Draft ¶
Validation Study Report¶

MCF-7 Cell Proliferation Test Method¶

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) ¶

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Table of Contents

1.0 Introduction

The purpose of this document is to describe the results of a validation study to evaluate the ability of the MCF-7:WS8 cell proliferation test method (MCF-7 CP TM) to identify substances with estrogen receptor (ER) agonist and/or antagonist activity. The study was organized and managed by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), the Japanese Center for the Validation of Alternative Methods (JaCVAM), and the Korean Center for the Validation of Alternative Methods (KoCVAM). Test method performance was assessed using substances recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (2011). A summary to test method performance is provided below:

1. Agonist
    - The accuracy at CertiChem Incorporated (CertiChem) was 95% (39/41), for the 41 agonist accuracy reference substances
    - The accuracy for those 17 reference substances that were tested at least once in all three laboratories was 100% (17/17) at CertiChem, 94% (16/17) at Hiyoshi Corporation (Hiyoshi), and 88% (15/17) at the Korea Food and Drug Administration (KFDA)
    - Intralaboratory (within lab) reproducibility was 100% (12/12) at CertiChem, Hiyoshi, and KFDA
    - Interlaboratory (between lab) reproducibility was 73% (19/26)
2. Antagonist
    - The accuracy at CertiChem was 92% (23/25) for the 25 antagonist accuracy substances
    - The accuracy for those 13 substances that were tested at least once in all three laboratories was 100% (13/13) at CertiChem, 69% (9/13) at Hiyoshi, and 62% (8/13) at KFDA
    - Intralaboratory (within lab) reproducibility was 67% (8/12) at CertiChem, 33% (4/12) at Hiyoshi, and 92% (11/12) at KFDA
    - Interlaboratory (between lab) reproducibility was 54% (14/26)

This validation study report was prepared for the MCF-7 CP TM study management team, which consisted of:

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The structure of this report follows the requested structure of the ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods (ICCVAM 2003c).

2.0 MCF-7:WS8 Cell Proliferation Test Method Protocol Components

2.1 Overview

This test method uses the MCF-7:WS8 cell line, which proliferates in response to estrogen exposure, to detect substances with *in vitro* ER agonist or antagonist activity. Estrogen-mediated proliferation is

measured by changes in DNA levels per well in 96-well tissue culture plates. DNA concentration per well is quantified using a micro-plate modification of the Burton diphenylamine assay (Burton 1956; Natarajan et al. 1994). A concentration–response curve can be established to provide qualitative and quantitative information regarding the *in vitro* estrogenic activity of a test substance.

The primary objective of this test method is to provide a qualitative assessment of *in vitro* estrogenic activity (i.e., whether a substance is positive or negative for estrogenic activity). Quantitative analysis is also performed to provide additional information on the estrogenic potency of test substances. For example, quantitative analysis can determine the half-maximal effective concentration (EC_{50}) or the half-maximal inhibitory concentration (IC_{50}). Separate protocols are used to identify substances that possess ER agonist or antagonist activity, although the two protocols share most major components.

In a 2003 evaluation, ICCVAM recommended minimum essential test method components for *in vitro* ER test method protocols (ICCVAM 2003a), which included the following considerations:

- A reference standard must be included to demonstrate the adequacy of the test method for detecting ER agonists or antagonists.
- Each study must include a set of concurrent solvent controls.
- Each study must include an evaluation of cytotoxicity.
- To demonstrate that the test method is functioning properly and is sufficiently sensitive to detect weak ER agonists, a weak positive agonist control with an EC_{50} two to three orders of magnitude higher than the reference estrogen must be included in each study.
- To demonstrate that the test method is functioning properly and is sufficiently sensitive to detect weak ER antagonists, each study must include a weak positive antagonist control that inhibits the reference estrogen response by 50% (IC_{50}) at a concentration two to three orders of magnitude higher than the reference anti-estrogen.
- The maximum test substance concentration must be 1 mM unless otherwise limited by solubility or cytotoxicity.
- At least seven concentrations in 10-fold intervals, up to the limit concentration, must be tested.
- EC_{50} or IC_{50} values must be calculated for all positive substances when possible.
- Protocols must contain established test plate acceptance criteria.

The ICCVAM-recommended test method components were incorporated into the MCF-7:WS8 cell proliferation test method (MCF-7 CP TM) protocols by CCi (**Appendices A1** and **A2**).

The remainder of this section provides details on the essential test method components and the rationale for their inclusion in the optimized protocols.

2.1.1 General Procedural Overview

Agonist and antagonist testing in the MCF-7 CP TM is conducted in four phases:

1. Solubility testing and dilution of test substances
2. Range finder testing and selection of starting concentrations and dilution factors of test substances to be used in comprehensive testing
3. Comprehensive testing, qualitative assessment of *in vitro* estrogenic activity, and, where appropriate, quantitative analysis to assess estrogenic or anti-estrogenic potency
4. Confirmation testing, i.e., confirmation that the observed positive response is driven by the ER.

2.2 Materials

2.2.1 MCF-7:WS8 Cells

The MCF-7:WS8 cell line is derived from MCF-7 immortalized human breast adenocarcinoma cells and endogenously express both human ERα and ERβ.

CCi provided cryopreserved MCF-7:WS8 cells from their cell bank to Hiyoshi and KFDA for the validation study. Hiyoshi and KFDA propagated and cryopreserved multiple ampoules of cells to establish their working cell banks for use throughout the study.

2.2.2 Cell Culture Reagents, Supplies, and Equipment.

The MCF-7 CP TM requires general cell culture materials, reagents, supplies and equipment (see **Appendices A1** and **A2** [protocols] for formulations, and concentrations of solutions and media). The participating laboratories independently acquired cell culture materials, reagents, and supplies, with the exception of fetal bovine serum (FBS)/calf serum (CS), which was obtained from CCi.

2.3 Cell Culture

Cryopreserved MCF-7:WS8 cells are thawed, resuspended in Roswell Park Memorial Institute (RPMI) media, transferred into 25-cm^2 tissue culture flasks, and incubated at 37°C ± 1°C, 90% ± 5% humidity, and 5% ± 1% CO_2/air for 48 to 72 hours (see **Appendices A1** and **A2** [protocols] for cell culture specifics). When cells reach 80% to 90% confluence (as estimated from a visual inspection of cell density), they are removed from the flask by trypsinization. A dissociated single-cell suspension is added to new flasks for propagation, and the cells are passaged/subcultured at least twice before conditioning in estrogen-free medium (EFM). Cells can be used for 16 passages before a new batch of cells must be thawed. Forty-eight to 72 hours after the second subculture, cells are trypsinized and pelleted. The RPMI media are removed. Cells are then resuspended in EFM, and the cell suspension is added to new flasks for conditioning. When cells are 80% to 90% confluent, they are trypsinized, counted, and seeded into 96-well plates for testing.

2.4 Reference Standards and Controls

ICCVAM recommends the use of appropriate reference standards and controls for ER TA test methods in order to maximize test method intra- and interlaboratory reproducibility and minimize the likelihood of erroneous results (ICCVAM 2003a).

2.4.1 Vehicle Control

- 1% ethanol (EtOH) in EFM is used as the concurrent vehicle control for all testing in agonist and antagonist protocols.

A concurrent vehicle control in ER agonist and antagonist test methods provides a measure of ER-mediated activity in the absence of the reference standard, control, or test substances. For the MCF-7 CP TM, the concurrent vehicle control is the baseline against which the extent of proliferative induction is determined.

2.4.2 Estrogenic Reference Standard

- In accordance with the ICCVAM recommendations (ICCVAM 2003a), 17β-estradiol (E2, CAS Registry Number [CASRN] 50-28-2) is used as the reference estrogen to demonstrate the adequacy of the ER test method. In the MCF-7 CP TM, adequacy is based on the ability of the E2 reference standard to induce cellular proliferation.

2.4.3 Weak Agonist Control

- Genistein (GNT, CASRN 446-72-0) is used as the weak positive control in agonist range finder and comprehensive testing. A weak positive control is not used during agonist confirmation testing.

ICCVAM recommends that a positive control with an EC_{50} two to three orders of magnitude higher than E2 (EC_{50} = 3.36 x 10^{-12} M) be included in each study to demonstrate that the test method is functioning properly and is sufficiently sensitive for detecting weak ER agonists (ICCVAM 2003a). However, given that the response range expected to be assessed during the validation study was greater than six orders of magnitude, the CCi and NICEATM staff concluded that a positive control with a higher EC_{50} would be more appropriate. GNT was selected for use as the weak positive control because it produced consistent response curves with an EC_{50} of 0.11 μM, approximately six orders of magnitude higher than E2 (EC_{50} = 3.36 x 10^{-12} M. The GNT concentration of 3.70×10^{-7} M was selected because it was the lowest concentration that gave the maximum response**.**

2.4.4 Negative Agonist Control (ICI control)

- ICI 182,780 (ICI, CASRN 129453-61-8) is used as a negative control on all ER agonist range finder and comprehensive test plates. 1.00×10^{-8} M ICI in 1% EtOH is added to EFM and used in each agonist range finder and comprehensive response testing. If ICI causes a significant reduction in DNA content per well compared with vehicle control (VC), the VC is deemed contaminated and the experiment must be repeated.
- ICI is used in all agonist confirmation tests at 1.00×10^{-8} M to confirm that observed agonist responses are ER-specific.
- The ICCVAM expert panel (ICCVAM 2002c) recommended that test substances which are positive for ER agonism be tested for specificity of response against ICI.

2.4.5 Toxicity Control

- Sodium dodecyl sulfate (SDS, CASRN 151-21-3) is used in all agonist range finder and comprehensive experiments at 3.47×10^{-4} M as a toxicity control. SDS is not used in agonist confirmation testing.

2.4.6 Anti-Estrogenic Reference Standard

- Raloxifene HCL (Ral, CASRN 82640-04-8) is used as the anti-estrogenic reference standard in all antagonist range finder, comprehensive, and confirmation experiments.

