 (high)	NSB	S6	1.00E-05	30	50	10	10	100	1.0E-06
S	E10	1	Buffer control	BC	BC1	-	-	100	-	-	100	-
S	E11	2	Buffer control	BC	BC2	-	-	100	-	-	100	-
S	E12	3	Buffer control	BC	BC3	-	-	100	-	-	100	-
S	F10	4	Buffer control	BC	BC4	-	-	100	-	-	100	-
S	F11	5	Buffer control	BC	BC5	-	-	100	-	-	100	-
S	F12	6	Buffer control	BC	BC6	-	-	100	-	-	100	-
S	G10*	1	Blank (for hot)	Hot	H1	-	90	-	10	-	100	-
S	G11*	2	Blank (for hot)	Hot	H2	-	90	-	10	-	100	-
S	G12*	3	Blank (for hot)	Hot	H3	-	90	-	10	-	100	-
S	H10*	4	Blank (for hot)	Hot	H4	-	90	-	10	-	100	-
S	H11*	5	Blank (for hot)	Hot	H5	-	90	-	10	-	100	-

S	H12	6	Blank (for hot)	Hot	H6	-	90	-	10	-	100	-

*: Note that the "hot" wells are empty during incubation. The 10 µl are added only for scintillation counting.

Competitive Binding Assay Well Layout

Plate	Position	Replicat	Well type	Well Code	Concentration	Competitor Initial Concentration	hrER stock (uL	Buffer Volume (uL)	Tracer (Hot E2) Volume	Volume from dilution plate(uL)	Final Volume (ul)	Competitor Final Concentration
P1	A1	1	Unknown 1	U1	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A2	2	Unknown 1	U1	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A3	3	Unknown 1	U1	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	B1	1	Unknown 1	U1	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B2	2	Unknown 1	U1	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B3	3	Unknown 1	U1	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	C1	1	Unknown 1	U1	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C2	2	Unknown 1	U1	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C3	3	Unknown 1	U1	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	D1	1	Unknown 1	U1	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D2	2	Unknown 1	U1	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D3	3	Unknown 1	U1	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	E1	1	Unknown 1	U1	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E2	2	Unknown 1	U1	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E3	3	Unknown 1	U1	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	F1	1	Unknown 1	U1	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F2	2	Unknown 1	U1	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F3	3	Unknown 1	U1	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	G1	1	Unknown 1	U1	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G2	2	Unknown 1	U1	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G3	3	Unknown 1	U1	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	H1	1	Unknown 1	U1	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H2	2	Unknown 1	U1	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H3	3	Unknown 1	U1	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	A4	1	Unknown 2	U2	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A5	2	Unknown 2	U2	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A6	3	Unknown 2	U2	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	B4	1	Unknown 2	U2	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B5	2	Unknown 2	U2	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B6	3	Unknown 2	U2	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	C4	1	Unknown 2	U2	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C5	2	Unknown 2	U2	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C6	3	Unknown 2	U2	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	D4	1	Unknown 2	U2	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D5	2	Unknown 2	U2	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D6	3	Unknown 2	U2	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	E4	1	Unknown 2	U2	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E5	2	Unknown 2	U2	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E6	3	Unknown 2	U2	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	F4	1	Unknown 2	U2	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F5	2	Unknown 2	U2	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F6	3	Unknown 2	U2	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	G4	1	Unknown 2	U2	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G5	2	Unknown 2	U2	7	1.00E-03	30	50	10	10	100	1.0E-04

