新規試験法提案書

エストロゲン受容体（ER）結合親和性化学物質の検出のための、ヒト組み換えエストロゲン受容体（hrER）*in vitro* 試験法に関する性能準拠試験法ガイドライン（TG493）

令和 2 年12月

国立医薬品食品衛生研究所

新規試験法提案書

令和 2 年 12 月 2 日

No.　2020-03

エストロゲン受容体（ER）結合親和性化学物質の検出のための、ヒト組み換えエストロゲン受容体（hrER）*in vitro* 試験法に関する性能準拠試験法ガイドライン（TG493）に関する提案

令和 2 年 6 月 25 日に国立医薬品食品衛生研究所にて開催された新規試験法評価会議（通称：JaCVAM 評価会議）において以下の提案がなされた。

提案内容： 本試験法は、ERへの結合活性を有する化学物質を同定するための簡便で迅速な*in vitro* のスクリーニング方法として活用することができると考える。

この提案書は、受容体結合試験資料編纂委員会によりまとめられた文書を用いて、JaCVAM 評価会議が評価および検討した結果、その有用性が確認されたことから作成された。

以上の理由により、行政当局の安全性評価方法としてエストロゲン受容体（ER）結合親和性化学物質の検出のための、ヒト組み換えエストロゲン受容体（hrER）*in vitro* 試験法に関する性能準拠試験法ガイドラインの使用を提案するものである。

大野泰雄

JaCVAM 評価会議　議長

平林容子

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

大 野 泰 雄 （公益財団法人 木原記念横浜生命科学振興財団）：座長
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横 関 博 雄 （日本皮膚免疫アレルギー学会）

任期：平成 30 年 4 月 1 日～令和 2 年 9 月 30 日
*：平成 30 年 4 月 1 日～令和 2 年 3 月 31 日
**：令和 2 年 4 月 1 日～令和 2 年 9 月 30 日

JaCVAM 運営委員会

平 林 容 子　（国立医薬品食品衛生研究所　安全性生物試験研究センター）：委員長
石 井 孝 司　（国立感染症研究所）
小川久美子　（国立医薬品食品衛生研究所　安全性生物試験研究センター　病理部）
諫 田 泰 成　（国立医薬品食品衛生研究所　安全性生物試験研究センター　薬理部）
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合 田 幸 広　（国立医薬品食品衛生研究所）
佐々木正広　（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）
杉 山 圭 一　（国立医薬品食品衛生研究所　安全性生物試験研究センター　変異遺伝部）
髙 橋 祐 次　（国立医薬品食品衛生研究所　安全性生物試験研究センター　毒性部　動物管理室）
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束 野 正 明　（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）
広 瀬 明 彦　（国立医薬品食品衛生研究所　安全性生物試験研究センター　安全性予測評価部）
笛 木　　修　（独立行政法人　医薬品医療機器総合機構）
横 田 雅 彦　（独立行政法人　医薬品医療機器総合機構）
足利太可雄　（国立医薬品食品衛生研究所　安全性生物試験研究センター　安全性予測評価部第二室）：事務局
小 島　　肇　（国立医薬品食品衛生研究所　安全性生物試験研究センター　安全性予測評価部第二室）：事務局

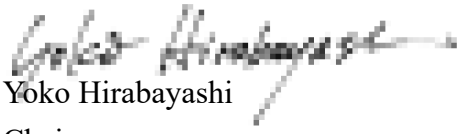
JaCVAM Statement on the Performance-Based Test Guideline (TG493) for Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assays to Detect Chemicals with ER Binding Affinity

At a meeting held on 25 June 2020 at the National Institute of Health Sciences (NIHS) in Kanagawa, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Proposal: We consider the Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assay to be possibly used as an *in vitro*, simple, and rapid screening method for detecting chemicals with ER binding affinity.

This statement was prepared, following the review prepared by the Receptor Binding Assay JaCVAM Editorial Committee, to acknowledge that the results of a review and study by the JaCVAM Regulatory Acceptance Board have confirmed the usefulness of this assay.
Based on the above, we propose the Performance-Based Test Guideline (TG493) for Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assays as useful means for estimating ER Binding Affinity of chemicals by regulatory agencies.

Yasuo Ohno
Chairperson
JaCVAM Regulatory Acceptance Board



Yoko Hirabayashi
Chairperson
JaCVAM Steering Committee

2 December 2020

The JaCVAM Regulatory Acceptance Board was established by the JaCVAM Steering Committee, and is composed of nominees from the industry and academia.

This statement was endorsed by the following members of the JaCVAM Regulatory Acceptance Board:

Mr. Yasuo Ohno (Kihara Memorial Yokohama Foundation for the Advancement of Life Sciences): Chairperson
Ms. Yoko Hirabayashi (Center for Biological Safety and Research: CBSR, National Institute of Health Sciences: NIHS)
Mr. Morihiko Hirota (Japan Cosmetic Industry Association)
Mr. Yoshiaki Ikarashi (NIHS)
Mr. Takanori Ikeda (Japan Pharmaceutical Manufacturers Association)**
Mr. Noriyasu Imai (Japanese Society for Alternatives to Animal Experiments)
Mr. Kunifumi Inawaka (Japan Chemical Industry Association)
Mr. Tomoaki Inoue (Japanese Society of Immunotoxicology) *
Mr. Yuji Ishii (CBSR, NIHS)
Ms. Yumiko Iwase (Japan Pharmaceutical Manufacturers Association)*
Mr. Fumihiro Kubo (Pharmaceuticals and Medical Devices Agency)*
Mr. Kenichi Masumura (Japanese Environmental Mutagen Society)
Ms. Ruriko Nakamura (National Institute of Technology and Evaluation)
Mr. Akiyoshi Nishikawa (CBSR, NIHS/ Saiseikai Utsunomiya Hospital)
Mr. Jihei Nishimura (Pharmaceuticals and Medical Devices Agency)
Mr. Satoshi Numazawa (Japanese Society of Toxicology)
Ms. Keiko Yamamoto (Pharmaceuticals and Medical Devices Agency)**
Mr. Hiroo Yokozeki (Japanese Society for Cutaneous Immunology and Allergy)

Term: From 1st April 2018 to 30th September 2020
*: From 1st April 2018 to 31st March 2020
**: From 1st April 2020 to 30th September 2020

This statement was endorsed by the following members of the JaCVAM steering Committee after receiving the report from JaCVAM Regulatory Acceptance Board:

Ms. Yoko Hirabayashi (CBSR, NIHS): Chairperson
Mr. Osamu Fueki (Pharmaceuticals and Medical Devices Agency)
Mr. Yukihiro Goda (NIHS)
Mr. Akihiko Hirose (Division of Risk Assessment, CBSR, NIHS)
Mr. Koji Ishii (National Institute of Infectious Diseases)
Mr. Yasunari Kanda (Division of Pharmacology, CBSR, NIHS)
Mr. Satoshi Kitajima (Division of Toxicology, CBSR, NIHS)
Ms. Kumiko Ogawa (Division of Pathology, CBSR, NIHS)
Mr. Masahiro Sasaki (Ministry of Health, Labour and Welfare)
Mr. Keiichi Sugiyama (Division of Genetics and Mutagenesis, CBSR, NIHS)
Mr. Masahiro Takahata (Ministry of Health, Labour and Welfare)
Mr. Yuhji Taquahashi (Animal Management Section of the Division of Toxicology, CBSR, NIHS)
Mr. Masaaki Tsukano (Ministry of Health, Labour and Welfare)
Mr. Masahiko Yokota (Pharmaceuticals and Medical Devices Agency)
Mr. Takao Ashikaga (Division of Risk Assessment, CBSR, NIHS): Secretary
Mr. Hajime Kojima (Division of Risk Assessment, CBSR, NIHS): Secretary

エストロゲン受容体（ER）結合親和性化学物質の検出のための、ヒト組み換えエストロゲン受容体（hrER）*in vitro* 試験法に関する性能準拠試験法ガイドライン（TG493）

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評価会議報告書

エストロゲン受容体(ER)結合親和性化学物質の検出のための、ヒト組み換えエストロゲン受容体(hrER)*in vitro* 試験法に関する性能準拠試験法ガイドライン(TG493)

JaCVAM 評価会議

令和 2 年(2020 年)6 月 25 日

JaCVAM 評価会議

大 野 泰 雄 （公益財団法人 木原記念横浜生命科学振興財団）：座長
五十嵐良明 （国立医薬品食品衛生研究所）
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任期：平成 30 年 4 月 1 日～令和 2 年 9 月 30 日
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経済協力開発機構（Organization for Economic Co-operation and Development: OECD）試験法ガイドライン（Test Guideline: TG）では、エストロゲン活性に関する試験法として、*in vitro* エストロゲン受容体転写活性化試験法（OECD TG455）や *in vivo* 試験法として子宮肥大試験（OECD TG440）が確立されているが、エストロゲン受容体（Estrogen Receptor: ER）を介した生体反応の起点となる化学物質と受容体・リガンド結合部位への特異的相互作用の簡便で迅速なスクリーニング法として組み換えタンパク質を用いた *in vitro* 結合試験法の開発が進められてきた。本試験法は、ER結合親和性化学物質の検出のための、ヒト組み換えエストロゲン受容体（Human Recombinant Estrogen Receptor Alpha: hrERα）*in vitro* 試験法に関する性能準拠試験法ガイドライン（Performance Based Test Guideline: PBTG）であり、*in vitro*で化学物質（競合物質）存在下での放射標識リガンド（[^{3}H]-17β-エストラジオール、[^{3}H]-E2）のERへの結合能を化学物質の濃度を上昇させながら測定するスクリーニング試験法を含むガイドラインである。本PBTGには、以下の2種類の試験法が参照試験法として掲載されている。

・完全長ヒト組み換えERαを用いたFreyberger-Wilson（FW）の *in vitro* hrERα 結合試験（以下、FWアッセイ）[1)]

・ヒト組み換えリガンド結合ドメイン（Ligand Binding Domain: LBD）タンパク質を用いた化学物質評価研究機構（Chemicals Evaluation and Research Institute: CERI）の *in vitro* hrERα-LBD 結合試験（以下、CERI アッセイ）[1)]

本試験法については、PBTG に掲載されている参照試験法について、日米欧 5 ～ 6 施設でバリデーション研究が実施され[2)]、施設内・施設間再現性が評価された。OECD 専門家会議でその正確性および信頼性が評価され、2015年に内分泌かく乱物質のスクリーニング評価に関する試験法として、OECD TG493が承認された[1)]。

Japanese Center for the Validation of Alternative Methods（JaCVAM）評価会議は、JaCVAM 受容体結合試験資料編纂委員会により作成された、「エストロゲン受容体（ER）結合親和性化学物質の検出のための、ヒト組換えエストロゲン受容体（hrER）*in vitro* 試験法に関する性能準拠試験法ガイドライン（TG493）の評価報告書」（令和2年2月18日）を用いて、本試験法の妥当性について検討した。

1. 試験法の定義

名称：

- 完全長ヒト組み換え ERα を用いた Freyberger-Wilson（FW）の *in vitro* hrERα 結合試験
- ヒト組み換えリガンド結合ドメイン(LBD)タンパク質を用いた化学物質評価研究機構（CERI）の *in vitro* hrERα-LBD 結合試験

代替する対象毒性試験：なし。本試験法はスクリーニングとして用いるものである。

試験法の概略：FW および CERI アッセイは、ER 結合親和性化学物質を検出することを目的として開発された試験法である。これら試験は、①受容体－リガンド相互作用パラメーターを特徴付ける飽和結合試験および②化学物質と標識リガンドとの間の競合

によって結合親和性を推定する競合結合試験の 2 つの主な要素から構成される。①飽和結合試験では、FW および CERI アッセイプロトコルで規定される過剰濃度の非標識リガンド(17β-estradiol)の存在下および非存在下で、放射標識リガンドの全結合量および非特異結合量が測定され、その差から特異的結合量が算出される。②競合結合試験では、飽和結合試験で得られた特異的結合量を踏まえ、FW アッセイでは 1 nM の放射標識リガンドが 20±5%、CERI アッセイでは 0.5 nM の放射標識リガンドが 40±10%の特異的結合を示す量の hrERα を用いて、化学物質存在下での放射標識リガンドの結合量が測定される。

2. 評価に用いた資料および評価内容の科学的妥当性

本試験法については、以下の①日米欧 5 ～ 6 施設による国際バリデーション研究 [2] および② その一環として実施された試験方法の正確性・信頼性を確認するためのバリデーション試験 [2] の結果を基に、OECD において評価された。その結果、OECD Task Force on Endocrine Disrupters Testing and Assessment で提案された OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters のレベル 2 に該当する内分泌かく乱物質のスクリーニング評価に有用な試験法として妥当性が認められ、OECD TG493 が承認された。

JaCVAM 受容体結合資料編纂委員会はこれらの資料を用いて評価し、報告書としてまとめたものを評価資料とした。

本試験法は、化学物質が hrERα に対し、アゴニスト作用を及ぼすかアンタゴニスト作用を及ぼすかの判定はできないものの、hrERα への結合に対し、極めて良好な特異性を有する試験法であると考えられることから、本試験法は hrERα への結合作用を有する化学物質をスクリーニングする方法として、科学的な妥当性があると考えられる。

① 国際バリデーション研究：

FW アッセイについては、Freyberger と Wilson により作成されたプロトコルに基づいて、CERI アッセイについては CERI により作成されたプロトコルに基づいて、米国 EPA の主導により、FW アッセイについては日米欧 6 施設、CERI アッセイについては日米欧 5 施設の参加による国際バリデーション研究が実施された。バリデーション研究は、飽和結合試験および競合結合試験が実施され、競合結合試験では 29 物質（陽性 21 物質、陰性 8 物質）について検討された。

② 試験方法の正確性・信頼性を確認するための検証試験:

ラット子宮のサイトゾルを用いた ER 結合試験（Estrogen Receptor Binding Assay Using Rat Uterine Cytosol: ER-RUC アッセイ）[3] の結果が入手可能かつ ER 反応性が明確な 22 物質（陽性 17、陰性 5）を用いて、検証試験が実施された。その結果、陽性 17 物質について、「判定不能」を除く一致率は、ER-RUC アッセイで 94.1%（16/17）、FW および CERI アッセイでいずれも 100%（16/16）であった。さらに、陰性 5 物質について、ER-RUC アッセイで陰性 1、偽陽性 1、評価不能 3 であったのに対し、FW および

CERI アッセイでは全て陰性であった。以上の結果、「判定不能」を除く各々の試験法の正確度について、ER-RUC アッセイは 94.4%（17/18）、FW および CERI アッセイは共に 100%（21/21）であった。

以上より、FW および CERI アッセイ共に、米国 Environmental Protection Agency（EPA）の現行の TG OPPTS 890.1250 と同等以上の性能標準を満たすことが示された。