Although ICCVAM recommends ICI 182,780 as a reference standard in *in vitro* ER antagonist assays, this substance has limited commercial availability (ICCVAM 2006b). As an alternative, the more commercially available Ral was selected for use. Ral is a strong ER antagonist also recommended by ICCVAM as a reference standard (ICCVAM 2006b).

2.4.7 Weak Antagonist Control

- Dibenzo[*a*,*h*]anthracene (DBA, CASRN 53-70-3) is used as the weak positive control for antagonist range finder and comprehensive testing. A weak positive control is not used for antagonist confirmation testing.

The use of a weak antagonist as a concurrent control in *in vitro* ER antagonist test methods provides a measure of the range of responses that can be detected by the test. ICCVAM recommends using a weak positive control with an IC_{50} at least three orders of magnitude higher than the reference antagonist (ICCVAM 2003a), Ral (IC_{50}= 2.24×10^{-9} M). CCi used DBA (3.59×10^{-6} M) as the weak antagonist control for the validation study.

2.4.8 E2 Control

- 17β-estradiol (E2, CAS Registry Number [CASRN] 50-28-2) is tested at a fixed concentration of 1.98×10^{-12} M in all wells except for VC wells in all antagonist range finder, comprehensive, and confirmation experiments.

The E2 control provides the estrogenic proliferative response against which any anti-estrogenic effects are measured.

2.4.9 E2 Antagonist Confirmation Control

Antagonist confirmation testing is a rescue-of-function experiment in which a higher concentration of E2 is tested to overcome the antagonistic effects of test substances.

- A low concentration of E2 (1.98×10^{-12} M) is utilized:
    1. As an E2 control, which is tested on the antagonist control plate. Induction of this E2 control must be greater than or equal to three-fold for an antagonist confirmation experiment to pass acceptance criteria.
    2. As a normalization factor. The replicate values for this E2 control are used to normalize all of the test substance plus low E2 concentration data.
    3. In combination with test substance. This serves as the standard response that the results of the test substance plus high E2 concentration results are compared.
- A high concentration of E2 (1.98×10^{-9} M) is utilized:
    1. As an E2 control, which is tested on the antagonist control plate.
    2. As a normalization factor. The replicate values for this E2 control are used to normalize all of the test substance plus high E2 concentration data.
    3. In combination with test substance, as a competitive ligand for ER.

2.5 Test Substance Concentrations

ICCVAM recommends that the maximum test substance concentration be 1 mM unless otherwise limited by solubility or cytotoxicity (ICCVAM 2003a).

2.5.1 Solubility Testing

- The starting concentration for range finder testing is established by determining the maximum test substance solubility in 1% EtOH in EFM.

For substances tested in Phases 2 and 3, the maximum solubility of each test substance in 1% EtOH in EFM was determined at 10-fold intervals up to the recommended 1 mg/mL limit concentration (ICCVAM 2003a). The maximum soluble concentration of each test substance was used as the highest concentration in the range finder test.

2.5.2 Cytotoxicity Testing

The assessment of cytotoxicity is incorporated into agonist and antagonist range finder, comprehensive, and confirmation testing. A qualitative method to assess cell viability was developed by CCi for use during the validation. This method relies on visual observation of cell density and morphology to assign cell viability scores in **Appendices A1** and **A2.** Test substance concentrations that generate a viability score of 2 or greater are deemed cytotoxic and not used for data analysis.

2.6 DNA Control Plate

A DNA control plate is run concurrently with all agonist and antagonist range finder, comprehensive, and confirmation experiments. The DNA control plate is used to generate a standard curve between 0 and 3 µg DNA per well, which is then used to calculate the DNA content per well in each test plate.

2.7 Cell Culture Control Plate

One cell culture control plate is run per experimental grouping (e.g., if running one range finder set or five comprehensive sets, a single cell culture control plate would accompany each set). The cell culture control plate contains VC, reference controls, cytotoxicity controls (SDS) and weak agonist or antagonist controls.

2.8 Volatility Testing

Volatility is of concern because highly volatile test chemicals can generate vapors during the test chemical incubation, which can then be absorbed by the media in adjacent wells, contaminating them and potentially causing false positive results. In addition to knowing the volatility characteristics of a substance, deviations from expected and historical values and consistent VC values can help identify volatility issues. Plate sealers and alternate plate layouts are used when testing a suspected volatile test substance (**Appendices A1** and **A2**). Volatility testing was conducted prior to initiation of Phases 2 and 3 at Hiyoshi and KFDA, as part of the demonstration of laboratory proficiency.

2.9 Tests for Evaluation of ER Agonism and Antagonism

All test substances underwent range finder and comprehensive testing, and if necessary, confirmation testing. Each test is described below.

2.9.1 Range Finder Testing

The purpose of range finder testing is to establish the concentration range of a test substance to be included in comprehensive testing. This involves identifying both an appropriate starting concentration and a dilution scheme. The starting concentration of a test substance is based on the highest soluble concentration that is not cytotoxic, as described in **Section 2.5**. Results from range finder testing are used to select a 1:5 or 1:2.5 dilution scheme for comprehensive testing (see **Table 2-1**). A 1:5 dilution covers a wider concentration range (approximately eight 10-fold dilutions), while a 1:2.5 dilution provides higher resolution over a smaller range (approximately four 10-fold dilutions). Procedures for range finder testing, along with the criteria used to determine the appropriate testing range, are provided in **Appendices A1** and **A2**.

2.9.2 Comprehensive Testing

Comprehensive tests are performed to determine if a test compound possesses ER agonist or antagonist activity. The range of doses used is determined by the range finder test results, as described in **Table 2-1**.

Table 2-1 Determination of Starting Concentrations and Dilutions for Agonist and Antagonist Comprehensive Testing

Range-finder Results	Concentration	Dilution
I: Negative	Maximum soluble	1:2.5
II: Negative + Cytotoxic	Lowest cytotoxic	1:2.5
III: Positive with concentration range < 4 orders of magnitude	Ten-fold higher than the concentration giving the highest value	1:2.5

Range-finder Results	Concentration	Dilution
IV: Positive with concentration range > 4 orders of magnitude	Ten-fold higher than the concentration giving the highest value	1:5
V: Positive with biphasic peaks with concentration range > 4 orders of magnitude	Ten-fold higher than the concentration giving the highest value in the peak associated with the higher concentration	1:5
	*Lowest cytotoxic concentration in the peak associated with the higher concentration	

*Antagonist assay only

2.9.3 Confirmation Testing

Confirmation testing is performed on all test substances that tested positive in agonist and antagonist comprehensive experiments. The competitive nature of the confirmation test ensures ER specificity and reduces the possibility of false positives. Criteria for determining whether a test substance is positive or negative are listed in **Sections 2.10.4** and **2.11.4** for agonist and antagonist testing, respectively.

2.10 ER Agonist Testing

2.10.1 Agonist Range Finder Testing

Reference Standard and Control Concentrations Used for Agonist Range Finder Testing

- E2, the reference estrogen, is tested at 10-fold dilutions in duplicate at eight concentrations (1.98×10^{-9} to 1.98×10^{-16} M).
- The GNT control is tested as eight replicates on each cell culture control plate at 3.70×10^{-7} M.
- The ICI control is tested as eight replicates on each test substance and the cell culture control plates at 1.00×10^{-8} M.
- The cytotoxicity control (SDS) is tested as eight replicates on each cell culture control plate at 3.47×10^{-4} M.
- The vehicle control (1% EtOH v/v in EFM) is tested as 16 replicates on the test substance plates, and as 80 replicates on the cell culture control plate.

Agonist Range Finder Test Plate Design

- One range finder test plate contains the lowest test substance concentrations, and a second range finder test plate contains the highest test substance concentrations.
- 80 of the 96 wells of the range finder test plates are used in testing.
- A maximum of seven substances can be tested at eight concentrations in duplicate on each pair of range finder test plates.
- Starting concentrations are determined during solubility testing.

Agonist Range Finder Plate Acceptance Criteria

- The mean VC value (DNA content per well) must be within 2.5 times the standard deviation (SD) of the historical VC value.
- E2 induction must be greater than or equal to three-fold. Induction is the average of the highest E2 reference value from both E2 concentration curves, divided by the average VC value.
- The E2 EC_{50} value must be within 2.5 times the SD of the historical E2 EC_{50}.
- The E2 curve must have an r^2 (coefficient of determination) value ≥ 0.9 calculated by Hill kinetics using GraphPad Prism® (GraphPad Software, Inc.).

- GNT induction must be greater than or equal to threefold. Induction is the average GNT value divided by the average VC value.
- Data from plates that fail any acceptance criterion must be discarded and the experiment repeated.

Interpretation of Results from Agonist Range Finder Testing

Criteria for determination of starting concentrations for agonist comprehensive testing are presented in **Table 2-1**.

2.10.2 Agonist Comprehensive Testing

Reference Standard and Control Concentrations Used for Agonist Comprehensive Testing

- E2, the reference estrogen, is tested at 1:2.5 or 1:5 dilutions (determined by agonist range finder results) in triplicate at twelve concentrations (**Table 2-1**) on four test plates.
- The GNT control is tested as eight replicates on each cell culture control plate at 3.70×10^{-7} M.
- The ICI control is tested as eight replicates on the cell culture control plates at 1.00×10^{-8} M.
- The cytotoxicity control (SDS) is tested as eight replicates on the cell culture control plate at 3.47×10^{-4} M.
- The VC (1% EtOH v/v in EFM) is tested as eight replicates on each test substance plate (32 replicates total), and as 72 replicates on the cell culture control plate.

Plate Acceptance Criteria for Comprehensive Agonist Testing

- Plate acceptance criteria for comprehensive testing is the same as those used for range finder testing (see **Section 2.10.1**).

2.10.3 Agonist Confirmation Testing

Reference Standard and Control Concentrations Used for Agonist Confirmation Testing

- E2, the reference estrogen, is tested at 1:2.5 or 1:5 dilutions (determined by agonist comprehensive results) in triplicate at six concentrations on two of the four test plates.
- E2 is tested at 1:2.5 or 1:5 dilutions (determined by agonist comprehensive results) in triplicate at six concentrations plus 1.00×10^{-8} M ICI on two of the four test plates.
- The VC is tested as eight replicates on two test substance plates (32 replicates total), and as 96 replicates on the cell culture control plate.