P1	G6	3	Unknown 2	U2	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	H4	1	Unknown 2	U2	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H5	2	Unknown 2	U2	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H6	3	Unknown 2	U2	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	A7	1	Unknown 3	U3	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A8	2	Unknown 3	U3	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A9	3	Unknown 3	U3	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	B7	1	Unknown 3	U3	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B8	2	Unknown 3	U3	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B9	3	Unknown 3	U3	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	C7	1	Unknown 3	U3	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C8	2	Unknown 3	U3	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C9	3	Unknown 3	U3	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	D7	1	Unknown 3	U3	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D8	2	Unknown 3	U3	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D9	3	Unknown 3	U3	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	E7	1	Unknown 3	U3	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E8	2	Unknown 3	U3	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E9	3	Unknown 3	U3	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	F7	1	Unknown 3	U3	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F8	2	Unknown 3	U3	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F9	3	Unknown 3	U3	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	G7	1	Unknown 3	U3	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G8	2	Unknown 3	U3	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G9	3	Unknown 3	U3	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	H7	1	Unknown 3	U3	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H8	2	Unknown 3	U3	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H9	3	Unknown 3	U3	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	A10	1	Control E2 (max)	S	E2max	1.00E-06	30	50	10	10	100	1.00E-07
P1	A11	2	Control E2 (max)	S	E2max	1.00E-06	30	50	10	10	100	1.00E-07
P1	A12	3	Control E2 (max)	S	E2max	1.00E-06	30	50	10	10	100	1.00E-07
P1	B10	1	Control E2 (IC_{50})	S	$E2IC_{50}$	$E2IC_{50}$x10	30	50	10	10	100	$E2IC_{50}$
P1	B11	2	Control E2 (IC_{50})	S	$E2IC_{50}$	$E2IC_{50}$x10	30	50	10	10	100	$E2IC_{50}$
P1	B12	3	Control E2 (IC_{50})	S	$E2IC_{50}$	$E2IC_{50}$x10	30	50	10	10	100	$E2IC_{50}$
P1	C10	1	Control NE (max)	S	Nemax	1.00E-3.5	30	50	10	10	100	1.00E-4.5
P1	C11	2	Control NE (max)	S	Nemax	1.00E-3.5	30	50	10	10	100	1.00E-4.5
P1	C12	3	Control NE (max)	S	Nemax	1.00E-3.5	30	50	10	10	100	1.00E-4.5
P1	D10	1	Control NE (IC_{50})	S	$NEIC_{50}$	$NEIC_{50}$	30	50	10	10	100	$NEIC_{50}$
P1	D11	2	Control NE (IC_{50})	S	$NEIC_{50}$	$NEIC_{50}$	30	50	10	10	100	$NEIC_{50}$
P1	D12	3	Control NE (IC_{50})	S	$NEIC_{50}$	$NEIC_{50}$	30	50	10	10	100	$NEIC_{50}$
P1	E10	1	cold E2 (high)	NSB	S1	1.00E-05	30	50	10	10	100	1.0E-06
P1	E11	2	cold E2 (high)	NSB	S2	1.00E-05	30	50	10	10	100	1.0E-06
P1	E12	3	cold E2 (high)	NSB	S3	1.00E-05	30	50	10	10	100	1.0E-06
P1	F10	4	cold E2 (high)	NSB	S4	1.00E-05	30	50	10	10	100	1.0E-06
P1	F11	5	cold E2 (high)	NSB	S5	1.00E-05	30	50	10	10	100	1.0E-06
P1	F12	6	cold E2 (high)	NSB	S6	1.00E-05	30	50	10	10	100	1.0E-06
P1	G10	1	total binding	TB	TB1	-	30	60	10	-	100	-
P1	G11	2	total binding	TB	TB2	-	30	60	10	-	100	-
P1	G12	3	total binding	TB	TB3	-	30	60	10	-	100	-
P1	H10	4	total binding	TB	TB4	-	30	60	10	-	100	-

P1	H11	5	total binding	TB	TB5	-	30	60	10	-	100	-
P1	H12	6	total binding	TB	TB6	-	30	60	10	-	100	-

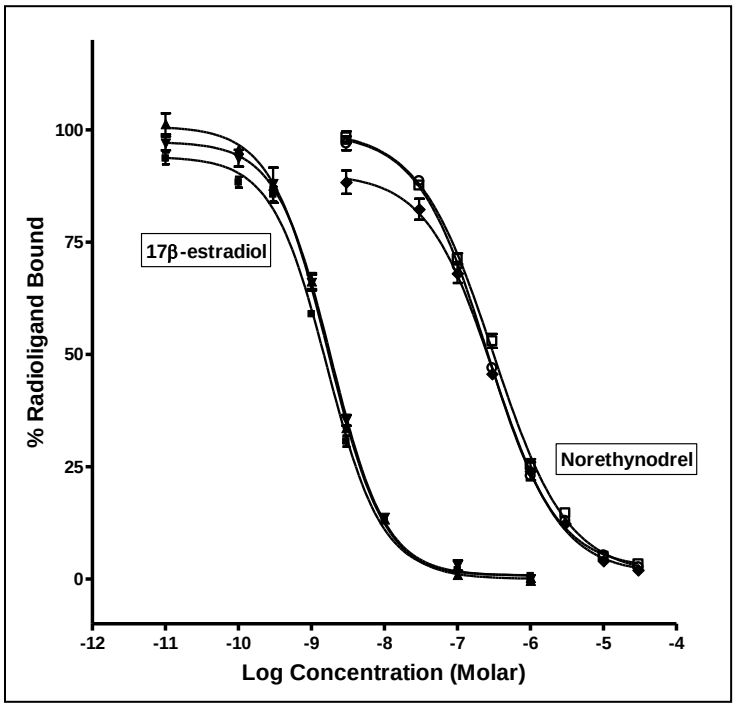
ANNEX 4: CONSIDERATIONS FOR THE ANALYSIS OF DATA FROM THE HRER COMPETITIVE BINDING ASSAY