3. 本試験法の有用性と適用限界

本試験法は、hrERα へ結合活性を良好な特異性をもって検出できる点が優れている。

バリデーション研究に用いた物質の内、ER-RUC アッセイとの比較に用いた物質において、ER との結合性が適切に評価できた化合物（equivocal と判定された 1 物質を除く 21 物質）では感度と特異度は良好な値を示しているが、例数を増やして正確な数値を求める必要がある。また、ER-RUC アッセイと同等以上の性能標準を満たすことも示されている。しかしながら、本試験法で評価できるのはあくまでも化学物質の hrERα への結合活性であり、化学物質のアゴニスト・アンタゴニスト活性を区別することは出来ず、本試験法で陽性と判定される化学物質が生体内でどのような影響を示すかについては評価することができない。なお、本試験法を行う上で、以下の点に留意する必要がある。

- 本試験法は放射性物質を取り扱う試験である。
- 未知の化学物質を試験する際に試験で用いる濃度は 1 mM を超えてはならない。

4. 目的とする物質又は製品の毒性を評価する試験法としての、社会的受け入れ性および行政上の利用の可能性

<u>社会的受け入れ性</u>：

本試験法は、放射標識リガンドを使用することから、放射性同位体の取り扱い可能な施設およびそれについての十分な知識を有した熟練した作業者による作業が必要であると考えるものの、①生きた動物を用いないという点で 3Rs の精神に合致していること、②内因性エストロゲンと競合して ER に結合する可能性のある化学物質を迅速に同定するための、有用かつ単純なツールであると考えられることから、本試験法の社会的受け入れ性は高いと考える。

<u>行政上の利用性：</u>

本試験法は、ER への結合活性を有する化学物質を同定するための簡便で迅速な *in vitro* のスクリーニング法として活用することができると考える。

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評価報告書

エストロゲン受容体（ER）結合親和性化学物質の検出のための、ヒト組み換えエストロゲン受容体（hrER）*in vitro* 試験法に関する性能準拠試験法ガイドライン（TG493）

受容体結合試験資料編纂委員会

令和 2 年（2020 年）2 月 18 日

受容体結合試験資料編纂委員会

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略語

CAB:	Chemical Advisory Board
CERI:	Chemicals Evaluation and Research Institute
CV:	Coefficient of Variation
DBP:	Di-*n*-Butyl Phthalate
E2:	17β-estradiol
EPA:	Environmental Protection Agency
ER:	Estrogen Receptor
ERα:	Estrogen Receptor Alpha
ERβ:	Estrogen Receptor Beta
FW:	Freyberger-Wilson
GLP:	Good Laboratory Practice
hERα:	Human Estrogen Receptor Alpha
hrER:	Human Recombinant Estrogen Receptor
hrERα:	Human Recombinant Estrogen Receptor Alpha
IC50:	The half maximal effective concentration of an inhibitory test chemical
ICCVAM:	Interagency Coordinating Committee on the Validation of Alternative Methods
LBD:	Ligand Binding Domain
MMTV:	Mouse Mammary Tumor Virus
NICEATM:	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD:	Organisation for Economic Co-operation and Development
OECD CF:	Conceptual Framework for Testing and Assessment of Endocrine Disrupters
OECD EDTA TF:	Endocrine Disruptor Testing and Assessment Task Force
OTES:	Octyltriethoxysilane
PBTG:	Performance Based Test Guideline
PS:	Performance Standard
RLU:	Relative Light Units
SD:	Standard Deviation
STTA:	Stably Transfected Transactivation Assay
TA:	Transactivation
TG:	Test Guideline
VMG-NA:	Validation Management Group for Non-Animal testing

要旨

性能標準(PS)に基づく性能準拠試験法ガイドライン(PBTG)として経済協力開発機構(OECD)で承認されたER結合親和性化学物質の検出のための、ヒト組み換えエストロゲン受容体(hrER) *in vitro* 試験法[1,2]は、*in vitro* で化学物質のエストロゲン受容体(ER)に対する結合活性を生体内リガンドであるエストラジオールとの競合結合活性を指標として検出するスクリーニング試験法であり、化学物質のERへの作用による生体反応の起点であるER結合活性を評価することが出来る。本PBTGには、国際的な多施設バリデーション研究により、試験法の妥当性と信頼性が証明された2種の試験法(FWアッセイおよび化学物質評価研究機構(CERI)アッセイ)が参照試験法として収載されている。また、本PBTGには、試験の目的である化学物質のhrER結合親和性を検出する類似試験法の開発とバリデーション研究のためのPSが設定されており、PSを満たすことが示された新規の類似試験法を追加することが可能である。本試験法に収載されている2つの試験法についての再現性や信頼性および試験法としての科学的妥当性と規制試験法としての妥当性については、米国環境保護庁(米国EPA)の主導により日米欧6施設の参加(CERIアッセイについてはうち5施設が参加)による国際バリデーション研究の結果をもとに、OECD第三者評価により評価され、OECD内分泌かく乱物質の試験と評価に関するタスクフォース(OECD EDTA TF)で提案されたOECD内分泌かく乱物質の試験法と評価に関する概念枠組み(OECD CF)のレベル2に該当する内分泌かく乱物質のスクリーニング評価に有用な *in vitro* 試験法としてOECD TG493が成立した。本試験法の課題として、本試験法は化学物質のERへの結合活性を評価できるのみであり化学物質のアゴニスト・アンタゴニスト活性を区別することは出来ず、本試験法で陽性と判定される化学物質が生体内でどのような影響を示すかについては評価できないという点があげられる。本試験法はあくまでもスクリーニング法であり、ERを介して惹起されると想定される有害影響を確定評価する試験法と組み合わせて評価を行うことで今後の化学物質管理に大きく貢献すると考えられる。

1. 本試験法の科学的妥当性と規制試験法としての妥当性

ER 結合親和性化学物質の検出のための、hrER *in vitro* 試験法[1, 2] に関する PBTGは、*in vitro* で化学物質の ER に対する結合親和性を検出するスクリーニング試験法である。本試験法ガイドライン（TG）は、OECD のガイダンス文書「Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment」[3] に規定されるバリデーション研究の原則に従った新たな類似試験または改良試験の開発を促進する PBTG であり、参照試験法として国際的な多施設バリデーション研究により[1] 試験法の妥当性と信頼性が証明された下記の 2 つの機序および機能的に同等の試験法が示されている。

・完全長ヒト組み換え ERα を用いた Freyberger-Wilson（FW）の *in vitro* hrERα 結合試験（以下、FWアッセイ）[1]
・ヒト組み換えリガンド結合ドメイン（LBD）タンパク質を用いた化学物質評価研究機構（CERI）の *in vitro* hrERα-LBD 結合試験（以下、CERIアッセイ）[1]

本 PBTG には、試験の目的である化学物質のヒト組み換えヒトエストロゲンアルファ受容体（hrERα）結合親和性を検出する類似試験法の開発とバリデーション研究のための PS[2] が設定されており、現行の PBTG を適時修正して新規類似試験法を追加することが可能である。ただし、類似試験法の追加は PS に示された参照物質 23 物質（表1-1）を用いたバリデーション研究により再現性や信頼性の基準を満たすことを示して合意された後にのみ可能である。

環境中や市場に流通する多くの化学物質が内分泌系に影響する生物活性を有することが示されており、そうした化学物質による内分泌系のかく乱による潜在的なヒト健康や環境に対する影響が指摘されている。OECD では、1998年に重点活動項目の 1 つとして、内分泌かく乱作用を有する可能性のある化学物質のスクリーニングおよび試験のための TG の整備のため、OECD EDTA TF を設置し、化学物質の内分泌かく乱作用評価のための試験法を OECD CF として整理し、既存 TG の改訂と、新規 TG の整備を進めている。OECD CF は、それぞれ生物学的複雑性の異なる 5 つのレベルから構成されており、本 TG は、レベル 2 の「機構に関する情報をもたらす *in vitro* 試験」に示されている該当するホルモン受容体結合試験法である。化学物質の内分泌かく乱作用のスクリーニングに有用な指標として、OECD CF では化学物質のホルモン受容体との結合性または受容体を介した内分泌機能の活性化および阻害を測定する方法が示されている。エストロゲン活性*に関する試験法としては†、これまでに *in vitro* エストロゲン受容体転写活性化試験法（OECD TG455）や *in vivo* 試験法として子宮肥大試験（OECD TG440）が確立されているが、ER を介した生体反応の起点となる化学物質と受容体リガンド結合部位への特異的相互作用の簡便で迅速なスクリーニング法として組み換えタンパク質を用いた *in vitro* 結合試験法の開発が進められてきた。

*エストロゲン活性：化学物質がエストロゲン受容体結合において 17β-エストラジオールを模す能力。hERα への結合は本 PBTG で検出することができる。

表 1-1　hrER α 結合試験(TG 493)の性能標準の参照物質 23 物質

No.	Chemical Name	CAS RN	Expected Response #	Concentration Range Tested(M)	FW Assay Classification	CERI Assay Classification	MESH Chemical Class	Product Class
1	17β-Estradiol	50-28-2	*Strong Binder*	1x10-11 – 1x10-6	Binder	Binder	Steroid	Pharmaceutical, Veterinary Agent
2	17α-ethynyl estradiol	57-63-6	*Strong Binder*	1x10-11 – 1x10-6	Binder	Binder	Steroid	Pharmaceutical, Veterinary Agent
3	Diethylstilbestrol (DES)	56-53-1	*Strong Binder*	1x10-11 – 1x10-6	Binder	Binder	Hydrocarbon, (Cyclic), Phenol	Pharmaceutical, Veterinary Agent
4	Meso-Hexestrol	84-16-2	*Strong Binder*	1x10-11 – 1x10-6	Binder	Binder	Hydrocarbon (cyclic), Phenol	Pharmaceutical, Veterinary Agent
5	Zearalenone	17924-92-4	*Strong Binder*	1x10-10 – 1x10-3	Binder	Binder	Hydrocarbon (heterocyclic), Lactone	Natural Product
6	Tamoxifen	10540-29-1	*Strong Binder*	1x10-10 – 1x10-3	Binder	Binder	Hydrocarbon, (Cyclic)	Pharmaceutical, Veterinary Agent
7	Norethynodrel or (Norethindrone) [a]	68-23-5 (68-22-4)a	*Moderate Binder*	3x10-9 – 30x10-4	Binder	Binder	Steroid	Pharmaceutical, Veterinary Agent
8	Genistein	446-72-0	*Moderate Binder*	1x10-10 – 1x10-3	Binder	Binder	Hydrocarbon (heterocyclic), Flavonoid	Natural Product
9	Equol	531-95-3	*Moderate Binder*	1x10-10 – 1x10-3	Binder	Binder	Phytoestrogen Metabolite	Natural Product
10	Butyl paraben (n-butyl-4-hydroxybenzoate)	94-26-8	*Weak Binder*	1x10-10 – 1x10-3	Binder	Binder	Paraben	Preservative
11	Nonylphenol (mixture)	84852-15-3	*Weak Binder*	1x10-10 – 1x10-3	Binder	Binder	Alkylphenol,	Intermediate Compound
12	o,p'-DDT [d]	789-02-6	*Weak Binder*	1x10-10 – 1x10-3	Binder	Binder	Organochlorine	Insecticide
13	5α- dihydrotestosterone	521-18-6	*Weak Binder*	1x10-10 – 1x10-3	Binder	Binder	Steroid, Nonphenolic	Natural Product
14	Bisphenol A (BPA)	80-05-7	*Weak Binder*	1x10-10 – 1x10-3	Binder	Binder	Phenol	Chemical Intermediate
15	4-n-heptylphenol	1987-50-4	*Weak Binder*	1x10-10 – 1x10-3	Equivocal	Binder	Alkylphenol	Intermediate
16	Kepone (Chlordecone)	143-50-0	*Weak Binder*	1x10-10 – 1x10-3	Binder	Binder	Hydrocarbon, (Halogenated)	Pesticide
17	Enterolactone	78473-71-9	*Weak Binder*	1x10-10 – 1x10-3	Binder	Binder	Phytoestrogen	Natural Product
18	* Di-n-butyl phthalate (DBP)	84-74-2	*Non-binder*	1x10-10 – 1x10-4	Non-Binder*	Non-Binder*	Hydrocarbon (cyclic), Ester	Plasticizer, Chemical
19	Octyltriethoxysilane	2943-75-1	*Non-binder*	1x10-10 – 1x10-3	Non-Binder	Non-Binder	Silane	Surface modifier
20	Corticosterone [c]	50-22-6	*Non-binder*	1x10-10 – 1x10-3	Non-binder	Non-Binder	Steroid	Natural Product
21	Benz(a)anthracene	56-55-3	*Non-Binder* [b]	1x10-10 – 1x10-3	Non-Binder	Non-Binder	Aromatic Hydrocarbon	Intermediate
22	Progesterone [c]	57-83-0	*Non-binder*	1x10-10 – 1x10-4	Non-Binder	Non-Binder	Steroid	Natural Product
23	Atrazine [c]	1912-24-9	*Non-binder*	1x10-10 – 1x10-4	Non-Binder	Non-Binder	Heterocyclic compound	Herbicide

Abbreviations: DES, diethylstilbestrol; HPTE, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane, an intermediate by-product of methoychlor; o,p'-DDT, 1,1,1-trichloro-2-2-[o- chlorophpenyl]-2-[p-chlorophenyl]ethane

#: The expected response for each chemical was based upon published data from in vitro studies and were reviewed by a Chemical Advisory Board whose members were not directly associated with the validation study for the FW and CERI hrER Binding Assays. Chemicals were selected to represent multiple chemical classes and cover a range of binding affinity potencies commonly associated with ER agonist activity. When tested in the FW or CERI hrER Assays, the LogIC50 for strong binders typically ranged from - 9 to -7 (M), moderate -7.1 to -6.0 (M) and weak < -5.9 (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 chemical was observed to be insoluble at 10-3M (e.g. turbidity) in some laboratories during the pre-validation studies. When DBP was tested up to 10-3M as a coded chemical, it was classified as 'equivocal' due to displacement of (3H)17b-estradiol at highest in 3/5 laboratories using the CERI assay and 5/6 laboratories using the FW assay.

a: Norethindrone is provided as an alternate for the control weak binder for cases when norethynodrel is unavailable.

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 chemical is primarily dependent upon its metabolic activation. Enzymatic metabolic activation of the chemical would not be anticipated in the cell free hrER assays as used in this inter-validation study. Thus, the correct classification for this chemical is a 'non-binder' when used under the experimental conditions for the FW and CERI assays.

c: Chemicals were observed to be insoluble at 10-3M (e.g.,turbidity) in some laboratories during the validation study.

d: Optional where o,p'-DDT is prohibited by regulatory authorities when replaced by chemical with comparable binding affinity.