Plate Acceptance Criteria for Agonist Confirmation Testing

- The mean VC value for wells not containing ICI must be within 2.5 times the SD of the historical VC mean value.
- E2 induction must be greater than or equal to three-fold. Induction is the average of the highest E2 reference value from both E2 concentration curves, divided by the average VC value.
- The E2 EC_{50} value must be within 2.5 times the SD of the historical E2 EC_{50}.
- The E2 curve must have an r^2 (coefficient of determination) value ≥ 0.9 calculated by Hill kinetics using GraphPad Prism®.
- Data from plates that fail any acceptance criterion must be discarded and the experiment repeated.

2.10.4 Interpretation of Agonist Results from Comprehensive and Confirmation Testing

The following requirements are necessary for a compound to be classified as positive or negative for ER agonism.

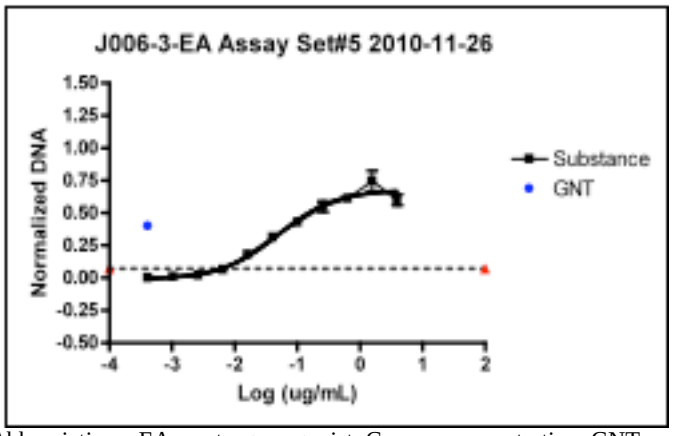
Positive Classification (Requires Confirmation)

A positive comprehensive test for agonism has (**Figure 2-1**)**:**

- A dose response with any point above the 3×SD threshold.

- Curves that do not demonstrate a dose response but have all points above 3×SD thresholds are deemed positive (inappropriate dose selection results causes the ascending portion of the curve to be missed).
- A positive comprehensive test for agonism must be followed by a confirmation test.

Figure 2-1 Example of a Positive Agonist Comprehensive Test



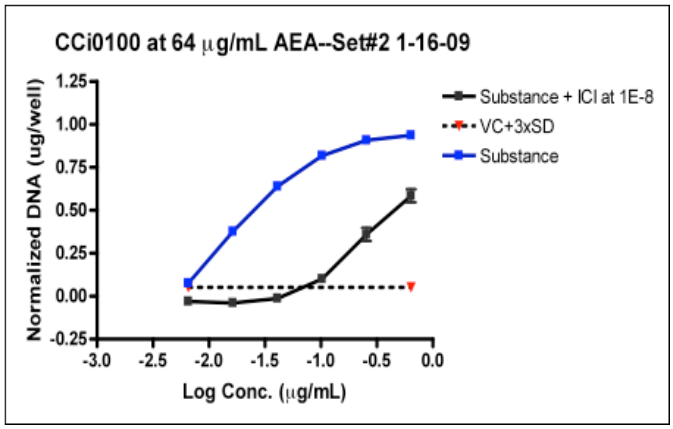
Abbreviations: EA = estrogen agonist; Conc. = concentration; GNT = genistein.

The test substance (solid line) is dose responsive with multiple points above the 3×SD threshold (dashed line).

A positive confirmation test has (**Figure 2-2**):

- Two points above the 3×SD threshold with two corresponding ICI-treated samples below the 3×SD threshold ("two differentiated points")
- At least two points on the test substance curve must be concentration-responsive.

Figure 2-2 Example of a Positive Agonist Confirmation Test

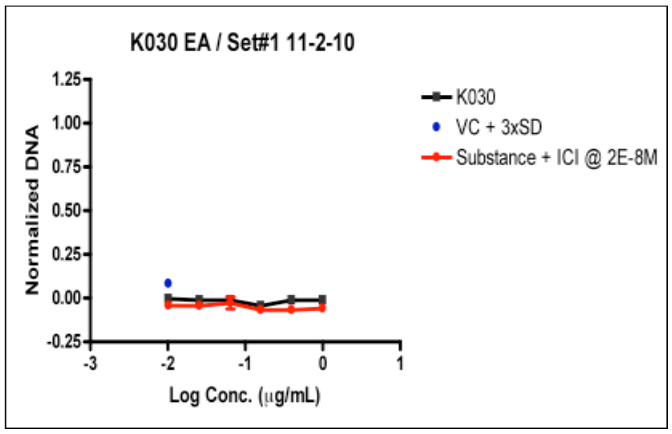


Abbreviations: AEA = estrogen antagonist; Conc. = concentration; ICI = ICI 182,780; SD = standard deviation; VC = vehicle control.

The test substance (blue line) is concentration-response and has points above the 3×SD threshold (dashed line). The ICI-treated samples (solid black line) have two corresponding points below the 3×SD threshold.

A negative confirmation test has less than two differentiated points (i.e., one differentiated point is not sufficient) (**Figure 2-3**).

Figure 2-3 Example of a Negative Agonist Confirmation Test



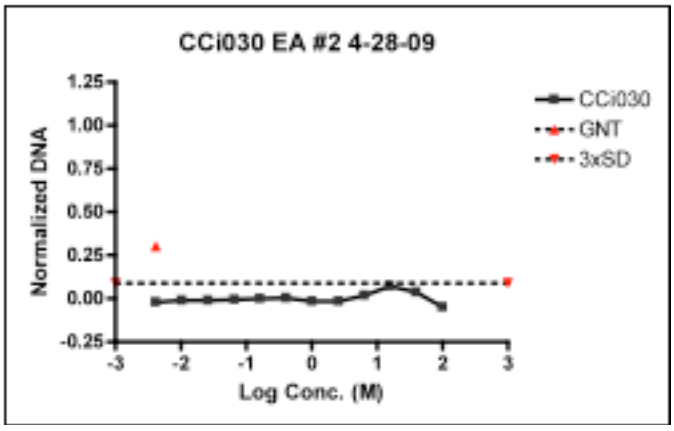
Abbreviations: EA = estrogen agonist; Conc. = concentration; ICI = ICI 182,780; SD = standard deviation; VC = vehicle control.
The test substance curve (black line) does not have any differentiated points when compared with the ICI-treated curve (red line).

Negative Classification (No Confirmation Required)

The negative comprehensive test for agonism has (**Figure 2-4**):

- All points below the 3×SD threshold
- Curves that do not demonstrate a dose response but have isolated (non-consecutive) points slightly above the 3×SD thresholds are deemed negative.

Figure 2-4 Example of a Negative Agonist Comprehensive Test



Abbreviations: EA = estrogen agonist; Conc. = concentration; GNT = genistein; SD = standard deviation
The test substance (solid line) lacks dose responsiveness and has no points above the 3×SD threshold (dashed line).

2.11 ER Antagonist Testing

2.11.1 Antagonist Range Finder Testing

Reference Standard and Control Concentrations Used for Antagonist Range Finder Testing

- A single concentration of E2 (1.98×10^{-12} M), intended to provide 80% of the maximum E2 induction, is tested as 16 replicates on the test substance plates and as 40 replicates on the cell culture control plate.
- Eight concentrations of the reference anti-estrogen, raloxifene HCl, are tested at 10-fold serial dilutions ranging from 9.80×10^{-7} to 9.80×10^{-14} in duplicate.
- The DBA control is tested as eight replicates at 3.59×10^{-6} M on each cell culture control plate.

- The cytotoxicity control (SDS) is tested as eight replicates at 3.47×10^{-4} M on each cell culture control plate.
- All reference anti-estrogen and test wells, except the VC wells, must contain E2 at the fixed concentration of 1.98×10^{-12} M.
- The VC (1% ETOH v/v in EFM) is tested as 16 replicates on the test substance plates and as 40 replicates on the cell culture control plate.

Antagonist Range Finder Plate Design

- Antagonist range finder experiments use two test plates containing test substances. One plate contains the lowest test substance concentrations and the second plate contains the highest test substance concentrations.
- An additional plate, referred to as a cell culture control plate, is tested together with the range finder test plates. The cell culture control plate contains DBA, E2, SDS, and VC replicates.
- 80 of the 96 wells of the range finder test plates are used during range finder testing.
- A maximum of seven substances can be tested at eight concentrations in duplicate on each pair of range finder test plates.
- Starting concentrations are determined during solubility testing.

Antagonist Range Finder Plate Acceptance Criteria

- The mean VC value (DNA content per well) for each plate must be within 2.5 times the standard deviation (SD) of the historical VC mean value.
- E2 control induction must be greater than or equal to three-fold. E2 induction is the average day control plate E2 control value divided by the averaged VC value from both the test plate and cell culture control plate.
- Ral reduction must be greater than or equal to 2.5-fold. Ral reduction is the averaged highest Ral values divided by the averaged lowest Ral values from the test plate and cell culture control plate.
- DBA reduction must be greater than or equal to 2.5-fold. DBA reduction is the average E2 value divided by the average DBA value.
- The Ral IC_{50} value must be within 2.5 times the SD of the historical Ral IC_{50}.
- The Ral curve must have an r^2 (coefficient of determination) value ≥ 0.85 calculated by Hill kinetics using GraphPad Prism®.
- Data from plates that fail any acceptance criterion must be discarded and the experiment repeated.

Interpretation of Results from Antagonist Range Finder Testing

Criteria for determination of starting concentrations for antagonist comprehensive testing are presented in **Table 2-1**.

2.11.2 Antagonist Comprehensive Testing

Reference Standard and Control Concentrations Used for Antagonist Comprehensive Testing

- A single concentration of E2 (1.98×10^{-12} M), intended to provide 80% of the maximum E2 induction, is tested as 16 replicates on the test substance plates and as 40 replicates on the cell culture control plate.
- Twelve concentrations of the reference anti-estrogen, raloxifene HCl, tested at either 1:2.5 or 1:5 dilutions (determined by agonist range finder results) in triplicate at twelve concentrations (**Table 2-2**) on the four test plates.
- The DBA control is tested as eight replicates on each cell culture control plate at 3.59×10^{-6} M.
- The cytotoxicity control (SDS) is tested as eight replicates on each cell culture control plate at 3.47×10^{-4} M.
- All reference anti-estrogen and test wells except the VC wells must contain a fixed concentration of E2 (1.98×10^{-12} M), intended to provide 80% of the maximum E2 induction.