1. The hrERα competitive binding assay measures the binding of a single concentration of [^{3}H]-17β-estradiol in the presence of increasing concentrations of a test chemical. The competitive binding curve is plotted as specific [^{3}H]-17β- estradiol binding versus the concentration (log10 units) of the competitor. The concentration of the test chemical that inhibits 50% of the maximum specific [^{3}H]-17β-estradiol binding is the IC_{50}.

Data Analysis for the Reference Estrogen and Weak Binder (1)

2. Data from the control runs are transformed (i.e. percent [^{3}H]-17β-estradiol specific binding and the log concentration of the control chemical) for further analysis. Estimates of log(IC_{50}) values for the positive controls (e.g. reference estrogen and weak binder) should be determined using an appropriate nonlinear curve fitting software to fit a four parameter Hill equation i.e.(e.g. BioSoft; GraphPad Prism) (2). The top, bottom, slope, and log(IC_{50}) can typically be left unconstrained when fitting these curves. Robust regression should be used when determining the best fit unless justification is given. The method chosen for robust regression should be stated. Correction for ligand depletion was not needed for the FW or CERI hrER test methods, but may be considered if needed. Following the initial analysis, each binding curve should be reviewed to ensure an appropriate fit to the model. The relative binding affinity (RBA) for the weak binder can be calculated as a percent of the log (IC_{50}) for the weak binder relative to the log (IC_{50}) for 17β-estradiol. Results for the positive controls and the non-binder control should be evaluated using measures of assay performance and acceptability criteria as described in the PBTG (paragraph 20), Annex 2 (FW Assay, paragraphs 41-51) and Annex 3 (CERI Assay, paragraphs 41-51). Examples of 3 runs for the reference estrogen and weak binder are shown in Figure 1.

Figure 1. Examples of the competitive binding curves for the reference estrogen and the control weak binder.



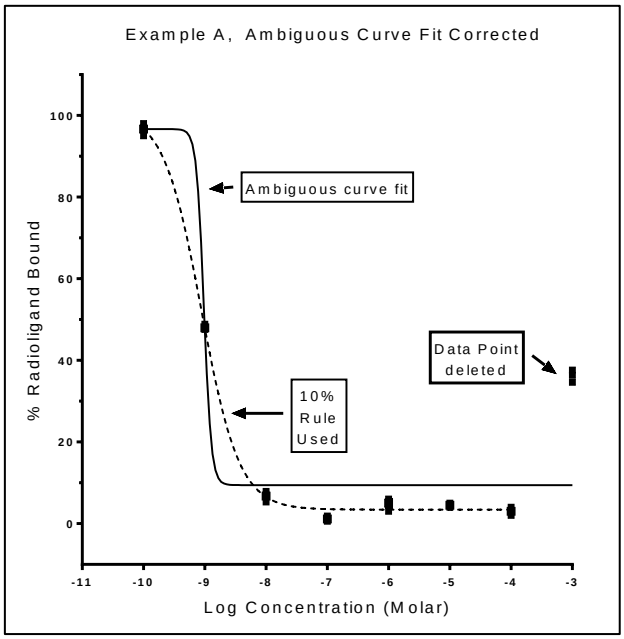
Data Analysis for Test Chemicals

3. Data for all test chemicals should be analyzed using a step-wise approach to ensure that data are appropriately analyzed and that each competitive binding curve is properly classified. Each run for a test chemical should initially undergo a standardized data analysis that is identical to that used for the reference estrogen and weak binder controls. Once completed, a technical review of the curve fit parameters as well as a visual review of how well the data fit the generated competitive binding curve for each run should be conducted. During this technical review, the observations of a concentration dependent decrease in the percent [^{3}H]-17β-estradiol specifically bound, low variability among the technical replicates at each chemical concentration, and consistency in fit parameters among the three runs are a good indication that the assay and data analyzes were conducted appropriately. Professional judgment should be applied when reviewing results from each run for a test chemical, and the data used to classify each test chemical as a binder or non-binder should be scientifically defensible.