参考文献(2) Table 1 を一部改変

本 PBTG は、ER のリガンド結合部位と被験物質との特異的結合を検出するため、被験物質（競合物質）存在下での放射標識リガンド（[^{3}H]-17β-エストラジオール : [^{3}H]-E2）の ER への結合能を被験物質の濃度を上昇させながら測定する競合結合試験としてデザインされている。ER に対して高い親和性を有する被験物質は、受容体親和性の低い化学物質と比べて、より低濃度で放射標識リガンドと競合する。ただし本試験法で評価できるのはあくまでも化学物質の ER への結合活性であり化学物質のアゴニスト・アンタゴニスト活性を区別することはできない。

参照試験法として示されている試験法のうち FW アッセイは完全長 hrERα への結合における被験物質の E2 に対する競合結合能を測定する手法であり、試験にはバキュロウイルスに感染させた昆虫細胞で作製、単離した完全長 hrERα を用いる。FW アッセイに用いる hrERα は、市販（Thermo Fisher Scientific, USA, A15674）で入手可能である。一方、CERI アッセイは hrERα の LBD を用いた *in vitro* hrERα-LBD 競合結合試験法であり、グルタチオン-S-トランスフェラーゼ融合タンパク質として *E. coli* で発現させた hrERα-LBD を用いる。CERI アッセイで用いる hrERα-LBD タンパク質は CERI によって作製されたものであり、入手可能である。CERI アッセイは FW アッセイと同様、hrERα へ結合する物質をスクリーニングする手法であり、hrERα-LBD への結合における被験物質の E2 に対する競合結合能を測定する。それぞれの試験プロトコルは 2 つの要素から構成されており、一つは試験に用いる組み換え受容体タンパク質と生理的リガンドである E2 との相互作用活性を明らかにする飽和結合試験であり、他の一つは、被験物質の ER への特異的結合性を評価するため放射標識リガンド（[^{3}H]-E2）と被験物質との ER 結合への競合作用を確認する競合結合試験である。いずれのアッセイ法とも、定量的評価結果として、IC50（50% の [^{3}H]-E2 を hrERα から解離させるのに必要な被験物質の濃度）、hrERα に関する被験物質の E2 に対する相対結合親和性などを求めることが可能である。化学物質のスクリーニングのための定性的評価結果として、結合曲線に関して設定された基準に従い被験物質を hrERα 結合物質、非結合物質、または判定不能に分類することが可能である。

これらの参照試験法として、FW アッセイについては、Freyberger と Wilson により作成されたプロトコルに基づいて、CERI アッセイについては CERI により作成されたプロトコルに基づいて、米国 EPA の主導により日米欧 6 施設の参加（CERI アッセイについてはうち 5 施設が参加）による国際バリデーション研究[1)] が実施され、試験法の使用目的に対する妥当性と信頼性が証明されている。バリデーション研究では、サブタスク 0 からサブタスク 4 まで、段階的に実施された。サブタスク 0 においては、飽和結合試験によりアッセイに用いるヒトエストロゲンアルファ受容体（hERα）タンパク質のリガンド結合能が試験実施性能の許容範囲を満たすことを全ての参加施設で確認した後、サブタスク 1 では、陽性・陰性対照の 3 物質（陽性 2、陰性 1 ）の測定、サブタスク 2 では陽性・陰性対照の 3 物質と非コード化 9 物質（陽性 8、陰性 1 ）、サブタスク 3 では陽性・陰性対照を含むコード化 14 物質（陽性 9、陰性 5 ）を被験物質として施設内および施設間再現性の評価が行われた。1 試験施設では、誤判定および不定判定、承認基準を満たせないケースが多く認められたが、施設特有の問題であると判断され、他の参加施設の測定結果の再現性は良好であった。ラット子宮のサイトゾルを使用した ER 結合試験（TG OPPTS 890.1250 : ER-RUC）結果が入手可能かつ ER 反応性が明確な 22 物質（陽性17、陰性 5 ）を用いた解析

の結果、CERI および FW アッセイの「判定不能」を除く分類結果は100%一致しており、各試験法での陽性物質（ER に対して結合性をもつことが予測される物質）の一致率は、CERI および FW アッセイは共に100%（16/16）、ER-RUC アッセイは94.1%（16/17）であった。一方、陰性物質（ER に対して結合性を示さないと予測される物質）については、CERI および FW アッセイは共に 5 物質全てを陰性と判定した。一方、ER-RUC アッセイの判定結果の内訳は陰性 1、偽陽性 1、評価不能 3 であった。最終的に、「判定不能」を除く各々の試験法の正確度は、CERI および FW アッセイは共に100%（21/21）、一方、ER-RUC アッセイは94.4%（17/18）であった。なお、比較に用いた物質の予想される結果の妥当性は、OECD VMG-NA のメンバーのうち本バリデーション研究に直接関わっていない 3 名から構成された CAB により確認された。

また、バリデーション研究開始時点のプロトコルで指定されていた弱結合陽性対照のノルエチノドレルおよび陰性対照（非結合性）のフタル酸ジ-n-ブチル（DBP）は、溶解性に問題があったため、追加のサブタスク 4 として 2 施設の参加によりコード化 7 物質を被験物質とした測定が実施され、弱結合性を示す陽性対照としてノルエチンドロンが、陰性対照としてオクチルトリエトキシシラン（OTES）が適切であることが示され、最終的なプロトコルでは、対照物質（基準エストロゲン E2、CAS 50-28-2）、弱結合物質（ノルエチノドレルやノルエチンドロンなど）、非結合物質（OTES）を用いて競合結合試験を実施することとされている。

なお、いずれの試験法とも放射性リガンドを用いるため、試験施設は放射性物質取扱に関する国の認可が必要であり、放射性同位元素および有害化学物質を扱う工程についてはすべて、国の法令で定められた規則および手順に従う必要がある。

2. 試験法の妥当性

2-1. 試験法の概略

2-1-1. 目的と原理

本試験法は内分泌かく乱物質のうち hrERα に作用（結合）する化学物質を検索し、その人体および自然界に対する有害影響を避けることを目的として開発された。本法は、hrERα に結合し、アゴニスト作用あるいはアンタゴニスト作用を示す化学物質のスクリーニング試験法であり、濃度を変えた試験物質（競合物質）の存在下において放射性同位元素［^{3}H］で標識した内因性のリガンド（E2）と hrERα との結合能を測定し、被験物質の hrERα に対する結合親和性を推測するものである。

2-1-2. 標準測定条件

結合試験は受容体-リガンド相互作用パラメーターを特徴付ける飽和結合試験および被験物質と標識リガンドとの間の競合によって結合親和性を推定する競合結合試験の 2 つの主な要素からなる。

飽和結合試験は、競合結合試験に用いる hrERα の結合親和性（Kd）および活性受容体部位数（Bmax）を特徴付ける目的で実施される。CERI および FW アッセイプロトコルに記載されている飽和結合実験では、それぞれ規定の過剰濃度の非標識リガンド（E2）の存在下および非存在下で、8 濃度の標識リガンドの結合量を評価する。活性結合部位を高濃度

の標識リガンドで飽和させた全結合および非特異的結合の両方を測定し、全結合から非特異的結合を差し引くことによって特異的結合量を計算する。Kd および Bmax は、非線形回帰分析とこれに続く Scatchard プロットにより求められる。

競合結合実験では、FW アッセイでは 1 nM の標識リガンドが20±5%、CERI アッセイでは 0.5 nM の標識リガンドが40±10%の特異的結合を示す量の hrERα を用いて異なる濃度の被験物質の存在下で標識リガンドの結合量を測定する。親和性は平衡状態で標識リガンドの特異的結合を50%阻害する化学物質の濃度（IC50）によって定量評価される。したがって、被験物質の結合曲線の特性が十分に示されるよう、Logistic 式における「頂点（TOP）」と「底値（BOTTOM）」を含まれるよう最終的な結果は LogIC50 の両側に十分な濃度範囲を確保する必要がある。

また、対照物質（基準エストロゲン E2）、弱結合物質（ノルエチノドレルまたはノルエチンドロン）、非結合物質（原則 OTES であるが、DBP を用いることもできる）の結合親和性を測定し、試験法の当該条件下での有効性および実験間での比較の指標とする。

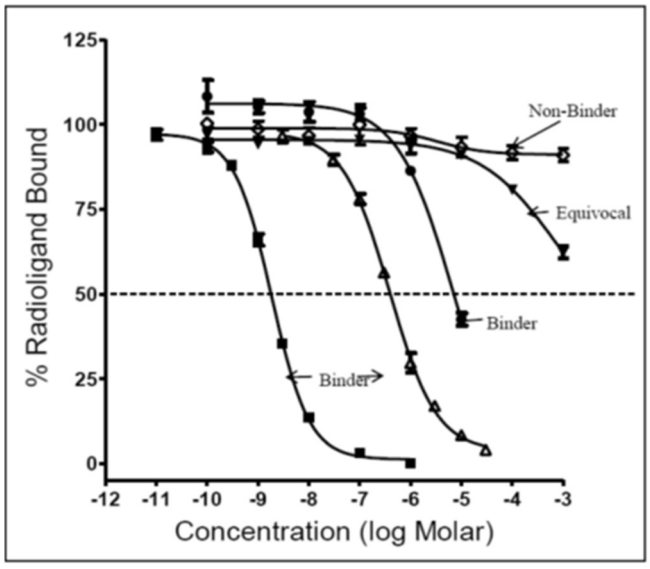


図2-1　競合結合試験における評価例

2-2. 生物学的妥当性

ER の内因性のリガンドである E2 の ER との結合は標的遺伝子の転写を活性化し、最終的には生理学的作用をもたらす。したがって、ER とリガンドの結合は一連の生物応答の起点であり、この試験法は生体系におけるエストロゲンシグナル伝達に必要な最初の分子的現象を反映している。また、不適切な ER シグナル伝達は、ホルモン依存性がん発症リスクの増加、生殖能力の低下および胎児の成長および発達の変化などの影響をもたらすことも報告されている[4)]。

検証試験の競合結合試験から得られた結果は、既存の論文、ER 結合および試験方法に関する総説等[5-10)] を基にまとめられた予想される結果と比較され、その妥当性が確認された。

ER 結合試験は、内因性エストロゲンと競合して ER に競合する可能性のある化学物質

を迅速に同定するための、有用かつ単純なツールであることが歴史的に証明されており、その実用性および *in vivo* の反応と密接に関連することが科学的資料に記載されている[11, 12]。

2-3. ER 結合試験法の注意点

結合試験に共通の注意点として、以下が挙げられる。

放射標識リガンドを使用することから、放射性同位体の取り扱いに十分な知識を有した熟練した作業者によって実施される必要がある。

結合試験の結果は被験物質の *in vitro* 系における受容体への結合に関するものであり、*in vivo* での内分泌システムへの影響に直接外挿することはできず、アゴニストとアンタゴニストの区別もできない。また、界面活性剤やアッセイ緩衝液の pH を変化させる物質では正しい結果を得ることができない可能性がある。

3. バリデーション研究に用いた物質の分類と結果の妥当性

両アッセイともに、飽和結合試験（サブタスク 0）では [^{3}H]-E2 について、競合結合試験（サブタスク 1 ～ 3）では陽性対照（ER 結合高親和性化学物質：E2、弱結合：ノルエチノドレル）および陰性対照（非結合 : DBP）を含むのべ 29 物質（表 3-1 および表 3-2 参照）について試験が実施された。また、適切な弱結合および非結合対照物質の探索のため、オプションとして 7 物質（表 3-1 および表 3-2 参照）が追加され競合試験（サブタスク 4）が実施された。

表3-1バリデーション研究に用いた物質数

	競合結合試験			
	サブタスク1	サブタスク2	サブタスク3	サブタスク4（オプション）
物質数	対照物質 3 物質	対照物質 3 物質＋ 9 物質	14 物質（対照物質 3 物質含む）	7 物質
コード化[1]/非コード化[2]の区別	非コード化	非コード化	コード化	コード化
陽性物質[3]数	2 物質	10 物質	9 物質	4 物質
陰性物質[4]数	1 物質	2 物質	5 物質	3 物質

[1] 試験に際し物質の本体が何であるかわからないように、また結果を予測できないようにするためにコードを付して試験施設に配付される被験物質。コード化には、試験を実施するに当たり、意図的、非意図的に結果を偏らせることを防ぐ役割がある。

[2] 試験に際し物質名が明記された状態で試験施設に配付される被験物質。

[3] ERに対して強い又は弱い結合性をもつことが予測される物質。

[4] ERに対して結合性を示さないと予測される物質。

表 3-2　バリデーション研究に用いた物質名

Subtask 1 (controls, uncoded)			
Test chemical	CAS #d	Expected binding affinity †	Expected Classification FW and CERI assays
17β-Estradiol	50-28-2	Strong	Binder
Norethynodrel	68-23-5	Moderate	Binder
Di-n-butyl phthalate (DBP)	84-74-2	Negative	Non-binder
Subtask 2 (uncoded chemicals)			
*17β-Estradiol	50-28-2	Strong	Binder
*Norethynodrel	68-23-5	Moderate	Binder
* Di-n-butyl phthalate (DBP)	84-74-2	Negative	Non-binder
Diethylstilbestrol (DES)	56-53-1	Very strong	Binder
17α-ethynyl estradiol	57-63-6	Very strong	Binder
Meso-Hexestrol	84-16-2	Strong	Binder
Genistein	446-72-0	Moderate	Binder
Equol	531-95-3	Moderate	Binder
Butyl paraben (n-butyl-4-hydroxybenzoate)	94-26-8	Weak	Binder
Nonylphenol (mixture)	84852-15-3	Weak	Binder
o,p'-DDT[a]	789-02-6	Weak	Binder
Corticosterone	50-22-6	Negative	Non-binder
Subtask 3 (coded chemicals)			
*17β-Estradiol	50-28-2	Strong	Binder
*Norethynodrel	68-23-5	Moderate	Binder
* Di-n-butyl phthalate (DBP)	84-74-2	Negative	Non-binder
Zearalenone	17924-92-4	Strong	Binder
Tamoxifen	10540-29-1	Strong	Binder
5α-dihydrotestosterone[b]	521-18-6	Weak	Binder
Bisphenol A	80-05-7	Weak	Binder
4-n-heptylphenol	1987-50-4	Weak	Binder
Kepone (Chlordecone)	143-50-0	Weak	Binder
Benz(a)anthracene	56-55-3	Weak	Non-binder1
Enterolactone	78473-71-9	Weak	Binder
Progesterone	57-83-0	Negative	Non-binder
Octyltriethoxysilane	2943-75-1	Negative	Non-binder
Atrazine	1912-24-9	Negative	Non-binder
Subtask 4*, ‡ (optional, coded chemicals)			
Bisphenol A	80-05-7	Weak	Binder
Genistein	446-72-0	Weak	Binder
HPTE[c]	2971-36-0	Weak	Binder
Norethindrone	68-22-4	Weak	Binder
Corticosterone	50-22-6	Negative	Non-binder
Dexamethasone	50-02-2	Negative	Non-binder
Octyltriethoxysilane	2943-75-1	Negative	Non-binder

*Controls (reference estrogen (17 β-estradiol, weak binder (norethynodrel), non-binder (DBP)) were used in each run, uncoded, for subtasks 1, 2, 3 and 4.
^Controls were also submitted to the laboratories as “coded test chemicals” (subtask 3) and were tested up to 1mM.
†Expected ER binding affinity as defined by the CAB for subtasks 1, 2 and 3 (Appendix D).
‡The CAB did not review the chemicals used in subtask 4. Chemicals were approved by the SMT based upon strong historical documentation in published literature.
[1] Benz(a)anthracene was reclassified as a non-binder based on additional information regarding its in vitro ER binding activity that became available after the review of the chemical list by the CAB. (See footnote 3 for additional information and references).
[a]o,p’-DDT: 1,1,1,-trichloro-2-[o-chlorophenyl]-2-[p-chlorophenyl]ethane
[b]5α-dihydrotestosterone: 5α-DHT or 5α-Androstan-17β-ol-3-one
19-Norethindrone (Norethindrone)
[c]HPTE: 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane, a metabolite of methoxychlor
[d]CAS: Chemical Abstracts Service Registry number.