- The vehicle control (1% EtOH v/v in EFM) is tested as eight replicates on each test substance plate (32 replicates total), and as 40 replicates on the cell culture control plate.

Antagonist Test Plate Design

- Seven substances can be tested at twelve concentrations, in triplicate, at concentrations and using a dilution determined based on range finder results (**Section 2.8.2**).

Plate Acceptance Criteria for Comprehensive Antagonist Testing

- Plate acceptance criteria for comprehensive testing is the same as for range finder testing (see **Section 2.11.1**).

2.11.3 Antagonist Confirmation Testing

Reference Standard and Control Concentrations Used for Antagonist Confirmation Testing

- A single low concentration of E2 (1.98×10^{-12} M), intended to provide 80% of the maximum E2 induction, is tested as 16 replicates on two of the test substance plates and as 32 replicates on the cell culture control plate.
- A single high concentration of E2 (1.98×10^{-9} M), intended to provide a maximum E2 induction, is tested as 16 replicates on two of the test substance plates and as 32 replicates on the cell culture control plate.
- Six concentrations of the reference anti-estrogen, Ral, are tested at either 1:2.5 or 1:5 dilutions (determined by comprehensive antagonist range finder results), in triplicate on two of the test plates.
- Six concentrations of the reference anti-estrogen, Ral, are tested at either 1:2.5 or 1:5 dilutions (determined by comprehensive antagonist range finder results), in triplicate on two of the test plates.
- The VC (1% EtOH v/v in EFM) is tested as 32 replicates on the cell culture control plate.

Plate Acceptance Criteria for Antagonist Confirmation Testing

- The mean VC DNA content per well must be within 2.5 times the SD of the historical VC mean DNA content per well.
- The low and high E2 control inductions must each be at least three-fold. E2 induction is the average cell culture control plate E2 control value divided by the averaged VC value from both the test plate and cell culture control plate.
- Plate reduction must be greater than or equal to 2.5-fold. Reduction is the averaged highest Ral + low E2 (1.98×10^{-12} M) reference value divided by the averaged lowest Ral value.
- The Ral IC_{50} value must be within 2.5 times the SD of the historical Ral IC_{50}.
- The Ral curve must have an r^2 (coefficient of determination) value ≥ 0.85 calculated by Hill kinetics using GraphPad Prism®.
- Data from plates that fail any acceptance criterion must be discarded and the experiment repeated.

2.11.4 Interpretation of Antagonist Results from Comprehensive and Confirmation Testing

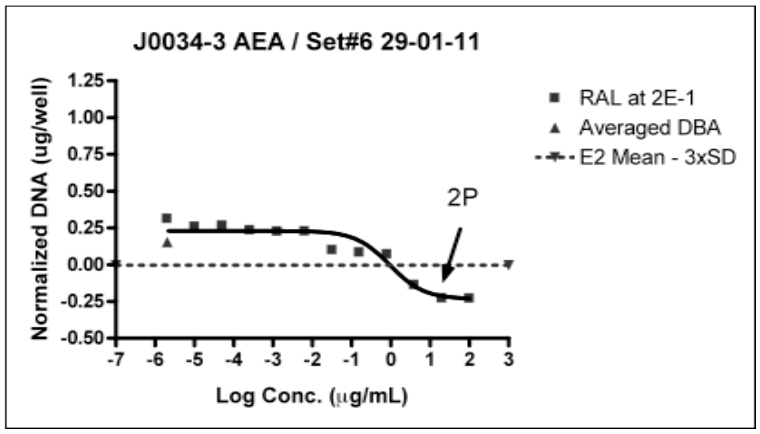
The following requirements are necessary for a substance to be classified as positive or negative for ER antagonism:

Positive Classification (Requires Confirmation)

A positive comprehensive test for antagonism has (**Figure 2-5**):

- Dose response with any point below the 3×SD threshold
- Curves that do not demonstrate a dose response but have all points below 3×SD are deemed positive (inappropriate dose selection resulted in descending portion of the curve being missed).
- A positive comprehensive test for antagonism must be followed by a confirmation test.

Figure 2-5 Example of a Positive Antagonist Comprehensive Test



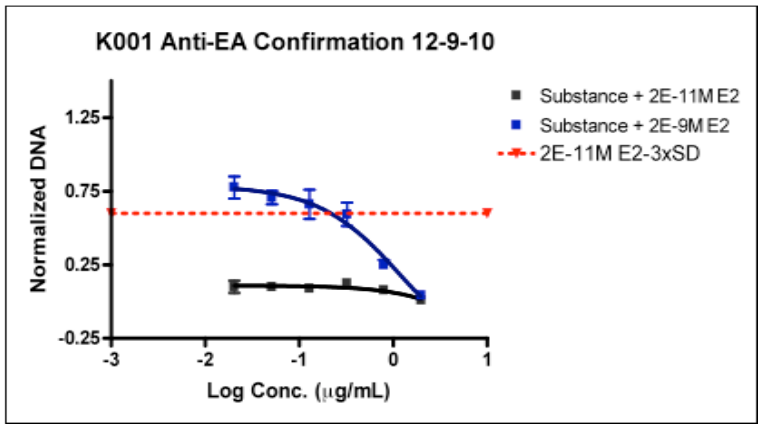
Abbreviations: AEA = estrogen antagonist; Conc. = concentration; DBA = dibenzo[a,h]anthracene; E2 = 17β-estradiol; 2P = cell viability score; RAL = raloxifene; SD = standard deviation.

Test substance (solid black line) is dose responsive and has one point below the 3XSD threshold (dashed black line).

A positive confirmation test for antagonism has (**Figure 2-6**):

- Any two low E2 points below the 3×SD threshold with two corresponding high E2-treated samples above the 3×SD threshold ("two differentiated points")
- At least two points on the low E2 curve must be concentration-responsive.

Figure 2-6 Example of a Positive Antagonist Confirmation Test

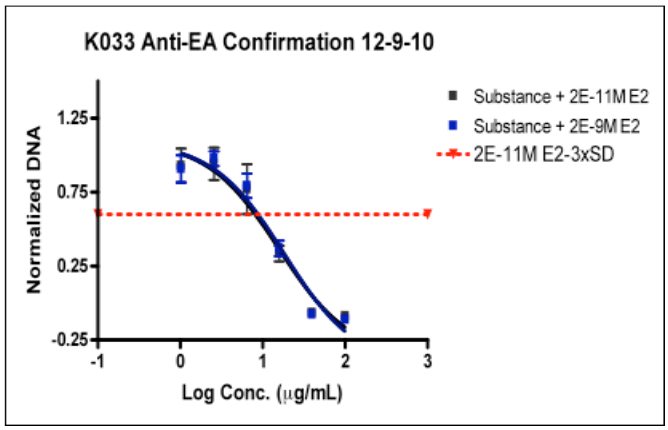


Abbreviations: EA = estrogen agonist; Conc. = concentration; E2 = 17β-estradiol; SD = standard deviation.

Test substance plus low E2 (solid black line) is dose response with points below the 3×SD threshold (dashed red line). The test substance plus high E2 (solid blue line) has multiple corresponding points above the 3×SD threshold.

A negative confirmation test for antagonism has less than two differentiated points (i.e., one differentiated point is not sufficient) (**Figure 2-7**).

Figure 2-7 Example of a Negative Antagonist Confirmation Test



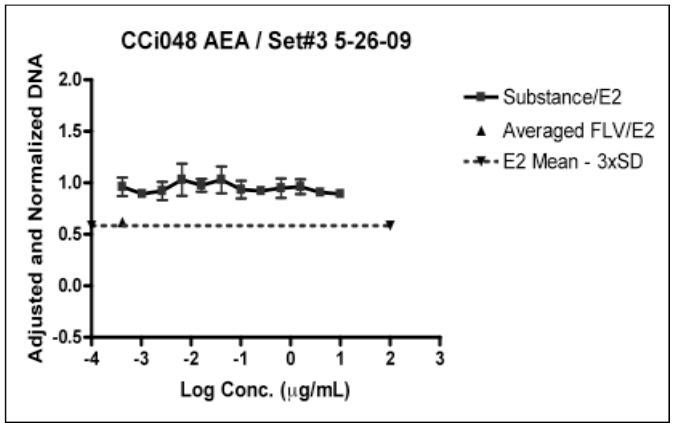
Abbreviations: EA = estrogen agonist; Conc. = concentration; E2 = 17β-estradiol; SD = standard deviation.
Test substance plus low E2 (solid black line) has no points that are differentiated from the test substance plus high E2.

Negative Classification (No Confirmation Required)

The negative comprehensive test for antagonism has (**Figure 2-8**):

- All points above the 3×SD threshold
- Curves that do not demonstrate a dose response but have isolated (non-consecutive) points slightly below the 3×SD thresholds are deemed negative.

Figure 2-8 Example of a Negative Antagonist Comprehensive Test



Abbreviations: AEA = estrogen antagonist; Conc. = concentration; E2 = 17β-estradiol; FLV = flavone; SD = standard deviation.
Test substance (solid black line) has all points above the 3×SD threshold (dashed black line).

2.13 Determination of Overall Classification for Agonist and Antagonist Results

- **Positive =** Positive Comprehensive + Positive Confirmation
- **Unconfirmed Positive =** Positive Comprehensive
- **Negative =** Negative Comprehensive
- **Negative =** Positive Comprehensive + Negative Confirmation

3.0 Substances used for the Validation of the MCF-7 CP TM

ICCVAM previously recommended a list of 78 substances for use in validation studies for *in vitro* ER and androgen receptor (AR) test methods (ICCVAM 2006a). These substances were selected based on information contained in the ICCVAM background review documents (BRD) for ER binding and transcriptional activation test methods (ICCVAM 2002b, 2002a), as well as information obtained from publications reviewed or published after completion of the ICCVAM BG1 BRD (ICCVAM 2011).