4. Occasionally, there may be examples of data that require additional attention in order to appropriately analyze and interpret the hrER binding data. Previous studies had shown cases where the analysis and interpretation of competitive receptor binding data can be complicated by an upturn of the percent specific binding when testing chemicals at the highest concentrations (Figure 2). This is a well-known issue that has been encountered when using protocols for a number of competitive receptor binding assays (3). In these cases, a concentration dependent response is observed at lower concentrations, but as the concentration of the test chemical approaches the limit of solubility, the displacement of [^{3}H]17β-estradiol no longer decreases. In these cases, data for the higher concentrations indicate that the biological limit of the assay has been reached. For example, this phenomena is many times associated with chemical insolubility and precipitation at high concentrations, or may also be a reflection of exceeding the capacity of the dextran-coated charcoal to trap the unbound radiolabeled ligand during the separation procedure at the highest chemical concentrations. Leaving such data points in when fitting competitive binding data to a sigmoid curve

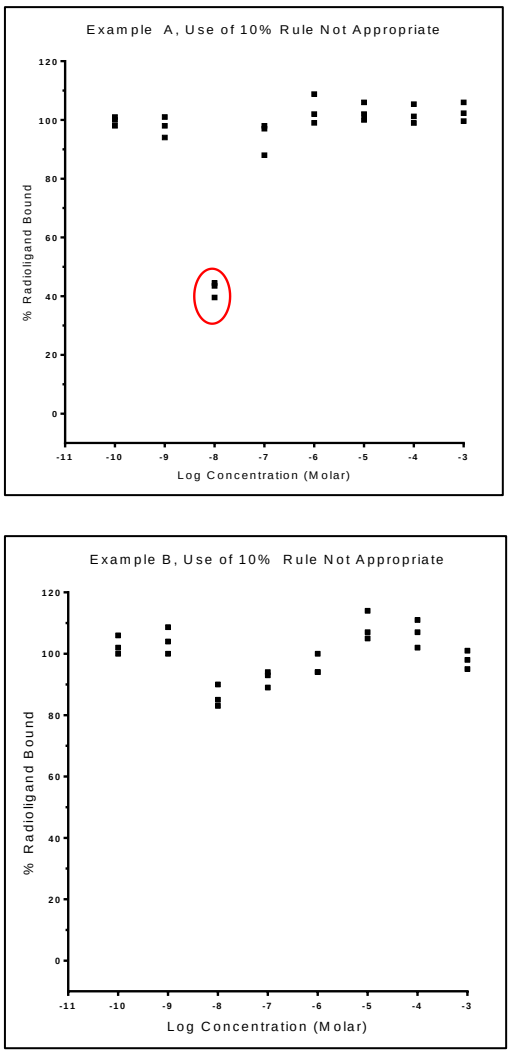
can sometimes lead to a misclassification of the ER binding potential for a test chemical (Figure 2). To avoid this, the protocol for the FW and CERI hrER binding assays includes an option to exclude from the analyses data points where the mean of the replicates for the percent [^{3}H]17β-estradiol specific bound is 10% or more above that observed for the mean value at a lower concentration (i.e. This is commonly referred to as the 10% rule). This rule can only be used once for a given curve, and there must be data remaining for at least 6 concentrations such that the curve can be correctly classified.

Figure 2. Examples, Competitive Binding Curves with and without Use of the 10% Rule.



5. The appropriate use of the 10% rule to correct these curves should be carefully considered and reserved for those cases where there is a strong indication of a hrER binder. During the conduct of experiments for the validation study of the FW hrER Binding Assay, it was observed that the 10% rule sometimes had an unintended and unforeseen consequence. Chemicals that did not interact with the receptor (i.e. true non-binders) often showed variability around 100% radioligand binding that were greater than 10% across the range of concentrations tested. If the lowest value happened to be at a low concentration, the data from all higher concentrations could potentially be deleted from the analysis by using the 10% rule, even though those concentrations could be useful in establishing that the chemical is a non-binder. Figure 3 show examples where the use of the 10% rule is not appropriate.

Figure 3. Examples, Competitive Binding Data Where Use of the 10% Rule is Not Appropriate.



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