競合試験（サブタスク 1 ～ 3 ）で用いられた 29 物質のうち、21 物質が陽性、8 物質が陰性物質であり、オプションの競合試験（サブタスク 4 ）では、4 物質が陽性、3 物質が陰性物質である。陽性物質には強結合性を有する物質とともに弱結合物質も含まれている。

これらバリデーション研究に用いられた物質には、多様な用途の化学物質から代表的な ER 強結合性、弱結合および非結合物質が含まれていることから選択は妥当と判断される。

なお、競合試験（サブタスク 1 ～ 3 ）に用いた物質の妥当性は、OECD VMG-NA のメンバーのうち本バリデーション研究に直接関わっていない 3 名から構成された CAB により確認された（Appendix 1 参照）。JaCVAM 受容体結合試験資料編纂委員会は、CAB の判断はいずれも妥当であり、バリデーションに用いられた化合物セットは ER 結合アッセイの評価に十分なケミカルスペースを確保出来ており、バリデーションは適切に実施されたと評価した。

4. 試験法のデータと結果の有用性

バリデーションでは競合試験（サブタスク 1 ～ 3 ）において 29 物質（陽性 21 物質、陰性 8 物質）の測定が実施され、さらに、オプションとして実施されたサブタスク 4 において、陽性 4 物質、陰性 3 物質の測定が実施された。

両アッセイのバリデーション研究に用いられた化学物質は、既知見情報や入手の容易さ等をもとに可能な限り広範な用途・構造の物質が選択されており、それら陽性物質には強結合性を有する物質とともに弱結合物質も含まれており、本バリデーション研究で得られた評価結果は各アッセイ系の特性を示す有用な情報である。

また、両アッセイのバリデーション試験で評価が行われた化学物質の既知見情報から想定される結合活性および構造による分類（ステロイド類、ベンゼン環を一つ有する化合物類等）については、TG493 の習熟度確認物質リスト（表 1-1）として試験濃度域および各アッセイでの評価結果とともに示されており、これらの情報は新たな関連試験系開発の参照物質として有用である。

5. 試験方法の再現性

CERI アッセイには 5 施設（A－E）が、FW アッセイには CERI アッセイに参加した 5 施設と施設 F を含めた 6 施設が、バリデーション研究に参加した。

（A : University of Missouri-Columbia、B : Lovelace Respiratory Research Institute、C： CERI、D：Bayer HealthCare, Bayer Pharma AG、E：CeeTox、F：University of Konstanz）

5-1. 飽和結合試験（サブタスク 0）

8 濃度段階の [^{3}H]-E2 の hrERα に対する全結合量、非特異的結合量および特異的結合量を測定し、測定結果をもとに再現性が評価された。両アッセイの全ての測定（CERI アッセイ：17、FW アッセイ：20）で、ER の標識リガンドによる飽和と強い結合性を有する単一のリガンド結合部位を有することが確認された。両アッセイ共に、全施設にて妥当な Kd 値が報告された（CERI アッセイ : 各施設平均=0.244～0.758 nM、全体平均±SD=0.451±0.16 nM ; FWアッセイ：各施設平均=0.230～0.707 nM、全体平均=0.423±0.20 nM）。特定の施設（FWアッセイ：施設 F）への輸送過程における受容体サンプルの分解が原因とされる場合を除き、殆どの施設が競合結合試験に必要なアッセイ条件を担保する活性受容体濃度を得た。CERI アッセイの場合、推奨される承認基準は（1）標識リガンド0.5 nM 存在下の全特異的結合量は40±10%、（2）標識リガンド 4.0 nM 使用時の非特異的結合量は全結合の35%未満であるのに対して、参加 5 施設の平均値±SDはそれぞれ33±5.4%、46±7.6%であった。FWアッセイの場合、推奨される承認基準は（1）標識リガンド1.0 nM 存在下の全特異的結合量は20±10%、（2）標識リガンド3.0 nM 使用時の非特異的結合量は全結合の35%未満であるのに対して、参加 6 施設の平均値±SDはそれぞれ20±7.7%、21±10.2%であった。従って、両アッセイで、共に参加施設の実施性能は概ね許容範囲内であった（表 5-1、5-2、5-3、5-4 参照）。

表 5-1　CERI アッセイ、hrER α への標識リガンド(0.5 nM)の特異的結合の割合

	CeeTox (%)	Lovelace (%)	Missouri (%)	Bayer (%)	CERI (%)
Run 1	21	27	23	40	45
Run 2	15	33	43	19	39
Run 3	21	27	33	34	43
Run 4	49		50		
Mean ± SD (n)	27 ± 15.3 (4)	29 ± 3.5 (3)	38 ± 12.2 (4)	31 ± 10.8 (3)	42 ± 3.1 (3)

参考文献(1) Table 14 を引用。

表 5-2　CERI アッセイ、hrER α への標識リガンド(4 nM)の非特異的結合の割合

	CeeTox (%)	Lovelace (%)	Missouri (%)	Bayer (%)	CERI (%)
Run 1	60	64	65	35	50
Run 2	50	53	25	42	48
Run 3	60	51	34	35	47
Run 4	22		37		
Mean ± SD (n)	48 ± 18.0 (4)	56 ± 7.6 (3)	40 ± 17.3 (4)	37 ± 4.0 (3)	48 ±1.5 (3)

参考文献(1) Table 15 を引用。

表 5-3　FW アッセイ、hrER α への標識リガンド(1.0 nM)の特異的結合の割合

	CeeTox (%)	Lovelace (%)	Missouri (%)	Bayer (%)	CERI (%)	Konstanz (%)
Run 1	27	33	22	23	22	6[a]
Run 2	21	30	17	20	21	5[a]
Run 3	23	23	16	24	16	5[a]
Run 4			15			8[b]
Mean ±SD	24 ± 3.1	29 ± 5.1	18 ± 3.1	22 ± 2.1	20 ± 3.2	6 ±1.4

[a] 実施施設から受容体分解の可能性が報告された（U. Konstanz）。
[b] 新しいバッチの受容体を使用して測定を実施した（U. Konstanz）。
参考文献(1) Table 18 を引用。

表 5-4　FW アッセイ、hrER α への標識リガンド(3 nM)の非特異的結合の割合

	CeeTox (%)	Lovelace (%)	Missouri (%)	Bayer (%)	CERI (%)	Konstanz (%)
Run 1	12	8	7	31	21	35[a]
Run 2	12	9	9	32	24	45[a]
Run 3	14	11	21	26	28	41[a]
Run 4			17			16[b]
Mean ±SD (n)	13 ± 1.2 (3)	9 ± 1.5 (3)	14 ± 6.6 (4)	30 ± 3.2 (3)	24 ± 3.5 (3)	34 ± 12.8 (3)

[a] 実施施設から受容体分解の可能性が報告された（U. Konstanz）。
[b] 新しいバッチの受容体を使用して測定を実施した（U. Konstanz）。
参考文献(1) Table 19 を引用。

5-2. 競合結合試験（サブタスク 1 ～ 3 ）

段階的に技術的難易度が高まる一連のサブタスクでは、8 濃度段階の被験物質の存在下、単一濃度の [^{3}H]-E2 の hrERα に対する結合量を測定する競合アッセイの測定結果をもとに施設内および施設間での再現性の評価が行われた。

5-2-1. サブタスク 1

3 つの非コード化被験物質陽性対照（強結合性：E2、弱結合性：ノルエチノドレル）および陰性対照（非結合性：DBP）の測定結果をもとに再現性が評価された。結合競合曲線の「頂点」、「底値」および「傾き」を注視するとの助言以外のデータ分析に関する定量的な承認基準の指示は無く、各施設は、アッセイパフォーマンスの評価尺度（表 5-5 参照）に基づき「承認」し得ると判断した 3 回の測定データと共に、全測定データの提出を求められた。両アッセイ共に、承認基準を満たした場合はいずれの被験物質も正しく判定し、アッセイ間で各々の陽性対照の LogIC50 が良く一致していた。CERI アッセイについて、E2 とノルエチノドレルおよび DBP の許容測定数の割合（許容測定数／全測定数）は、それぞれ27/28、27/28および25/28であった。一方、FW アッセイについて、E2 は20/23、ノルエチノドレルは21/22、DBP は24/24であった。全施設の全体平均値 LogIC50（平均値±SD）と R^2 値（平均値±SD）は、CERI アッセイについて、E2 は-9.03±0.20、0.97±0.06（許容測定数：27）、ノルエチノドレルは -6.14±0.28、0.98±0.02（許容測定数：27）であった（表 5-6 参照）。また、施設毎の LogIC50 の SD 値は E2 とノルエチノドレルそれぞれ、最大で0.442

と0.259であった。一方、FW アッセイについて、E2 は -8.89±0.18、0.97±0.03（許容測定数：20）、ノルエチノドレルについては -6.25±0.34、0.98±0.02（許容測定数：21）であった（表 5-6 参照）。さらに、施設毎の LogIC50 の SD 値は E2 とノルエチノドレルそれぞれ、最大で0.255と0.401であった。なお、両アッセイの承認基準を満たさない測定結果の多くの場合においても、競合結合曲線の「頂点」が100%予測値と著しく乖離していたが、それらは明確な標識リガンドの ER 結合に対する濃度依存的な阻害を示し、適切な LogIC50 を得ていた。従って、両アッセイで、共に施設内と施設間それぞれにおける定性的および定量的な再現性が示された。

表 5-5　飽和結合試験および競合結合試験のアッセイパフォーマンスの評価尺度

Saturation Binding	
1.	Did the specific binding curve reach a plateau with increasing concentrations of [^{3}H]17β-estradiol)?
2.	Was the ratio of specific binding to total radioligand used in the assay within the limits recommended for the assay?
3.	Was non-specific binding within the limits as recommended for the assay?
4.	When data were analyzed using the recommended non-linear regression model with a correction for ligand depletion, was fit for the data linear when displayed using a Scatchard (Rosenthal) plot?
Competitive Binding	
1.	Did increasing concentrations of the reference estrogen (17β-estradiol) displace [^{3}H]17β-estradiol from the hrERα in a manner consistent with a one-site competitive binding model?
2.	Was the IC_{50} for the reference estrogen consistent across multiple runs?
3.	Was the ratio of specific binding to total radioligand used in the assay within the recommended range for the assay?
4.	Did the negative control (Di-n-butyl phthalate) displace less than 25% of the [^{3}H]17β- estradiol?
5.	Did comparison of solvent and buffer controls confirm that the solvent does not have any effect on ER binding?

参考文献(1) Table 9 を引用。

表 5-6　CERI アッセイと FW アッセイの比較、サブタスク 1（対照物質）

	CERI Assay Mean ± SD (n)	FW Assay Mean ± SD (n)
Reference estrogen (17β-estradiol)		
LogIC50	-9.03 ± 0.20 (27)	-8.88 ±0.18 (23)
R^2	0.97 ± 0.06 (27)	0.97 ± 0.03 (23)
Weak Binder (Norethynodrel)		
Log IC50	-6.14 ± 0.28 (27)	-6.22 ± 0.34 (24)
R^2	0.98 ± 0.02 (27)	0.98 ± 0.02 (24)
Non-binder Di-n-butyl phthalate[a]		
Non-binder	27/29 total runs[b]	24/24 total runs[b]

[a] 対照非結合物質として、最大 10^{-4}M まで試験した。
[b] 全測定回数に対する DBP を非結合物質と判定した測定回数を記す。
参考文献(1) Table 31 を引用。

5-2-2. サブタスク 2

ER 結合性が明確な 9 つの非コード化被験物質の測定結果をもとに再現性が評価された。測定データの分析は、(1) 陽性対照の曲線フィットパラメータに基づく許容判別と (2) 各試験施設の許容判定を共に満たす測定結果に限定され、測定毎に被験物質を分類判定した。なお、技術的検討を受け、「判定不能」に分類された被験物質に対して10%ルール‡を適用させた後§、再分析された測定結果を含む。両アッセイにおいて、共に 9 物質の各施設における分類結果を総合すると、結合物質と非結合物質を問わず予想結果と一致しており、両アッセイで、共に施設間における非コード化被験物質の定性的な再現性が示された(表 5-7、5-8 参照)。施設 B では、両アッセイ共に分類判定に不正確な点があったが、施設特有の問題と判断された。詳細な技術的検討の結果、分類判定の不正確さの要因として、(1) 10%ルールの不適切な適用、(2) 4 パラメータロジスティック回帰分析へのデータの不適合性、③最適数に満たない許容可能な測定結果を基にした分類の限界、などが挙げられ、これらの理由から、最終的な分類前に段階的な分析アプローチの導入が推奨された。

表 5-7　CERI アッセイ、サブタスク 2（非コード化被験物質の分類判定）

Chemical	*Expected Result*	CeeTox	Lovelace	Missouri	Bayer	CERI	Simple Average
Butyl paraben	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*
Corticosterone	*Non-Binder*	Non-Binder	Equivocal	Non-Binder	Non-Binder	Non-Binder	*Non-Binder*
o, p'-DDT	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*
Diethylstilbestrol	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*
Ethynyl estradiol	*Binder*	Binder	Equivocal	Binder	Binder	Binder	*Binder*
Equol	*Binder*	Binder	Equivocal	Binder	Binder	Binder	*Binder*
Genistein	*Binder*	Binder	Equivocal	Binder	Binder	Binder	*Binder*
Meso-Hexestrol	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*
Nonylphenol	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*

緑色背景は、予想される結果との不一致を示す。
参考文献(1) Table 32 を引用。

‡ 10%ルール：[^{3}H]-17β-エストラジオールの特異的結合の割合について、反復試験の平均値が低濃度で認められた平均値よりも 10%以上大きい場合、解析のデータポイントから除外する選択肢。

表 5-8　FW アッセイ、サブタスク 2（非コード化被験物質の分類判定）

Chemical	*Expected Result*	CeeTox	Lovelace	Missouri	Bayer	CERI	Konstanz	*Simple Average*
Butyl paraben	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Corticosterone	*Non-Binder*	Non-Binder	Binder	Non-Binder	Non-Binder	Non- Binder	Non-binder	*Non-Binder*
o, p'-DDT[b]	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Diethylstibesterol	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Ethynyl estradiol	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Equol	*Binder*	Binder	§	Binder	Binder	Binder	Binder	*Binder*
Genistein	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Meso-Hexestrol	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Nonylphenol	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*