3.1 Test Substance Procurement, Coding, and Distribution

On behalf of NICEATM, the National Toxicology Program Substance Inventory (NTPSI) procured and distributed all reference standards and controls to the participating laboratories. Reference substances that required a license from the U.S. Drug Enforcement Agency (i.e., 4-androstenedione, 5α-dihydrotestosterone, methyl testosterone, testosterone, and phenobarbital) were distributed only to CCi. Reference substances were coded with a laboratory-specific unique identifier, and aliquots were sent in coded vials to participating laboratories. Reference standards and controls were provided with material safety data sheets (MSDS) and coded reference substances were provided with a sealed envelope containing the identity of each test substance as well as its MSDS to be opened in the event an accident occurred (e.g., chemical spill). The NTPSI also obtained Certificates of Analysis for reference standards, controls, and reference substances.

Substances were packaged so as to minimize damage during transit, and shipped under appropriate storage conditions and according to the appropriate regulatory transportation procedures. The NICEATM validation study project manager maintained certificates of analysis for all test substances and the participating laboratories were notified upon shipment in order to prepare for receipt. Information regarding weight or volume and storage conditions for each coded reference substance was also provided to each laboratory in advance of shipment. The shipment contained instructions for the participating laboratories to:

- Contact the NTPSI and the validation study project manager upon receipt of test substances.
- Contact the validation study project manager if test facility personnel opened the health and safety packet at any time, for any reason, during the study.

No laboratories opened the health and safety packet during the study.

4.0 Test Method Data And Results

This section summarizes the results from testing of 26 coded reference substances in the three participating laboratories (CCi, KFDA, and Hiyoshi), as well as an additional 52 coded reference substances tested in the lead laboratory (CCi), using the agonist and antagonist protocols for the MCF-7 CP TM.

4.1 Agonist and Antagonist Test Substance Results

Summary results of the MCF-7 CP TM for comprehensive and confirmation agonist tests, as well as an overall classification by each laboratory, are shown in **Tables 4-1** and **4-2**. Antagonist results are shown in **Tables 4-3** and **4-4**.

Table 4-1 Summary of Agonist Comprehensive, Confirmation, and Overall Results at Hiyoshi and KFDA[a]

Phase	Substance Name	Comprehensive		Confirmation		Overall	
		Hiyoshi	KFDA	Hiyoshi	KFDA	Hiyoshi	KFDA
2	17α-Ethinyl estradiol	POS (3/3)	POS (3/3)	POS (1/1)	POS (1/1)	POS	POS
2	Atrazine	POS (3/3)	NEG (3/3)	POS (1/1)	NT	POS	NEG
2	Bisphenol A	POS (3/3)	POS (3/3)	POS (1/1)	POS (1/1)	POS	POS
2	Bisphenol B	POS (3/3)	POS (3/3)	POS (1/1)	POS (1/1)	POS	POS
2	Butylbenzyl phthalate	POS (4/4)	POS (3/3)	POS (1/2)	POS (1/1)	POS	POS
2	Corticosterone	POS (3/3)	NEG (3/3)	NEG (1/1)	NT	NEG	NEG
2	Diethylstilbestrol	POS (3/3)	POS (3/3)	POS (1/1)	POS (1/1)	POS	POS
2	Flavone	NEG (3/3)	NEG (3/3)	NT	NT	NEG	NEG
2	Genistein	POS (3/3)	POS (3/3)	POS (1/1)	POS (1/1)	POS	POS
2	*p,p'* -DDE[b]	POS (3/3)	POS (3/3)	POS (1/1)	NEG (1/1)	POS	NEG
2	Progesterone[b]	NEG (3/3)	NEG (3/3)	POS (1/1)	NT	NEG	NEG
2	Vinclozolin	NEG (3/3)	NEG (3/3)	NT	NT	NEG	NEG
3	Clomiphene citrate	POS (3/3)	NEG (1/1)	POS (1/1)	NT	POS	NEG
3	Coumestrol	POS (3/3)	POS (1/1)	POS (1/1)	POS (1/1)	POS	POS
3	Daidzein	POS (1/1)	POS (1/1)	POS (1/1)	POS (1/1)	POS	POS
3	Dibenzo[*a,h*]anthracene	NEG (1/1)	NEG (1/1)	NT	NT	NEG	NEG
3	Diethylhexyl phthalate	NEG (1/1)	NEG (1/1)	NT	NT	NEG	NEG
3	Estrone	POS (3/3)	POS (1/1)	POS (1/1)	POS (1/1)	POS	POS

Phase	Substance Name	Comprehensive		Confirmation		Overall	
		Hiyoshi	KFDA	Hiyoshi	KFDA	Hiyoshi	KFDA
3	Ethyl paraben	POS (1/1)	POS (1/1)	POS (1/1)	POS (1/1)	POS	POS
3	Hydroxyflutamide	NEG (1/1)	NEG (1/1)	NT	NT	NEG	NEG
3	Kepone	NEG (1/1)	POS (1/1)	POS (2/2)	POS (1/1)	NEG	POS
3	Morin	POS (1/1)	POS (1/1)	POS (1/1)	POS (1/1)	POS	POS
3	Norethynodrel	POS (3/3)	POS (1/1)	POS (1/1)	POS (1/1)	POS	POS
3	Propylthiouracil	NEG (1/1)	NEG (1/1)	NT	NT	NEG	NEG
3	Raloxifene HCl	NEG (1/1)	NEG (1/1)	NT	NT	NEG	NEG
3	Sodium azide	NEG (1/1)	NEG (1/1)	NT	NT	NEG	NEG

Abbreviations: CCi = CCi, Inc., KFDA = Korea Food and Drug Administration; POS = positive, NEG = negative, NT = not tested. Numbers in parentheses indicate number test results (POS or NEG)/number of plates tested.

[a] *p*-*n*-Nonylphenol and *o*,*p'*-DDT were tested in Phase 2 by CCi only; Hiyoshi and KFDA did not test these substances.

[b] *p*,*p'*-DDE and progesterone were tested in Phase 2 by Hiyoshi and KFDA only; CCi tested them during Phase 3.

Table 4-2 Summary of Agonist Comprehensive, Confirmation, and Overall Results at CCi

Phase	Substance Name	Comprehensive	Confirmation	Overall
2	17α-Ethinyl estradiol	POS (7/7)	POS (1/1)	POS
2	Apigenin	POS (3/3)	POS (1/1)	POS
2	Atrazine	NEG (3/3)	NT	NEG
2	Bisphenol A	POS (2/2)	POS (1/1)	POS
2	Bisphenol B	POS (2/2)	POS (1/1)	POS
2	Butylbenzyl phthalate	POS (4/4)	POS (1/1)	POS
2	Corticosterone	NEG (3/3)	NT	NEG
2	Diethylstilbestrol	POS (7/7)	POS (1/1)	POS
2	Flavone	POS (3/3)	POS (1/1)	POS
2	Genistein	POS (3/3)	POS (1/1)	POS
2	*p*-n-Nonylphenol[a]	POS (3/3)	POS (1/1)	POS
2	*o*,*p'* -DDT[a]	POS (3/3)	POS (1/1)	POS
2	*p*,*p'* -DDE[b]	POS (1/1)	POS (1/1)	POS

Phase	Substance Name	Comprehensive	Confirmation	Overall
2	Progesterone[b]	NEG (1/1)	NT	NEG
2	Resveratrol	POS (2/2)	POS (1/1)	POS
2	Tamoxifen	NEG (1/1)	NT	NEG
2	Vinclozolin	POS (3/3)	POS (1/1)	POS
3	12 –*O*-Tetradecanoylphorbol-13-acetate	NEG (1/1)	NEG (1/1)	NEG
3	17α-Estradiol	POS (2/2)	POS (1/1)	POS
3	17ß-Estradiol	POS (2/2)	POS (1/1)	POS
3	2-*sec*-Butylphenol	POS (1/1)	POS (1/1)	POS
3	2,4,5-Trichlorophenoxy acetic acid	NEG (1/1)	NEG (1/1)	NEG
3	4-Androstenedione	NEG (1/1)	NT	NEG
3	4-Cumylphenol	POS (1/1)	POS (1/1)	POS
3	4-Hydroxytamoxifen	NEG (1/1)	NT	NEG
3	4-*tert*-Octylphenol	POS (1/1)	POS (1/1)	POS
3	5α-Dihydrotestosterone	POS (1/1)	POS (1/1)	POS
3	Actinomycin D	NEG (1/1)	NT	NEG
3	Clomiphene citrate	POS (2/2)	POS (1/1)	POS
3	Coumestrol	POS (1/1)	POS (1/1)	POS
3	Daidzein	POS (1/1)	POS (2/2)	POS
3	Dexamethasone	NEG (1/1)	NEG (1/1)	NEG
3	Di-*n*-butyl phthalate	POS (1/1)	POS (1/1)	POS
3	Dibenzo[*a*,*h*]anthracene	NEG (1/1)	NT	NEG
3	Dicofol	POS (1/1)	POS (1/1)	POS
3	Diethylhexyl phthalate	POS (1/1)	POS (1/1)	POS
3	Estrone	POS (1/1)	POS (1/1)	POS
3	Ethyl paraben	POS (1/1)	POS (1/1)	POS
3	Fluoranthene	POS (1/1)	POS (1/1)	POS
3	Hydroxyflutamide	NEG (1/1)	NT	NEG
3	Kaempferol	POS (1/1)	POS (2/2)	POS

Phase	Substance Name	Comprehensive	Confirmation	Overall
3	Kepone	POS (1/1)	POS (1/1)	POS
3	*meso*-Hexestrol	POS (2/2)	POS (1/1)	POS
3	Methyl testosterone	POS (1/1)	POS (1/1)	POS
3	Morin	POS (1/1)	POS (1/1)	POS
3	Norethynodrel	POS (1/1)	POS (1/1)	POS
3	*p,p'*-Methoxychlor	POS (1/1)	POS (1/1)	POS
3	Phenolphthalin	POS (1/1)	POS (1/1)	POS
3	Propylthiouracil	NEG (1/1)	NT	NEG
3	Raloxifene HCl	NEG (1/1)	NT	NEG
3	Sodium azide	NEG (1/1)	NEG (1/1)	NEG
3	Testosterone	POS (2/2)	POS (2/2)	POS
4	17ß-Trenbolone	POS (1/1)	POS (1/1)	POS
4	19-Nortestosterone	POS (1/1)	POS (1/1)	POS
4	4-OH Androstenedione	NEG (1/1)	NT	NEG
4	Ammonium perchlorate	NEG (1/1)	NT	NEG
4	Apomorphine	NEG (1/1)	NT	NEG
4	Bicalutamide	NEG (1/1)	NT	NEG
4	Chrysin	POS (1/1)	POS (1/1)	POS
4	Cycloheximide	NEG (1/1)	NT	NEG
4	Cyproterone acetate	NEG (1/1)	NT	NEG
4	Fenarimol	POS (1/1)	NEG (1/1)	NEG
4	Finasteride	NEG (1/1)	NT	NEG
4	Fluoxymestrone	NEG (1/1)	NT	NEG
4	Flutamide	NEG (1/1)	NT	NEG
4	Haloperidol	NEG (1/1)	NT	NEG
4	Ketoconazole	NEG (1/1)	NT	NEG
4	L-Thyroxine	NEG (1/1)	NT	NEG
4	Linuron	NEG (1/1)	NT	NEG
4	Medroxyprogesterone acetate	NEG (1/1)	NT	NEG
4	Mifepristone	NEG (1/1)	NT	NEG
4	Nilutamide	NEG (1/1)	NT	NEG
4	Oxazepam	NEG (1/1)	NEG (1/1)	NEG

Phase	Substance Name	Comprehensive	Confirmation	Overall
4	Phenobarbital	NEG (1/1)	NT	NEG
4	Pimozide	NEG (1/1)	NT	NEG
4	Procymidone	NEG (1/1)	NEG (1/1)	NEG
4	Reserpine	NEG (1/1)	NT	NEG
4	Spironolactone	NEG (1/1)	NT	NEG

Abbreviations: CCi = CCi, Inc., KFDA = Korea Food and Drug Administration; POS = positive, NEG = negative, NT = not tested. Numbers in parentheses indicate number test results (POS or NEG)/number of plates tested.

[a]*p-n*-Nonylphenol and *o,p'*-DDT were tested in Phase 2 by CCi only; Hiyoshi and KFDA did not test these substances.

[b]*p,p'*-DDE and progesterone were tested in Phase 2 by Hiyoshi and KFDA only; CCi tested them during Phase 3.

Table 4-3 Summary of Antagonist Comprehensive, Confirmation, and Overall Results at Hiyoshi and KFDA

Phase	Substance Name	Comprehensive		Confirmation		Overall	
		Hiyoshi	KFDA	Hiyoshi	KFDA	Hiyoshi	KFDA
2	Apigenin	NEG (4/4)	NEG (3/3)	NT	NT	NEG	NEG
2	Atrazine	NEG (2/4)	POS (3/3)	NEG (1/1)	NEG (1/1)	NEG	NEG
2	Bisphenol B[a]	POS (3/3)	POS (3/3)	NEG (1/1)	NEG (1/1)	NEG	NEG
2	Butylbenzyl phthalate	NEG (2/3)	POS (3/3)	NEG (1/1)	NEG (1/1)	NEG	NEG
2	Corticosterone	POS (4/5)	POS (3/3)	NEG (1/1)	NEG (1/1)	NEG	NEG
2	Dibenzo[*a,h*]anthracene	POS (3/4)	POS (3/3)	NEG (1/1)	NEG (1/1)	NEG	NEG
2	Flavone	POS (4/4)	POS (3/3)	NEG (1/1)	NEG (1/1)	NEG	NEG
2	Genistein	NEG (2/4)	POS (3/3)	NEG (1/1)	NEG (1/1)	NEG	NEG
2	*p,p'* -DDE[a]	POS (2/3)	POS (2/3)	POS (1/1)	NT	POS	POS
2	Progesterone	POS (2/4)	POS (3/3)	POS (1/1)	POS (1/1)	POS	POS
2	Resveratrol	POS (3/3)	POS (3/3)	NEG (1/1)	NEG (1/1)	NEG	NEG
2	Tamoxifen	POS (3/4)	POS (3/3)	POS (1/1)	POS (1/1)	POS	POS
3	4-Hydroxytamoxifen	POS (2/2)	POS (1/1)	NEG (1/1)	NEG (1/1)	NEG	NEG

Phase	Substance Name	Comprehensive		Confirmation		Overall	
		Hiyoshi	KFDA	Hiyoshi	KFDA	Hiyoshi	KFDA
3	Clomiphene citrate	POS (2/2)	POS (1/1)	NEG (1/1)	NEG (1/1)	NEG	NEG
3	Coumestrol	NEG (1/2)	POS (1/1)	NEG (1/1)	NEG (1/1)	NEG	NEG
3	Daidzein	NEG (1/1)	POS (1/1)	NT	NEG (1/1)	NEG	NEG
3	Dexamethasone	NEG (1/1)	POS (1/1)	NT	POS (1/1)	NEG	POS
3	Diethylhexyl phthalate	POS (1/1)	POS (1/1)	POS (1/1)	POS (1/1)	POS	POS
3	Ethyl paraben	NEG (2/2)	POS (1/1)	NEG (1/1)	NT	NEG	POS
3	Fluoranthene	NEG (1/1)	NEG (1/1)	NT	NT	NEG	NEG
3	Hydroxyflutamide	NEG (1/1)	NEG (1/1)	NEG (1/1)	NEG (1/1)	NEG	NEG
3	Norethynodrel	NEG (1/1)	POS (1/1)	NEG (1/1)	NEG (1/1)	NEG	NEG
3	*p,p'*- Methoxychlor	POS (1/1)	POS (1/1)	NEG (1/1)	NEG (1/1)	NEG	NEG
3	Phenolphthalin	NEG (1/1)	POS (1/1)	NEG (1/1)	NEG (1/1)	NEG	NEG
3	Raloxifene HCl	POS (2/2)	NEG (1/1)	POS (1/1)	NT	POS	NEG
3	Sodium azide	POS (1/1)	POS (1/1)	NEG (1/1)	NEG (1/1)	NEG	NEG

Abbreviations: CCi = CCi, Inc., KFDA = Korea Food and Drug Administration; POS = positive, NEG = negative, NT = not tested. Numbers in parentheses indicate number test results (POS or NEG)/number of plates tested.

[a] Bisphenol B and *p,p'*-DDE were tested in Phase 2 by Hiyoshi and KFDA only; CCi tested them during Phase 3.

Table 4-4 Summary of Antagonist Comprehensive, Confirmation, and Overall Results at CCi

Phase	Substance Name	Comprehensive	Confirmation	Overall
2	Apigenin	NEG (4/7)	NEG (1/1)	NEG
2	Atrazine	NEG (6/7)	NT	NEG
2	Butylbenzyl phthalate	NEG (3/6)	NEG (1/1)	NEG
2	Corticosterone	POS (3/3)	NEG (2/2)	NEG
2	Dibenzo[*a,h*]anthracene	POS (6/6)	POS (1/1)	POS
2	Flavone	POS (6/6)	POS (1/1)	POS
2	Genistein	POS (3/4)	NEG (1/1)	NEG
2	*p-n*-Nonylphenol[b]	POS (3/3)	NEG (1/1)	NEG

Phase	Substance Name	Comprehensive	Confirmation	Overall
2	*o,p'* –DDT[b]	POS (3/3)	NEG (1/1)	NEG
2	Progesterone	POS (3/3)	NEG (1/1)	NEG
2	Resveratrol	POS (7/7)	NEG (1/1)	NEG
2	Tamoxifen	POS (6/6)	POS (1/1)	POS
3	12 –*O*-Tetradecanoylphorbol-13-acetate	POS (1/1)	NEG (1/1)	NEG
3	17α-Estradiol	NEG (1/1)	NT	NEG
3	17α-Ethinyl estradiol	NEG (1/1)	NEG (1/1)	NEG
3	17ß-Estradiol	NEG (1/1)	NEG (1/1)	NEG
3	2-*sec*-Butylphenol	NEG (1/1)	NEG (1/1)	NEG
3	2,4,5-Trichlorophenoxyacetic acid	NEG (1/1)	NEG (1/1)	NEG
3	4-Androstenedione	POS (2/2)	POS (1/1)	POS
3	4-Cumylphenol	NEG (1/1)	NT	NEG
3	4-Hydroxytamoxifen	POS (2/2)	POS (1/2)	POS
3	4-*tert*-Octylphenol	NEG (1/1)	NEG (1/1)	NEG
3	5α-Dihydrotestosterone	POS (1/2)	POS (1/1)	POS
3	Actinomycin D	POS (1/1)	NEG (1/1)	NEG
3	Bisphenol A	POS (1/1)	NEG (1/1)	NEG
3	Bisphenol B[a]	POS (1/1)	NEG (1/1)	NEG
3	Clomiphene citrate	POS (1/1)	POS (1/1)	POS
3	Coumestrol	NEG (1/1)	NT	NEG
3	Daidzein	NEG (1/1)	NT	NEG
3	Dexamethasone	POS (1/1)	NEG (1/1)	NEG
3	Diethylstilbestrol	POS (1/1)	NEG (1/1)	NEG
3	Di-*n*-butyl phthalate	NEG (1/1)	NEG (1/1)	NEG
3	Dicofol	NEG (1/1)	NEG (1/1)	NEG
3	Diethylhexyl phthalate	NEG (1/1)	NEG (1/1)	NEG
3	Estrone	NEG (1/1)	NT	NEG
3	Ethylparaben	NEG (1/1)	NT	NEG
3	Fluoranthene	NEG (2/2)	POS (1/1)	NEG
3	Hydroxyflutamide	POS (1/1)	POS (1/1)	POS
3	Kaempferol	NEG (1/1)	NT	NEG
3	Kepone	NEG (1/1)	NEG (1/1)	NEG
3	*meso*-Hexestrol	NEG (1/1)	NEG (1/1)	NEG