緑色背景は、予想される結果との不一致を示す。
§ 許容基準を満たす測定結果の提出なし。
[b]o,p'-DDT: 1,1,1,-trichloro-2-[o-chlorophenyl]-2-[p-chlorophenyl]ethan
参考文献(1) Table 33 を引用。

5-2-3. サブタスク3

ER 結合性が明確な14のコード化被験物質の測定結果をもとに施設内および施設間再現性の評価が行われた。データ分析と分類判定はサブタスク 2 と同様に実施した。全体として、両アッセイにおいて、共に14 物質の各施設における分類結果の単純平均は、結合物質と非結合物質を問わず予想結果と良く一致しており（CERI アッセイ：12 物質で一致、FW アッセイ：12 物質で一致）、両アッセイで、共に施設間におけるコード化被験物質の良好な定性的な再現性が示された（表 5-9、5-10 参照）。なお、これらの単純平均は、測定回数を考慮した加重平均と一致していた。特記すべき点として、両アッセイにおいてコード化被験物質として評価した場合、DBP（陰性）は「判定不能 : equivocal」と分類され、各施設で出された分類判定の総合結果は予測結果と一致しなかった。この要因として、(1) 反応液中の溶解度限界と (2) 最大溶解濃度での ER に対する結合性が、依然として不確定であることが挙げられた。ヘプチルフェノール（弱結合性）は CERI アッセイにおいて分類判定の総合結果は「判定不能」と分類されたが、非常に弱い陽性物質では、十分な高濃度域で評価ができず、完全な競合結合曲線が得られないため正しく分類できない場合が指摘された。なお、本物質は FW アッセイでは当初、各施設における分類結果の単純平均に基づいて「結合物質 : Binder」と判定されていたが、CERI アッセイと同様に十分な高濃度域で評価ができず、完全な競合結合曲線が得られないため、「判定不能 : equivocal」に変更された。また、E2 の様に非常に強い陽性物質では、完全な結合曲線を得るためにより低濃度の結果が含まれるべきであると指摘された。これらの結果を受けて、分類判定の不正確さについてサブタスク 2 と同様の要因が挙げられた他、最終的な分類前に段階的な分析アプローチの導入が強く推奨された。また、施設 B では誤判定および不定判定、承認基準を満たせないケースが多く認められたが、施設特有の問題であると判断された。

表 5-9　CERI アッセイ、サブタスク 3（コード化被験物質の分類判定）

Chemical	*Expected Result*	CeeTox	Lovelace	Missouri	Bayer	CERI	*Simple Average*
Atrazine	*Non-Binder*	Non-Binder	Equivocal	Non-Binder	Non-Binder	Non-Binder	*Non-Binder*
Bisphenol A	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*
Benz(a)anthracene	*Non-Binder*	Non-Binder	Non-binder	Non-Binder	Non-Binder	Non-Binder	*Non-Binder*
Dibutyl phthalate-TC	*Non-Binder*	Binder	Equivocal	Non-Binder	Binder	Non-Binder	*Equivocal*
Dihydrotestosterone	*Binder*	Binder	Binder	Equivocal	Binder	Binder	*Binder*
[a]17B-estradiol-TC	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*
Enterolactone	*Binder*	Binder	Binder	Equivocal	Binder	Equivocal	*Binder*
Heptylphenol	*Binder*	Binder	Equivocal	Equivocal	Binder	Equivocal	*Equivocal*
Kepone	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*
[a]Norethynodrel-TC	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*
Octyltriethoxysilane	*Non-Binder*	Non-Binder	Non-Binder	Non-Binder	Non-Binder	Non-Binder	*Non-Binder*
Progesterone	*Non-Binder*	Non-Binder	Non-Binder	Non-Binder	Non-Binder	Non-Binder	*Non-Binder*
Tamoxifen	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*
Zearalenone	*Binder*	Binder	Binder	Binder	Binder	Binder	*Binder*

緑色背景は、予想される結果と不一致を示す。
[a] 対照物質をコード化被験物質として試験した。
参考文献(1) Table 34 を引用。

表 5-10　FW アッセイ、サブタスク 3（コード化被験物質の分類判定）

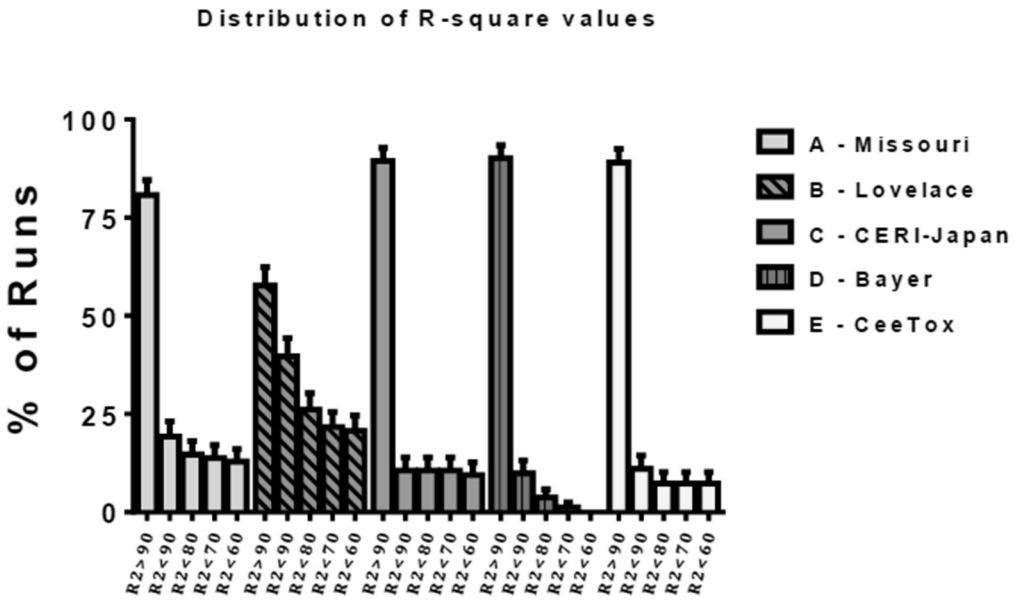
Chemical	*Expected Result*	CeeTox	Lovelace	Missouri	Bayer	CERI	Konstanz	*Simple Average*
Atrazine	*Non-Binder*	Non-Binder	§	Non-Binder	Non-Binder	Non-Binder	Equivocal	*Non-Binder*
Bisphenol A	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Benz(a)anthracene _DMSO	*Non-Binder*[b]	Equivocal	§	Non-Binder		Non-Binder		*Non-Binder*
Benz(a)anthracene _EtOH	*Non-Binder*[b]			Non- Binder	Non- Binder		Non- Binder	*Non- Binder*
Di-n-butylphthalate[†]	*Non-Binder*	Non-Binder	Non-Binder	Non-Binder	Non-Binder	Non-Binder	Non-Binder	*Non-Binder*
Di-n-butyl phthalate - TC[a]	*Non-Binder*	Equivocal	§	Equivocal	Equivocal	Equivocal	Equivocal	*Equivocal*
Dihydrotestosterone	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
17-β-estradiol-TC[a]	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Enterolactone	*Binder*	Binder	§	Binder	Binder	Binder	Binder	*Binder*
Heptylphenol	*Binder*	Equivocal	Binder	Non-Binder	Binder	Binder	Binder	*Equivocal*
Kepone	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Norethynodrel-TC[a]	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Octyltriethoxysilane	*Non-Binder*	Equivocal	§	Non-Binder	Non-Binder	Non-Binder	Non-Binder	*Non-Binder*
Progesterone	*Non-Binder*	Non-Binder	Binder	Non-Binder	Non-Binder	Non-Binder	Non-Binder	*Non-Binder*
Tamoxifen	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*
Zearalenone	*Binder*	Binder	Binder	Binder	Binder	Binder	Binder	*Binder*

緑色背景は、予想される結果と不一致を示す。
[a] 対照物質をコード化被験物質として、通常、溶解限界濃度または最大 10^{-3}M まで試験した。
[§] 許容基準を満たす測定結果の提出なし。
[†]DBP は陰性対照（非結合物質）として、最大 10^{-4}M まで試験した。
参考文献(1) Table 35 を引用。

5-2-4. R^2値に基づいた評価

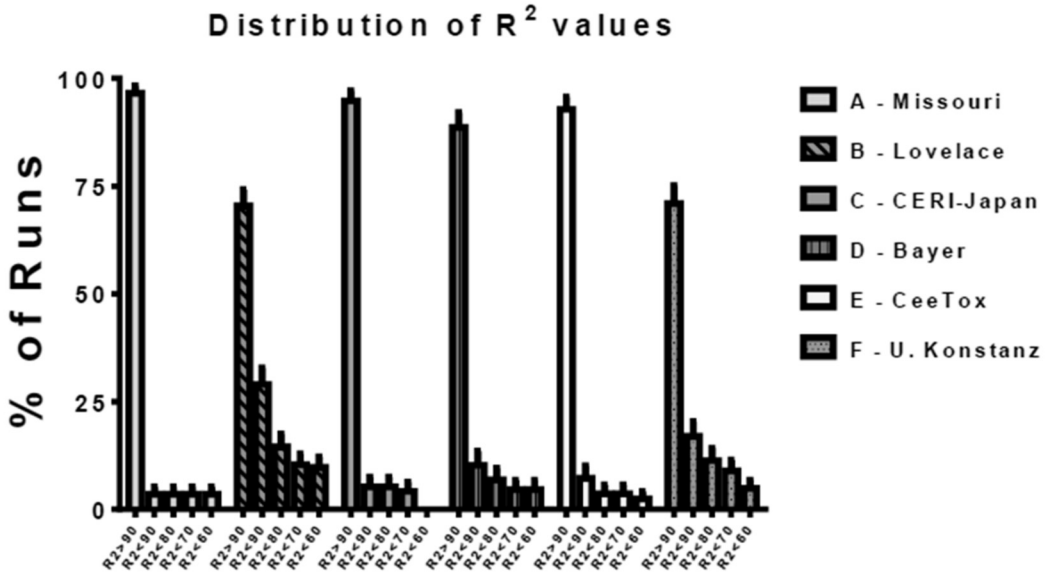
より包括的な施設内および施設間における再現性の評価を目的として、陽性対照のデータ許容の有無を問わず、実施施設が各プロトコルの承認基準に基づき除外したものを除く全ての測定データを適用させて、測定データのモデルへの適合の指標となる R^2 値を用いた評価が行われた。データ分析において、各測定の R^2 値が0.9（または90%）以上の場合を「良好」と判定した。

CERI アッセイについて、施設毎の全測定結果の R^2 値の分布を比較すると、施設 B を除く 4 施設では、8 割超が「良好」と判定され、平均値が各々95%以上、変動係数（CV）値は6.5%以下であった（図 5-1 参照）。施設 B では、「良好」は 6 割弱にとどまり、平均値と CV 値はそれぞれ91.8%と11.9%であった。施設毎の予測陽性物質の正判定率（R^2>0.9以上）の比較では、施設 A では80%以上、施設 C－E では90%超であったが、施設 B では～70%と他 4 施設と比べて偽陰性率が有意に高かった。また、施設 A および C では偽陽性判定は無く、他 3 施設の偽陽性率は約5－12%の範囲であった。特筆すべき点として、DBP を被験物質とした場合（10^{-3} M まで試験）が「偽陽性」の約半分を占めた。FW アッセイでは、4 施設において「良好」判定が高い割合を占めたが、施設 B と F では 7 割程度にとどまった（A：96.6%、B：70.3%、C：94.8%、D：88.6%、E：92.8%、F：71.0%）（図 5-2 参照）。施設毎の平均 R^2 値は、施設 B を除く 4 施設で95%以上、施設 B は93%であった。また、それらの施設毎の CV 値は、最低値0.8%（施設 A）、最大値9.4%（施設 B）、残り 4 施設では5.5～8.4%の範囲に収まった。施設毎の予測陽性物質の正判定率（R^2>0.9以上）を比較すると、4 施設では90%を超えた（偽陰性率、2－7%）一方、施設 B と施設 F では、それぞれ85%（偽陰性率、15%）、75%（偽陰性率、25%）と有意に低かった。また、施設 A および F では偽陽性判定は無く、他 4 施設の偽陽性も2.3－7.6%の範囲に収まった。従って、R^2 値に基づく分析の結果、一部の施設で技術的問題が見受けられたが、全体として、両アッセイでは、共にコード化を問わず、良好な定性的な再現性が示された。



参考文献(1) Figure 14(A)を抜粋引用。

図 5-1　CERI アッセイ、施設毎における全測定結果の R^2 値分布比較



参考文献(1) Figure 16(A)を抜粋引用。

図 5-2　FW アッセイ、施設毎における全測定結果の R^2 値分布比較

5-2-5. サブタスク4

両アッセイの実施に適用可能な弱結合性および非結合性対照物質の探索を目的として、2 施設（施設 A および E）において 7 物質（6 物質に各々 2 つのコード、および 1 物質に 4 つのコードを付した）が合計 16 の独立したコード化被験物質として提供され、それらの競合結合試験が実施された。両施設は共に、どちらのアッセイを使用した場合においても、各々コード化被験物質を正しく分類判定した。なお、幾つかの被験物質で溶解度の問題が認められたものの、弱結合性物質の分類判定には影響が無かった。サブタスク 4 の結果から、アッセイに最適な弱結合性物質としてノルエチンドロンが選択され、DBP の代替えとなる最も有望な陰性対照として OTES が同定された。これらの結果を受けて、両アッセイを使用する場合のより適切なデータの分析と解釈には、(1) 被験物質の溶解限界の決定とその分類判定への影響の確認と (2) 10%ルールが適用されるケースの適切な判別が重要であることが示された。

6.試験方法の正確性・信頼性

CERI および FW アッセイによる評価結果の信頼性の検証のため、各々の施設で実施された全被験物質の hrERα への結合能の評価を基にした最終分類結果を用いて、米国 EPA の現行 Tier I スクリーニング試験群に含まれるラット子宮のサイトゾルを使用した ER 結合試験（TG OPPTS 890.1250：ER-RUC）の結果が比較された（表 6-1 参照）。なお、比較には、3 種の試験法全てにおいて結合試験データが入手可能であった ER 結合性が明確な 22 物質（予測陽性：17、予測陰性：5）を用いた。「判定不能」を除き、3 アッセイの分類判定結果を比較したところ、それぞれ CERI および FW アッセイの結果は、予想された ER に対する結合親和性の強弱を問わず、全ての被験物質の分類において100%一致していた。各々の試験法の陽性物質の一致率は、CERI および FW アッセイは共に100%（16/16）、ER-RUC アッセイは94.1%（16/17）であった。陰性物質については、CERI および FW アッセイは共に 5 物質全てを陰性と判定した一方、ER-RUCアッセイの判定結果の内訳は陰性 1、偽陽性 1、評価不能 3 であった。また、「判定不能」を除く各々の試験法の正確度は、CERI および FW アッセイは共に100%（21/21）、ER-RUC アッセイは94.4%（17/18）であった。従って、CERI および FW アッセイ共に、米国 EPA の現行の TG OPPTS 890.1250と同等以上の性能標準を満たすことが示された。

表 6-1　FW アッセイ、CERI アッセイおよびテストガイドライン OPPTS 890. 1250 を用いた化学物質分類判定の性能比較

Chemical Name	CAS RN	Expected Response	FW Assay	CERI Assay	TG OPPTS 890.1250	MESH Chemical Class	Product Class
17β-Estradiol	50-28-2	*Binder*	Binder	Binder	Binder	Steroid	Pharmaceutical, Veterinary Agent
Norethynodrel	68-23-5	*Binder*	Binder	Binder	Binder	Steroid	Pharmaceutical, Veterinary Agent
*Di-n-butyl phthalate	84-74-2	*Non-binder*	Non-Binder*†	Non-Binder*†	Not Tested	Ester, Phthalic Acid	Plasticizer, Chemical Intermediate
DES	56-53-1	*Binder*	Binder	Binder	Binder	Hydrocarbon, (Cyclic)	Pharmaceutical, Veterinary Agent
17α-ethynylestradiol	57-63-6	*Binder*	Binder	Binder	Binder	Steroid	Pharmaceutical, Veterinary Agent
Meso-Hexestrol	84-16-2	*Binder*	Binder	Binder	Binder	Hydrocarbon, (Cyclic)	Pharmaceutical, Veterinary Agent
Genistein	446-72-0	*Binder*	Binder	Binder	Binder	Flavonoid, Heterocyclic Compound	Natural Product
Equol	531-95-3	*Binder*	Binder	Binder	Binder	Phytoestrogen Metabolite	
Butyl paraben (n butyl-4-hydroxybenzoate)	94-26-8	*Binder*	Binder	Binder	Binder	Paraben	
Nonylphenol (mixture)	84852-15-3	*Binder*	Binder	Binder	Binder	Alkylphenol, Intermediate Compund	
o,p'-DDT	789-02-6	*Binder*	Binder	Binder	Binder	Organochlorine	
Corticosterone	50-22-6	*Non-binder**	Non-binder	Non-Binder	Binder	Steroid	Natural Product
Zearalenone	17924-92-4	*Binder*	Binder	Binder	Binder	Resorcyclic Acid Lactone, Mycotoxin	
Tamoxifen	10540-29-1	*Binder*	Binder	Binder	Binder	Antiestrogen	Pharmaceutical, Veterinary Agent
5a-dihydrotestosterone	521-18-6	*Binder*	Binder	Binder	Equivocal	Steroid, Nonphenolic	
Bisphenol A	80-05-7	*Binder*	Binder	Binder	Binder	Phenol	Chemical Intermediate
4-n-heptylphenol	1987-50-4	*Binder*	Equivocal	Equivocal	Binder	Alkylphenol	
Kepone (Chlordecone)	143-50-0	*Binder*	Binder	Binder	Binder	Hydrocarbon, (Halogenated)	Pesticide
Benz(a)anthracene	56-55-3	*Non-Binder**	Non-Binder	Non-Binder	Equivocal	Aromatic Hydrocarbon	
Enterolactone	78473-71-9	*Binder*	Binder	Binder	Binder	Phytoestrogen	
Progesterone	57-83-0	*Non-binder**	Non-Binder	Non-Binder	Equivocal	Steroid	Natural Product
Octyltriethoxysilane	2943-75-1	*Non-binder*	Non-Binder	Non-Binder	Non-Binder	Silane	
Atrazine	1912-24-9	*Non-binder**	Non-Binder	Non-Binder	Equivocal	Triazine	Herbicide

濃いグレーの背景は、予想される結果と不一致を示す。
*†10^{-4}M まで試験した場合、非結合物質に分類された。10^{-3}M まで試験した場合、最大濃度で標識リガンドとの競合が検出されたことから「判定不能」に分類された（CERI アッセイ—5 施設中 3 施設、FW アッセイ—6 施設中 5 施設、表 5-8 および 5-9 参照）。
参考文献(1) Table 40 を改編引用。

7.データの質

本試験法のバリデーション研究における多施設間試験は、GLP（Good Laboratory Practice）に準拠した精神で試験責任者による科学的指導および管理下で実施されたものである。また、すべてのデータは取り纏めを行った米国 EPA に提出される前に、それぞれの試験施設において独立した品質保証担当者による査察が実施されており、データの質および信頼性は確保されていると考えられる。

8. 試験法の有用性、限界および提言

1) CERI および FW アッセイは原理の項でも述べた様に、放射性標識リガンド（[^{3}H]-E2）と被験物質との hrERα 結合への競合作用を確認することにより、化学物質と hrERα との直接的な結合を検出できる。しかし、被験物質がアゴニスト作用を及ぼすかアンタゴニスト作用を及ぼすかの判定はできない。特異性は極めて良好である。混合物への応用については検討されていない。
2) 本試験法では放射性リガンドを用いるため、試験施設は放射性物質取り扱いに関する認可を要する。放射性同位元素および有害化学物質を扱う工程についてはすべて、国の法令で定められた規則および手順に従う必要がある。
3) 標準的な品質管理を実施し、受容体の活性、化学物質濃度の正しさ、信頼限界が複数の反復試験を通じて安定な状態に保たれ、かつ試験期間中、期待される hrERα 結合反応を示す性能が保持されていることを確認することが必要である。
4) 新たに試験を実施する試験施設は、予め hrERα 標品の特異性と活性を確認する飽和試験および E2 と TG に示された習熟度確認物質（弱結合物質および非結合物質）を用いた競合結合試験を実施し、当該試験法を使用する習熟度のあることを証明する必要がある。さらに、異なる日に実施した 3 ～ 5 試験より得られた基準エストロゲン（E2）および対照物質の結果を含む履歴データベースを作成する必要がある。
5) 未知の化学物質を試験するには、予備試験を実施し、各被験物質の溶解限界を確認するとともに、試験実施に用いる適切な濃度範囲を特定する必要がある。各被験物質の溶解度はまず溶媒で決定し、さらに試験条件下で混濁や沈殿の有無を確認する。試験で用いる最高濃度は 1 mM を超えてはならない。
6) 望ましい溶媒として FW アッセイではエタノールが、CERI アッセイでは DMSO がそれぞれ示されているが、両アッセイともエタノール及び DMSO が使用可能である。各溶媒の試験ウエル内の最終濃度は、FW アッセイでは1.5%（最大 2 %を超えてはならない）、CERI アッセイでは、2.05%（最大2.5%を超えてはならない）とされている。
7) hrERα 結合試験（CERI および FW アッセイ）は、スクリーニングのための試験法であり、化学物質の安全性評価に用いる際には、他の *in vitro* および *in vivo* 評価系等との結果と併せて実際の生体影響について総合的に判断を行うべきである。

9.その他の試験方法の科学的な報告

内分泌かく乱に係ると考えられる ER に関わる試験法としては、被験物質の受容体への結合実験がまず考えられる。実際、本 PBTG（OECD TG493）で取り上げられている、hrER 結合親和性化学物質の検出のための、hrER *in vitro* 試験法は、二つの機序および機

能的に同等と評価されたhrERα結合物質確認試験法から成るが、いずれも非細胞系(cell-free)において、RI標識リガンドに対する被験物質の競合的な結合阻害により、hrERαとの結合能を調べる、というものである。結合実験では、化学物質との相互作用が容易となるように、受容体が水相に露出していることが望ましいが、この目的には細胞を破壊したcell-freeが有利であり、実際、この系を用いた結合実験が本TGにて提案されている。

一方、受容体と相互作用を示す化学物質には、受容体に結合して本来結合すべき生体内物質と同様の細胞内情報伝達系を作動する(活性化する)「アゴニスト」と、受容体に結合はするが本来結合すべき生体物質と異なり生体反応を起こさず、その結合によって本来結合すべき生体内物質と受容体の結合を阻害することで生体応答反応を起こさない「アンタゴニスト」があり、上述した結合実験のみでは、アゴニスト・アンタゴニスト両者の区別が困難である。さらに、非細胞系における結果は、細胞系の場合と一致しないという報告も多く、この理由として、細胞内への移行性による差異や細胞内に特有の結合を制御する因子の存在が示唆され、したがって、受容体との相互作用の検討には、細胞系を利用して調べることが望ましい。

この点、hERαの転写活性を指標とするレポーターアッセイでは、アゴニスト・アンタゴニスト作用の有無を判定できる。現時点でhERαのレポーターアッセイに関するOECD TGとしてOECD TG455が成立している。OECD TG455もまたPBTGであり、参照試験法としてER STTA法(hERα-HeLa-9903細胞を用いたER恒常発現系転写活性化試験法)とVM7LucER TA法(VM7Luc4E2細胞を用いるER転写活性化(TA)試験)の2種の方法が示されている。

両方法の相違は、(1)ER STTA法がhERαをHeLa細胞に組み込んだものであるのに対し、VM7LucER TA法ではVM7細胞に内在するhERを利用する、(2)ER STTA法では、組み込まれた受容体はhERαのみであるが、VM7細胞ではERαとERβの双方を発現している。ただし、2種のERアイソフォーム(hERα、hERβ)に対して完全な選択性を示す化学物質は、知られておらず、定性的評価において両測定系の結果は、同等と考えられる。(3)VM7LucER TA法のアンタゴニスト試験では、バリデーション研究において高濃度領域でのルシフェラーゼ活性の非特異的阻害が認められたことから、評価可能な被験物質の最大濃度は、20μg/mL(約10μM)に制限されているのに対して、ER STTA法では、媒体への溶解性が良好かつ細胞毒性が認められない場合、アゴニスト・アンタゴニスト試験とも最大1 mMまで評価可能である。

ER STTA法については、CERIが、安定的形質移入による転写活性化試験の妥当性確認研究を、ヒトの子宮頚癌に由来するhERα-HeLa-9903細胞株を用いて実施し、hERαを介してエストロゲンアゴニスト活性を検出する試みを行い、実施目的に関する試験の適合性および信頼性を証明している。この細胞株には、次の2種類を安定的に挿入されている:(i)hERα発現ベクターおよび(ii)マウスのメタロチオネイン(MT)遺伝子 のプロモーター部位であるTATAエレメントによって駆動される、アフリカツメガエルのビテロゲニン由来のエストロゲン応答エレメント(ERE)のタンデムリピートを5個含むホタル・ルシフェラーゼのレポーター遺伝子。

もう一方のVM7LucER TA法は、NICEATMおよびICCVAMによって妥当性の確認がなされてきた。ヒト卵巣腺癌細胞株VM7において安定的に形質移入したhER応答ルシ

フェラーゼ・レポーター遺伝子を用いて *in vitro* hER アゴニスト活性またはアンタゴニスト活性を有する物質に関する濃度反応データを提供する。具体的には、マウス乳癌ウイルス（MMTV）プロモーターの上流に位置する 4 つの ERE の制御の下でルシフェラーゼ・レポーター遺伝子を安定的に形質移入した ER 応答ヒト卵巣腺癌細胞株 VM7 を利用して、*in vitro* の ER アゴニストまたはアンタゴニスト活性を有する物質を検出する。アッセイには安定的に形質移入した VM7 細胞株を使用する。さらに PS を満たす類似（me too）試験法として、ERα CALUX アッセイ（Stably Transfected Human Estrogen Receptor-α Transactivation Assay for Detection of Estrogenic Agonist and Antagonist Activity of Chemicals using the ERα CALUX cell line）が収載されている。このアッセイは、BioDetection Systems BV（オランダ）によって妥当性の確認がなされてきた。CALUX とは、Chemical-Activated Luciferase Expression の略であり、ヒト骨肉腫細胞株 U-2 OS において安定的に形質移入した ERα CALUX 細胞株を使用し、3 つの ERE 制御による、hER 応答ルシフェラーゼ・レポーター遺伝子を用いて、*in vitro* hER アゴニスト活性またはアンタゴニスト活性を、ルシフェラーゼの活性（発光量）を測定することにより検出する。内因性のレポーター活性はほとんど検出されないと報告されている。

内分泌かく乱作用としては他にも、リガンド結合ポケット以外の部位での ERαとの相互作用や、エストロゲンシグナル伝達に関連する他の受容体である ERβ、Gタンパク質共役エストロゲン受容体およびその他の受容体あるいは、内分泌系内の酵素系との相互作用なども考慮する必要があるが、現時点でこうした点を考慮したバリデーション研究が終了している試験系はない。

なお、2012年に改訂された OECD CF は 5 つのレベル（level 1 ～ 5 ）から構成され、それぞれの異なったレベルの生物学的複雑性に対応している。上記のレポーターアッセイは、レベル 2 の「選択された内分泌機構/経路に関する情報をもたらす *in vitro* アッセイ（非哺乳類の方法）」に相当する。

10. 結論

ER 結合親和性化学物質の検出のための、hrER *in vitro* 試験法に関する PBTG は、*in vitro* で化学物質の ER に対する結合親和性を生体内リガンドであるエストラジオールとの競合結合により検出するスクリーニング試験法である。現在、本 PBTG には、2 種の試験法（CERI および FW アッセイ）が参照試験法として掲載されている。また、本 PBTG には、類似試験法の開発とバリデーション研究のための PS が設定されており、PS を満たすことが示された新規の試験法を追加することが可能である。

現在、本 PBTG に参照試験法として掲載されている 2 種の試験法についての科学的妥当性と規制試験法としての妥当性については、日米欧 6 施設の参加によるバリデーション研究において陽性 21 物質、陰性 8 物質を用いた評価が行われ、施設内・施設間再現性とも良好な結果が示されており、OECD の第三者評価では、それらバリデーション研究の結果をもとに本試験法の正確性・信頼性が評価され、OECD EDTA TF で提案された OECD CF のレベル 2 に該当する内分泌かく乱物質のスクリーニング評価に有用な試験法として OECD TG493 が成立した。

本試験法による評価の課題として、本試験法で評価できるのはあくまでも化学物質の

ER への結合活性であり化学物質のアゴニスト･アンタゴニスト活性を区別することは出来ず、本試験法で陽性と判定される化学物質が生体内でどのような影響を示すかについては評価出来ないというという点があげられる。本試験法はあくまでもスクリーニング法であり、ER を介して惹起されると想定される有害影響を確定評価する試験法と組み合わせて評価を行うことで今後の化学物質管理に大きく貢献すると考えられる。