Phase	Substance Name	Comprehensive	Confirmation	Overall
3	Methyl testosterone	POS (1/2)	NEG (1/1)	NEG
3	Morin	NEG (1/1)	NT	NEG
3	Norethynodrel	NEG (1/1)	NT	NEG
3	*p,p'* -DDE[a]	NEG (2/2)	NEG (1/1)	NEG
3	*p,p'*- Methoxychlor	NEG (1/1)	NT	NEG
3	Phenolphthalin	POS (1/1)	POS (1/1)	POS
3	Propylthiouracil	NEG (1/1)	NT	NEG
3	Raloxifene HCl	POS (2/2)	POS (1/1)	POS
3	Sodium azide	POS (1/1)	NEG (1/1)	NEG
3	Testosterone	POS (1/2)	NEG (1/1)	NEG
3	Vinclozolin	NEG (1/1)	NT	NEG
4	17ß-Trenbolone	POS (1/1)	NEG (2/2)	NEG
4	19-Nortestosterone	POS (1/1)	NEG (1/1)	NEG
4	4-OH Androstenedione	POS (2/2)	POS (2/2)	POS
4	Ammonium perchlorate	POS (1/1)	NEG (1/1)	NEG
4	Apomorphine	POS (1/1)	NEG (1/1)	NEG
4	Bicalutamide	POS (1/1)	NEG (1/1)	NEG
4	Chrysin	POS (1/1)	POS (1/1)	POS
4	Cycloheximide	POS (1/1)	NEG (1/1)	NEG
4	Cyproterone acetate	POS (1/1)	NEG (1/1)	NEG
4	Fenarimol	POS (1/1)	NEG (1/1)	NEG
4	Finasteride	POS (1/1)	NEG (1/1)	NEG
4	Fluoxymestrone	POS (1/1)	NEG (1/1)	NEG
4	Flutamide	POS (1/1)	NEG (1/1)	NEG
4	Haloperidol	NEG (1/1)	NEG (1/1)	NEG
4	Ketoconazole	POS (1/1)	NEG (1/1)	NEG
4	L-Thyroxine	POS (1/1)	NEG (1/1)	NEG
4	Linuron	POS (1/1)	NEG (1/1)	NEG
4	Medroxyprogesterone acetate	POS (1/1)	NEG (1/1)	NEG
4	Mifepristone	POS (1/1)	NEG (1/1)	NEG
4	Nilutamide	POS (1/1)	NEG (1/1)	NEG
4	Oxazepam	POS (1/1)	NEG (1/1)	NEG
4	Phenobarbital	POS (1/1)	POS (1/1)	POS
4	Pimozide	NEG (1/1)	NEG (1/1)	NEG
4	Procymidone	POS (1/1)	NEG (1/1)	NEG
4	Reserpine	POS (1/1)	NEG (1/1)	NEG

Phase	Substance Name	Comprehensive	Confirmation	Overall
4	Spironolactone	POS (1/1)	NEG (1/1)	NEG

[a] Bisphenol B and *p,p'*-DDE were tested in Phase 2 by Hiyoshi and KFDA only; CCi tested them during Phase 3.
[b] *p-n*-Nonylphenol and *o,p'*-DDT were tested in Phase 2 by CCi only; Hiyoshi and KFDA did not test these substances.

5.0 Accuracy of the MCF-7 CP TM

This section discusses the accuracy of the MCF-7 CP TM in the multi-laboratory validation effort. Accuracy is evaluated by assessing the following:

- Accuracy: The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and one aspect of "relevance". The term is often used interchangeably with accuracy. Concordance is highly dependent on the prevalence of positives in the population being examined.
- Sensitivity: The proportion of all positive substances that are classified correctly as positive in a test method. It is a measure of test method accuracy.
- Specificity: The proportion of all negative substances that are classified correctly as negative in a test method. It is a measure of test method accuracy.
- False positive rate: The proportion of all negative (inactive) substances that are falsely identified by a test method as positive. It is one indicator of test method accuracy.
- False negative rate: The proportion of all positive (active) substances falsely identified by a test method as negative. It is one indicator of test method accuracy.

Each of these parameters can be calculated as follows (**Table 5-1**):

Table 5-1 Two-by-Two Table used for Accuracy Analysis

		Test Method Outcome		
		Positive	**Negative**	**Total**
Reference Test Classification	**Positive**	a	c	a+c
	Negative	b	d	b+d
	Total	a+b	c+d	a+b+c+d

a = positive in assay and positive by reference test classification
b = positive in assay and negative by reference test classification
c = negative in assay and positive by reference test classification
d = negative in assay and negative by reference test classification

Accuracy = ([a+d]/[a+b+c+d])
Sensitivity = (a/[a+c])
Specificity = (d/[b+d])
False positive rate = (b/[b+d])
False negative rate (c/[a+c])

The MCF-7 CP TM was evaluated for its ability to correctly classify ER agonists and antagonists both within and across laboratories as an aggregate measure of test method performance.

For these evaluations, test substances were assigned positive, or negative results. Classification of test substances is described in depth in **Section 2.12**. Briefly:

- Positive test substances are classified as positive in both comprehensive and confirmation tests.

- Negative test substances are those that were negative in comprehensive tests, or were positive in comprehensive tests, but did not pass confirmation standards.

For these analyses, test substance classifications (positive or negative for ER agonist/antagonist activity) obtained during the validation study (new test outcome) were compared with the classification of the same substance based on a preponderance of published data (reference test classification).

5.1 Substances Used for Accuracy Analysis

As reviewed in **Section 3.1**, in 2011, NICEATM completed a comprehensive literature review of available *in vitro* data (ICCVAM 2011) and identified 48 unique substances on the ICCVAM recommended list of 78 (ICCVAM 2003a, 2006b) that could be considered unequivocally positive or negative for ER agonist or antagonist activity. NICEATM/ICCVAM recommended these 48 substances (ICCVAM 2011) for use in the evaluation of test method accuracy for all future ER transcriptional activation and proliferation assays. **Table 5-2** lists the 41 substances tested to evaluate ER agonist accuracy (32 positive, 9 negative). **Table 5-3** lists the 25 substances tested to evaluate ER antagonist activity (3 positive, 22 negative). Nineteen substances were tested in both evaluations.

Table 5-2 41 ICCVAM-Recommended Substances Used to Evaluate ER Agonist Accuracy

Substance	CASRN	Classification			
		ICCVAM Consensus	CCi	Hiyoshi	KFDA
17∝-Estradiol	57-91-0	POS	Positive	NT	NT
17∝-Ethinyl estradiol	**57-63-6**	**POS**	**Positive**	**Positive**	**Positive**
17ß-Estradiol	50-28-2	POS	Positive	NT	NT
19-Nortestosterone	434-22-0	POS	Positive	NT	NT
4-Cumylphenol	599-64-4	POS	Positive	NT	NT
4-*tert*-Octylphenol	140-66-9	POS	Positive	NT	NT
5∝-Dihydrotestosterone	521-18-6	POS	Positive	NT	NT
Apigenin	520-36-5	POS	Positive	NT	NT
Atrazine	**1912-24-9**	**NEG**	**Negative**	**Positive**	**Negative**
Bicalutamide	90357-06-5	NEG	Negative	NT	NT
Bisphenol A	**80-05-7**	**POS**	**Positive**	**Positive**	**Positive**
Bisphenol B	**77-40-7**	**POS**	**Positive**	**Positive**	**Positive**
Butylbenzyl phthalate	**85-68-7**	**POS**	**Positive**	**Positive**	**Positive**
Chrysin	480-40-0	POS	Positive	NT	NT
Clomiphene citrate	**50-41-9**	**POS**	**Positive**	**Positive**	**Negative**
Corticosterone	**50-22-6**	**NEG**	**Negative**	**Negative**	**Negative**
Coumestrol	**479-13-0**	**POS**	**Positive**	**Positive**	**Positive**
Daidzein	**486-66-8**	**POS**	**Positive**	**Positive**	**Positive**
Dicofol	115-32-2	POS	Positive	NT	NT

Substance	CASRN	Classification			
		ICCVAM Consensus	CCi	Hiyoshi	KFDA
Diethylstilbestrol	**56-53-1**	**POS**	**Positive**	**Positive**	**Positive**
Estrone	**53-16-7**	**POS**	**Positive**	**Positive**	**Positive**
Ethylparaben	**120-47-8**	**POS**	**Positive**	**Positive**	**Positive**
Fenarimol	60168-88-9	POS	Negative	NT	NT
Flutamide	13311-84-7	NEG	Negative	NT	NT
Genistein	**446-72-0**	**POS**	**Positive**	**Positive**	**Positive**
Hydroxyflutamide	**52806-53-8**	**NEG**	**Negative**	**Negative**	**Negative**
Kaempferol	520-18-3	POS	Positive	NT	NT
Kepone	**143-50-0**	**POS**	**Positive**	**Positive**	**Positive**
Linuron	330-55-2	NEG	Negative	NT	NT
meso-Hexestrol	84-16-2	POS	Positive	NT	NT
Methyl testosterone	58-18-4	POS	Positive	NT	NT
Norethynodrel	**68-23-5**	**POS**	**Positive**	**Positive**	**Positive**
o,p'-DDT	789-02-6	POS	Positive	NT	NT
p-n-Nonylphenol	104-40-5	POS	Positive	NT	NT
***p,p'*-DDE**	**72-55-9**	**POS**	**Positive**	**Positive**	**Negative**
p,p'-Methoxychlor	72-43-5	POS	Positive	NT	NT
Phenobarbital	50-06-6	NEG	Negative	NT	NT
Procymidone	32809-16-8	NEG	Negative	NT	NT
Resveratrol	501-36-0	POS	Positive	NT	NT
Spironolactone	52-01-7	NEG	Negative	NT	NT
Tamoxifen	10540-29-1	POS	Negative	NT	NT

Abbreviations: MCF-7 CP TM = MCF-7 cellular proliferation test method; CASRN = CAS Registry Number (American Chemical Society); ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; KFDA = Korean Food and Drug Agency Laboratory; NEG = negative; NT = not tested; POS = positive.