11. 利益相反(COI)について

利益相反の観点から、CERI アッセイが適切に評価されたことを受容体結合資料編纂委員会は確認した。

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Appendix 1

2006 年 8 月 1 日付

化学品承認委員会（Chemical Approval Board, CAB）報告書の概要

CAB 委員名簿

Dr. Taisen Iguchi, Okazaki Institute for Integrative Bioscience, NIBB

Dr. William Kelce, Pozen Pharmaceuticals

Dr. Weida Tong, NCTR, US FDA

1996年、US Environmental Protection Agency (EPA)は、食品品質保護法 (Food Quality Protection Act: FQPA)により、内分泌かく乱物質スクリーニングプログラム(Endcrine Disruptor Screening Program: EDSP)の開発を義務付けられた。EDSP プログラムの一環として、EPA はエストロゲン受容体（Estrogen Receptor: ER）と相互作用する化学物質のスクリーニングに ER 結合アッセイを使用することを提案した。EPA は2000年に、代替法の妥当性確認に関する ICCVAM に対し、ER 結合アッセイに関する文献をレビューし、公表された文献のデータに基づいてアッセイの妥当性を確認するよう依頼した。Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) は、多くの ER 結合アッセイが文献で報告されているが、ER 供給源（組換え、組織、細胞株）は共通のものが使用されておらず、共通のプロトコールも使用されていないことから、どの ER 結合アッセイも検証するための情報が不十分であるとの結論に至った。ICCVAM は、2003年に「ラットまたはヒト組み換え ER（a および b サブタイプ）結合アッセイが、試験方法の標準化、事前検証、および検証のための最優先事項である」と勧告した（ICCVAM, 2003）。

これを受けて、2003 年 3 月に、米国 EPA は、Organisation for Economic Co-operation and Development (OECD)の非動物試験用バリデーション管理グループ（Validation Management Group for Non-Animal Tests: VMG-NA）が、組換え ER 結合アッセイの開発を主導し、調整することに合意した。この目的のために、2 種類のヒト組み換え ERα タンパク質 (human recombinant (hr) ERa) と、2 つの異なる結合試験のプロトコルが検証に用いられた。2 種類の hrERa タンパク質は、Pan Vera hrERa（PV-ER；バキュロウイルス発現全長 ERa 受容体）と CERI タンパク質（CERI-ER；大腸菌産生 ER リガンド結合ドメインに Glutathion S-Transferase (GST)融合タンパク質を連結したもの）である。2 つのプロトコルは、CERI アッセイ（日本 CERI が開発）と Freyberger-Wilson Assay（FW）アッセイ（Alexius Freyberger 氏が開発し、Vickie Wilson と Alexius Freyberger の両者によって最適化）である。FW アッセイは PV-ER を使用し、CERI プロトコールは CERI-ER を使用した。

これらの hrER 結合アッセイの使用を検証するための国際的な取り組みが、OECD を通じて行われた。バリデーションのステップには、標準化されたプロトコルの開発、他の検査機関研究室へのプロトコルの技術移転性の検証、プロトコルが施設内および施設間で、同一化学物質について再現性のある結果を出すことの証明、および化学物質のアッセイの信頼性の検証が含まれる。プロトコールの標準化に次いで、プロトコルの移転性と適用性を検証す

るために、施設間の検証で使用する一連の化学物質のセットを決めることが必要である。OECD の hrER 結合アッセイ検証グループが、結合アッセイにおける過去の使用実績、結合強度、化学構造の多様性に基づいて一連の化学物質を提案した。ER に対する親和性が知られている化学物質を、構造的に多様であるが ER 結合試験履歴のない化学物質よりも優先的に選択した。既知および報告されている結合親和性値と一致する結合活性に基づいている。OECD の hrER バリデーショングループは、化学品承認委員会（CAB）に対して、タスク I、II、III にノミネートされた化学物質をレビューし、修正を提案することを任務としている。

施設間試験は、両方のプロトコルとそれぞれの hrER タンパク質を用いて、3 段階のタスクで実施されることになった。施設間バリデーションに参加する施設には、FW アッセイと CERI アッセイの両方での経験がある 2 つの施設（ヨーロッパ 1、日本 1）と、結合アッセイの経験はあるが FW アッセイや CERI アッセイを特別に実行したことがない 3 つの施設（ヨーロッパ 1、米国 2）が含まれている。3 つのタスクについては、下記の通り。

タスク I： 試験実施施設は、標準化学物質と陰性化学物質を使用してアッセイを実施する能力を実証する。タスク I の化学物質は、高結合親和性の標準物質（17b-Estradiol）、中程度結合親和性の標準物質（Norethynodrel）、および陰性物質（Dibutyl phthalate）である。これら 3 物質は、参加施設の試験の試験能力が基準を満たすことを検証するために、すべての試験参加研究室で評価する。17 b -Estradiol は内因性リガンドであり、Norethynodrel は、中等度のエストロゲン作用物質であり、Dibutyl phthalate は、2 つのアッセイプロトコルの両方で陰性である。これらの 3 つの基準物質は、内部コントロールとして、タスク II および III のすべての実験で試験した（すなわち、それぞれの実験で、またはアッセイ実施日毎に）。

タスク II：タスク 2 は、以前に FW アッセイと CERI アッセイで試験され、 EU Reference Laboratory for alternatives to animal testing (EURL ECVAM)でレビューした 12 の物質を用いて、施設内および施設間で再現性のある結果を得るための参加施設の能力を試験する。これらの化学物質は、化学物質をコード化することなく試験した。このタスクでの試験物質の総数は、9 種類の化学物質（超強結合物質 DES, 17 b -Ethynylestradiol； 強結合物質 Meso-Hexestrol； 中結合物質 Genistein, Equal； 弱結合物質 Butyl paraben, n-butyl 4-hydrobezoate, Nonylphenol, o.p’-DDT；非結合物質 Corticosterone）に 3 つの標準物質(17 b -Estradiol, Norethynodrel, Dibutyl phthalate)を加えた 12 種類である。

タスク III：タスク 3 は、過去に他の結合アッセイプロトコル（ラット子宮サイトゾールまたは hrER のいずれか）で試験したことがあるが、まだ FW アッセイまたは CERI アッセイでは試験の経験がない施設の、9 物質を用いた試験で、施設内および施設間で再現性の高い実験結果を得るための参加施設の能力を調べるため、試験物質をコード化した。既知の結合親和性に対応した結合結果が得られると期待された。これらの化学物質は、期待される結

合親和性とその構造的多様性に基づいて選択された。リストには多数の弱結合物質や非結合の陰性の物質が含まれており、施設間での再現が最も困難である。

この提案に対して、CAB は、タスク I および II に指定された化学物質が適切であることに合意した。タスク I および II の化学物質はそれぞれのタスクで示した。

タスク III については、CAB は、特異度、感度、正確度の点でアッセイの性能をより良く評価するために、結合活性が弱い、または全くない化学物質に重きを置くことを提案した。具体的には、CAB は Estrone（強結合剤）を外し、Enterolactone と Benz(a)anthracene（弱結合剤）と Atrazine（非結合剤）を追加することを提案した。Enterolactone の追加は、化学物質リストの構造の多様性を高め、食品の腸内代謝物であるリグナンを追加することになる。Benz(a)anthracene は、既知の弱結合物質の追加であり、Atrazine は、ER を介さないエストロゲン活性化合物の追加となる。このことは、ER 結合がエストロゲン活性化合物の作用モードを区別するためのメカニズムツールとして使用できることを示すことになる。結果として、タスク III の化学物質は、標準の 3 物質に加えて、強結合物質（Zearalenone, Tamoxifen）、弱結合物質(5a-Dihydrotestosterone, Bisphenol A, 4-n-Heptylphenol, Kepone (Chlordecone), Benz(a)anthracene, Enterolactone)、非結合（陰性）物質(Progesterone, Octyltriethoxysilane, Atrazine)の 11 物質を試験物質として承認した。

CAB は、Equol（Apin 社より提供）がラセミ混合物であることを認め、承認した。天然の Equol は S(-)エナンチオマーであるが、両方のエナンチオマーは hrER に対して類似の結合親和性を有しており、そのため CAB はこれが被験物質選択上の障害になるとは考えていない。

OECD/OCDE 493

Adopted:
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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 (ERα).

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.

13. 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

14. 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

15. 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

16. 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

17. 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.

18. 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

19. 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

20. 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

21. 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).

22. 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

23. 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

24. 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

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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 parameters of the competitive binding curves for the control substances should be within the limits of the 95%confidence interval (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)	95% Confidence Intervals[b]	
				Lower Limit	Upper Limit
17β-estradiol	Top (%)	100.44	10.84 (67)	97.8	103.1
	Bottom (%)	0.29	1.25 (67)	-0.01	0.60
	Hill Slope	-1.06	0.20 (67)	-1.11	-1.02
	$LogIC_{50}$ (M)	-8.92[c]	0.18 (67)	-8.97	-8.88
Norethynodrel	Top (%)	99.42	8.90 (68)	97.27	101.60
	Bottom (%)	2.02	3.42 (68)	1.19	2.84
	Hill Slope	-1.01	0.38 (68)	-1.10	-0.92
	LogIC50 (M)	-6.39	0.27 (68)	-6.46	-6.33
Norethindrone[c]	Top (%)	96.14	8.44 (27)	92.80	99.48
	Bottom (%)	2.38	5.02 (27)	0.40	4.37
	Hill Slope	-1.41	0.32 (27)	-1.53	-1.28
	$LogIC_{50}$(M)	-5.73	0.27 (27)	-5.84	-5.62

[a]Mean (n) ± Standard Deviation (SD) were calculated using curve fit parameter estimates (4-parameter Hill Equation) for control runs conducted in four laboratories during the validation study (see Annex N of Reference 2).
[b] The 95% confidence intervals are provided as a guide for acceptability criteria.
[c] Testing of norethindrone was optional for Subtask 4 during validation study (see Reference 2, see Subtask 4). Thus, the mean ± SD (n) were calculated using curve fit estimates (4-parameter Hill equation) for control runs conducted in two laboratories.

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 + 1.0 µM E_2			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 + 1.0 µM E_2			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 (Annex N of Reference 2). 95% confidence intervals are provided for the mean (n) +/- SD for all control runs across the laboratories participating in the validation study. 95% confidence intervals were calculated for the curve fit parameters (i.e. top, bottom, Hillslope, logIC_{50}) for the reference estrogen and weak binders

and for the log_{10}RBA of the weak binders relative to the reference estrogen and are provided as performance criteria for the positive controls. Table 1 provides expected ranges for the curve fit parameters that can be used as performance criteria. 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:

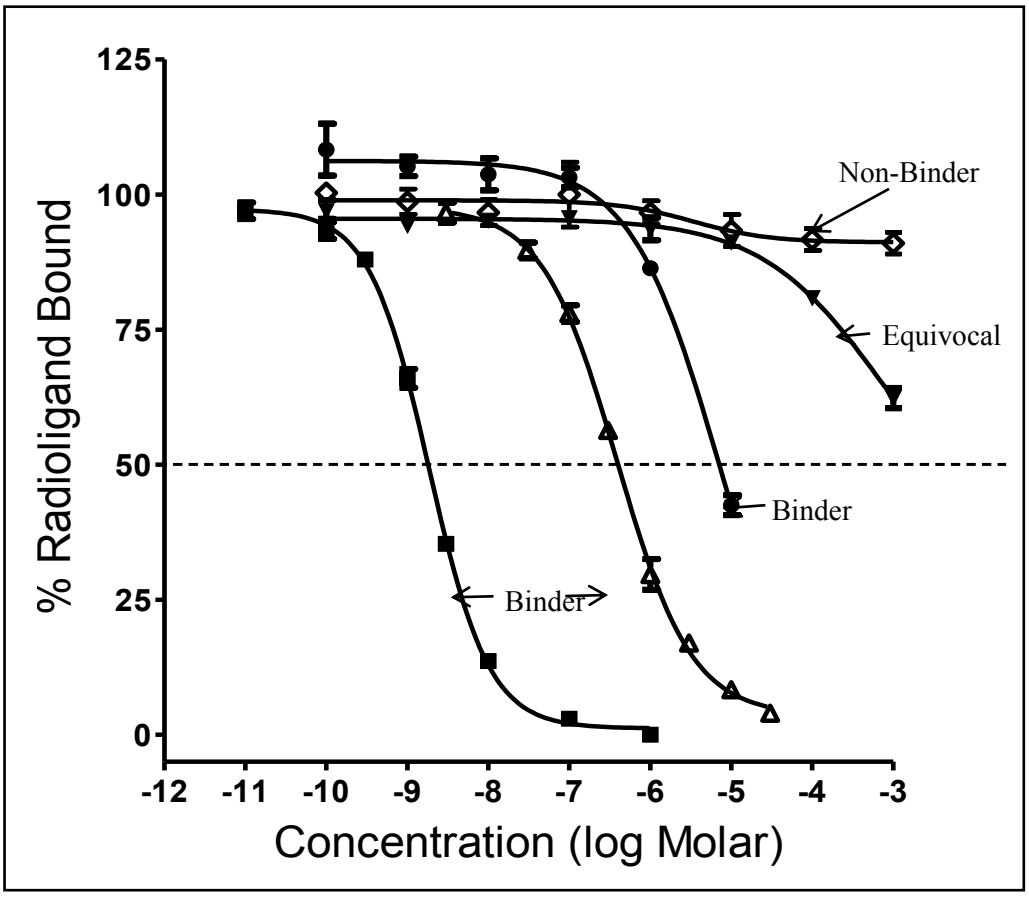
- A binding curve can be fit and the lowest point on the fitted response curve within the range of the data is above 75%, or
- A binding curve cannot be fit and the lowest unsmoothed average percent binding among the concentration groups in the data is above 75%.

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]E2: 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	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	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
S	G4	1	OTES	N	OTES6	2.00E-08	40	-	40	80	160	1.0E-08
S	G5	2	OTES	N	OTES6	2.00E-08	40	-	40	80	160	1.0E-08
S	G6	3	OTES	N	OTES6	2.00E-08	40	-	40	80	160	1.0E-08
S	G7	1	OTES	N	OTES7	2.00E-09	40	-	40	80	160	1.0E-09
S	G8	2	OTES	N	OTES7	2.00E-09	40	-	40	80	160	1.0E-09
S	G9	3	OTES	N	OTES7	2.00E-09	40	-	40	80	160	1.0E-09
S	G10	1	OTES	N	OTES8	2.00E-10	40	-	40	80	160	1.0E-10
S	G11	2	OTES	N	OTES8	2.00E-10	40	-	40	80	160	1.0E-10
S	G12	3	OTES	N	OTES8	2.00E-10	40	-	40	80	160	1.0E-10
S	H1	1	hot	H	H1	-	-	-	40	-	40	-
S	H2	2	hot	H	H2	-	-	-	40	-	40	-
S	H3	3	hot	H	H3	-	-	-	40	-	40	-
S	H4	1	hot	H	H4	-	-	-	40	-	40	-
S	H5	2	hot	H	H5	-	-	-	40	-	40	-
S	H6	3	hot	H	H6	-	-	-	40	-	40	-
S	H7	1	buffer control	BC	BC1	-	40	80	40	-	160	-
S	H8	2	buffer control	BC	BC2	-	40	80	40	-	160	-
S	H9	3	buffer control	BC	BC3	-	40	80	40	-	160	-
S	H10	1	buffer control	BC	BC4	-	40	80	40	-	160	-
S	H11	2	buffer control	BC	BC5	-	40	80	40	-	160	-
S	H12	3	buffer control	..BC	BC6	-	40	80	40	-	160	-

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	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)
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 Concentration (M)
P1	E41	Test Chemical 2		TC2	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	E52	Test Chemical 2		TC2	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	E63	Test Chemical 2		TC2	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	E71	Test Chemical 2		TC2	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	E82	Test Chemical 2		TC2	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	E93	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	F11	Test Chemical 3		TC3	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	F22	Test Chemical 3		TC3	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	F33	Test Chemical 3		TC3	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	F41	Test Chemical 3		TC3	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	F52	Test Chemical 3		TC3	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	F63	Test Chemical 3		TC3	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	F71	Test Chemical 3		TC3	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	F82	Test Chemical 3		TC3	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	F93	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 ERα 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 parameters of the competitive binding curves for the control substances should be within the limits of the 95% confidence interval (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)	95% Confidence Intervals[b]	
				Lower Limit	Upper Limit
17β-estradiol	Top	104.74	13.12 (70)	101.6	107.9
	Bottom	0.85	2.41 (70)	0.28	1.43
	HillSlope	-1.22	0.20 (70)	-1.27	-1.17
	$LogIC_{50}$	-8.93	0.23 (70)	-8.98	-8.87
Norethynodrel	Top	101.31	10.55 (68)	98.76	103.90
	Bottom	2.39	5.01 (68)	1.18	3.60
	HillSlope	-1.04	0.21 (68)	-1.09	-0.99
	$LogIC_{50}$	-6.19	0.40 (68)	-6.29	-6.10
Norethindrone[C]	Top	92.27	7.79 (23)	88.90	95.63
	Bottom	16.52	10.59 (23)	11.94	21.10
	Hill Slope	-1.18	0.32 (23)	-1.31	-1.04
	$LogIC_{50}$	-6.01	0.54 (23)	-6.25	-5.78

[a] *Mean ± Standard Deviation (SD) with (sample size (n) were calculated using curve fit estimates (4-parameter Hill equation) for control runs conducted in four laboratories during the validation study (see Annex N of reference 2).*

[b] *The 95% confidence are provided as a guide for acceptability criteria.*

[c] *Testing of norethindrone was optional for Subtask 4 during validation study (see Reference 2, see Subtask 4). Thus, the mean ± SD (n) were calculated using curve fit estimates (4-parameter Hill equation) for control runs conducted in two laboratories.*

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 hrERα

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	[^{3}H]E_2 dilutions for plate column 1-9
A	0.0313 nM [^{3}H] E_2 + ER			0.0313 nM [^{3}H] E_2 + 0.0625 µM E_2 + ER			0.0313 nM				0.625 µM	0.313 nM
B	0.0625 nM [^{3}H] E_2 + ER			0.0625 nM [^{3}H] E_2 + 0.125 µM E_2 + ER			0.0625 nM				1.25 µM	0.625 nM
C	30.125 nM [^{3}H] E_2 + ER			0.125 nM [^{3}H] E_2 + 0.25 µM E_2 + ER			0.125 nM				2.5 µM	1.25 nM
D	0.250 nM [^{3}H] E_2 + ER			0.250 nM [^{3}H] 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 [^{3}H] E_2 + 1 µM E_2 + ER			0.50 nM				10 µM	5 nM
F	1.00 nM [^{3}H] E_2 + ER			1.00 nM [^{3}H] E_2 + 2 µM E_2 + ER			1.00 nM				20 µM	10 nM
G	2.00 nM [^{3}H] E_2 + ER			2.00 nM [^{3}H] E_2 + 4 µM E_2 + ER			2.00 nM				40 µM	20 nM
H	4.00 nM [^{3}H] E_2 + ER			4.00 nM [^{3}H] E_2 + 8 µM E_2 + ER			4.00 nM				80 µM	40 nM

TB: total binding,
NSB: non-specific binding
[^{3}H] E_2: [^{3}H]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 [^{3}H]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 (Annex N of reference 2). 95% confidence intervals are provided for the mean ± SD (n) of all control runs across four laboratories that participated in the validation study. 95% conference intervals were calculated for the curve fit parameters (i.e. top, bottom, Hillslope and Log IC_{50}) for the reference estrogen and weak binders, and the $Log_{10}RBA$ of the weak binders relative to the reference estrogen. Table 1 provides expected ranges for the curve fit parameters that can be used as performance criteria. 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:

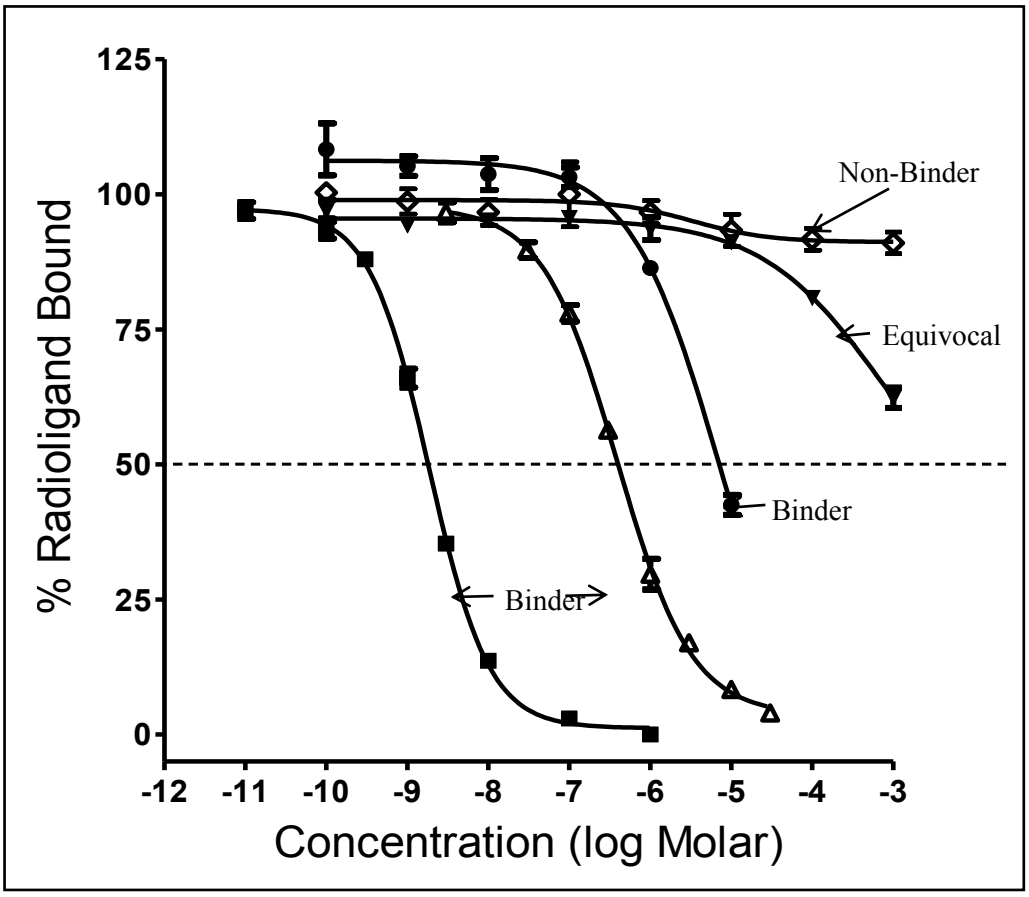
- A binding curve can be fit and the lowest point on the fitted response curve within the range of the data is above 75%, or
- A binding curve cannot be fit and the lowest unsmoothed average percent binding among the concentration groups in the data is above 75%.

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]E2: 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	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	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	Replicate	Well type	Well Code	Concentration Code	Competitor Initial Concentration (M)	hrER stock (uL)	Buffer Volume (uL)	Tracer (Hot E2) Volume	Volume from Dilution plate(uL)	Final Volume (ul)	Competitor Final Concentration (M)
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	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)
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	E2max1	1.00E-06	30	50	10	10	100	1.00E-07
P1	A11	2	Control E2 (max)	S	E2max2	1.00E-06	30	50	10	10	100	1.00E-07
P1	A12	3	Control E2 (max)	S	E2max3	1.00E-06	30	50	10	10	100	1.00E-07
P1	B10	1	Control E2 (IC_{50})	S	$E2IC_{50}1$	$E2IC_{50}$x10	30	50	10	10	100	$E2IC_{50}$
P1	B11	2	Control E2 (IC_{50})	S	$E2IC_{50}2$	$E2IC_{50}$x10	30	50	10	10	100	$E2IC_{50}$
P1	B12	3	Control E2 (IC_{50})	S	$E2IC_{50}3$	$E2IC_{50}$x10	30	50	10	10	100	$E2IC_{50}$
P1	C10	1	Control NE (max)	S	Nemax1	1.00E-3.5	30	50	10	10	100	1.00E-4.5
P1	C11	2	Control NE (max)	S	Nemax2	1.00E-3.5	30	50	10	10	100	1.00E-4.5
P1	C12	3	Control NE (max)	S	Nemax3	1.00E-3.5	30	50	10	10	100	1.00E-4.5
P1	D10	1	Control NE (IC_{50})	S	$NEIC_{50}1$	$NEIC_{50}$ x10	30	50	10	10	100	$NEIC_{50}$
P1	D11	2	Control NE (IC_{50})	S	$NEIC_{50}2$	$NEIC_{50}$ x10	30	50	10	10	100	$NEIC_{50}$
P1	D12	3	Control NE (IC_{50})	S	$NEIC_{50}3$	$NEIC_{50}$ x10	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

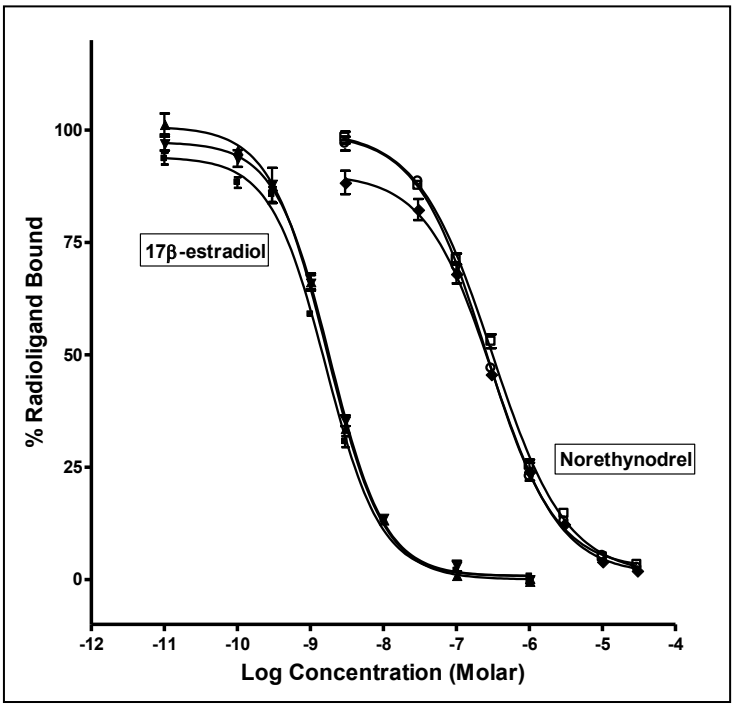
Considerations for the Analysis of Data from the hrER Competitive Binding Assay

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.

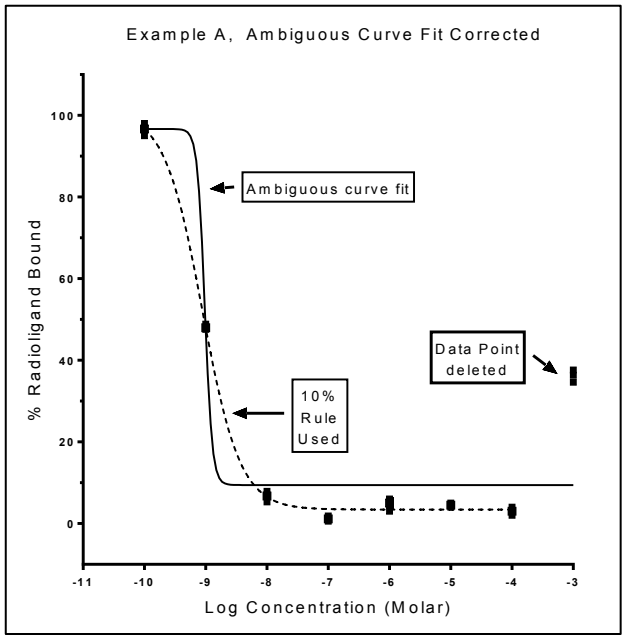


Data Analysis for Test Chemicals

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.

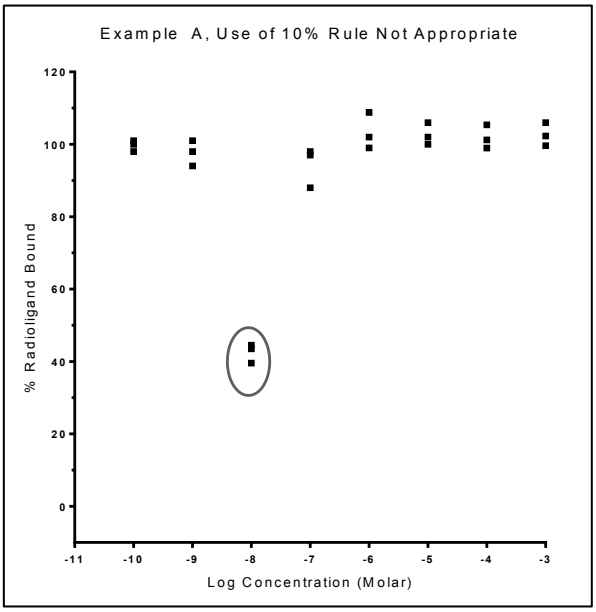
4. Occasionally, there may be examples of data that require additional attention in order to appropriately analyze and interpret the hrER binding data. Previous studies had shown cases where the analysis and interpretation of competitive receptor binding data can be complicated by an upturn of the percent 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.

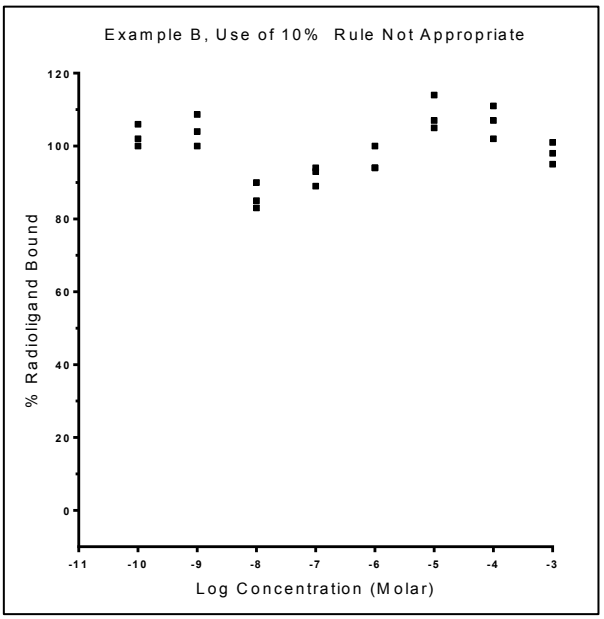
Figure 2. Examples, Competitive Binding Curves with and without Use of the 10% Rule.



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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