Bolded text highlights the 17 accuracy substances that were tested in all three laboratories.

Table 5-3 25 ICCVAM-Recommended Substances Used to Evaluate ER Antagonist Accuracy

Substance	CASRN	Classification			
		ICCVAM Consensus	CCi	Hiyoshi	KFDA
17α–Ethinyl estradiol	57-63-6	NEG	Negative	NT	NT
4-Hydroxytamoxifen	**68047-06-3**	**POS**	**Positive**	**Negative**	**Negative**
5α-Dihydrotestosterone	521-18-6	NEG	Positive	NT	NT

Substance	CASRN	Classification			
		ICCVAM Consensus	CCi	Hiyoshi	KFDA
Apigenin	**520-36-5**	**NEG**	**Negative**	**Negative**	**Negative**
Bisphenol A	80-05-7	NEG	Negative	NT	NT
Butylbenzyl phthalate	**85-68-7**	**NEG**	**Negative**	**Negative**	**Negative**
Chrysin	480-40-0	NEG	Positive	NT	NT
Coumestrol	**479-13-0**	**NEG**	**Negative**	**Negative**	**Negative**
Daidzein	**486-66-8**	**NEG**	**Negative**	**Negative**	**Negative**
Di-*n*-butyl phthalate	84-74-2	NEG	Negative	NT	NT
Dicofol	115-32-2	NEG	Negative	NT	NT
Diethylhexyl phthalate	**117-81-7**	**NEG**	**Negative**	**Positive**	**Positive**
Diethylstilbestrol	56-53-1	NEG	Negative	NT	NT
Genistein	**446-72-0**	**NEG**	**Negative**	**Negative**	**Negative**
Kaempferol	520-18-3	NEG	Negative	NT	NT
Kepone	143-50-0	NEG	Negative	NT	NT
Mifepristone	84371-65-3	NEG	Negative	NT	NT
Norethynodrel	**68-23-5**	**NEG**	**Negative**	**Negative**	**Negative**
o,p'-DDT	789-02-6	NEG	Negative	NT	NT
p-n-Nonylphenol	104-40-5	NEG	Negative	NT	NT
***p,p'*-DDE**	**72-55-9**	**NEG**	**Negative**	**Positive**	**Positive**[a]
Progesterone	**57-83-0**	**NEG**	**Negative**	**Positive**	**Positive**
Raloxifene HCl	**82640-04-8**	**POS**	**Positive**	**Positive**	**Negative**
Resveratrol	**501-36-0**	**NEG**	**Negative**	**Negative**	**Negative**
Tamoxifen	**10540-29-1**	**POS**	**Positive**	**Positive**	**Positive**

Abbreviations: MCF-7 CP TM = MCF-7 cellular proliferation test method; CASRN = CAS Registry Number (American Chemical Society); ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; KFDA = Korean Food and Drug Agency Laboratory; NEG = negative; NT = not tested; POS = positive

Bolded text highlights the 13 accuracy substances that were tested in all three laboratories.

[a] Because no confirmation test was performed at KFDA, this substance was classified as positive.

5.2 Accuracy Analyses of the MCF-7 CP TM for ER Agonists

Accuracy of the MCF-7 CP TM for ER agonists was calculated for each laboratory. **Table 5-4** presents the individual laboratory accuracy results CCi tested all of the accuracy substances. Separate accuracy results for CCi are shown for all 41 accuracy substances and for the subset of 17 accuracy substances that were tested in all three laboratories. For the 17 accuracy substances, accuracy at the individual laboratories ranged from 88% (15/17) to 100% (17/17).

Table 5-4 Accuracy of the MCF-7 CP TM for ER Agonism

Laboratory	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
CCi	41[a]	95% (39/41)	94% (30/32)	100% (9/9)	0% (0/9)	6% (2/32)
CCi	17[b]	100% (17/17)	100% (14/14)	100% (3/3)	0% (0/14)	0% (0/3)
Hiyoshi	17[b]	94% (16/17)	100% (14/14)	67% (2/3)	33% (1/3)	0% (0/14)
KFDA	17[b]	88% (15/17)	86% (12/14)	100% (3/3)	0% (0/3)	14% (2/14)

Abbreviations: MCF-7 CP TM = MCF-7 cellular proliferation test method; N = number of substances.

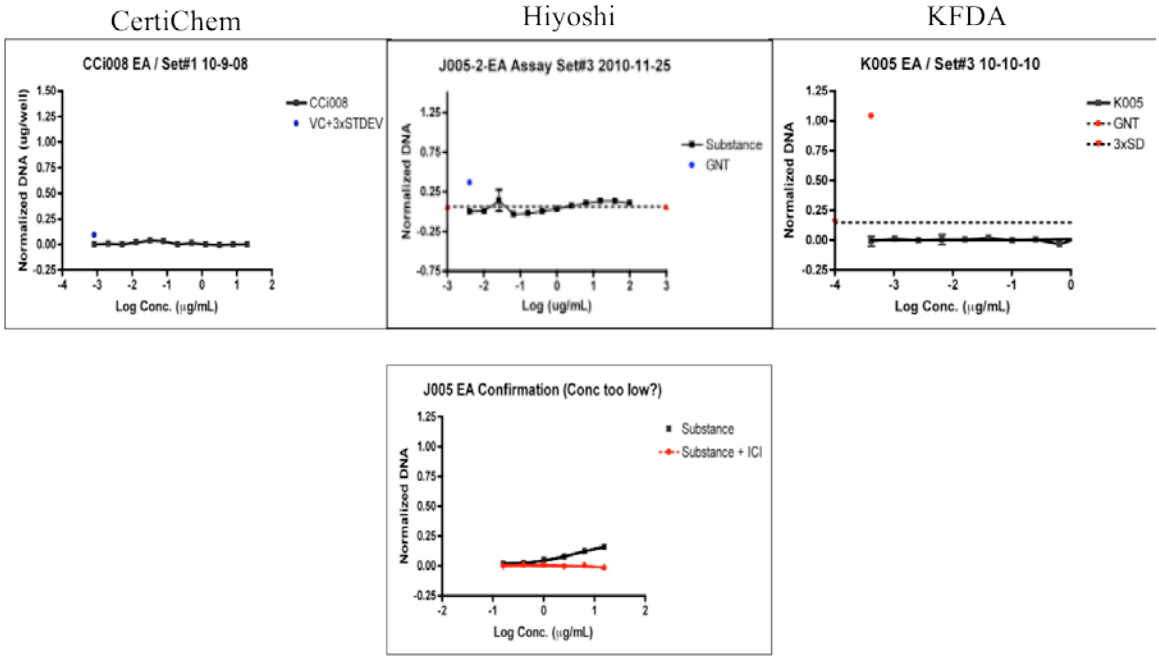
[a] The 41 accuracy substances were evaluated in the MCF-7 CP TM for ER agonists at CCi.

[b] Of the 41 accuracy substances, 17 substances were evaluated in the MCF-7 CP TM for ER agonists at all three laboratories.

5.2.1 Discordant Substances for the Accuracy Analysis of the MCF-7 CP TM for ER Agonists

Of the 17 accuracy substances that were tested at all three laboratories, 3 substances yielded results that were discordant with the ICCVAM reference result. Discordant results were obtained for atrazine at Hiyoshi (false positive) and for clomiphene citrate (false negative) and *p,p'*-DDE (false negative) at KFDA. Comprehensive, and, where applicable, confirmation graphs are presented in **Figures 5-1** to **5-3**. Where multiple comprehensive tests were conducted in a laboratory, a representative comprehensive graph has been selected.

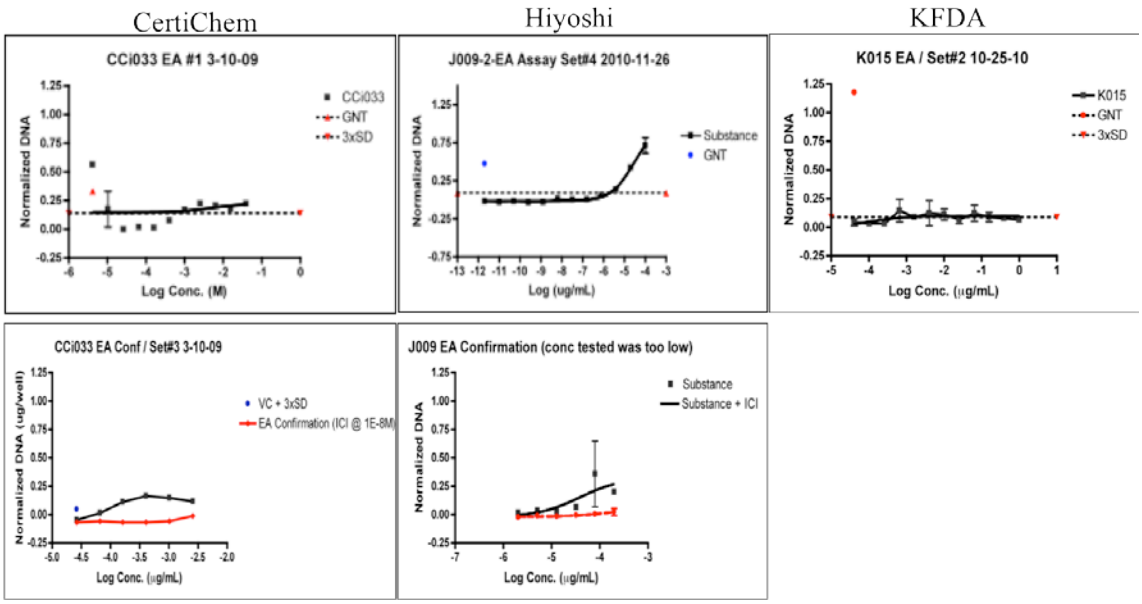
Figure 5-1 Atrazine Agonist Graphs



Abbreviations: Conc. = concentration; EA = estrogen agonist; GNT = genistein; ICI = ICI 182,780; SD = standard deviation; STDEV = standard deviation; VC = vehicle control.

Comprehensive tests at CCi and KFDA were negative. No confirmation testing was performed. Atrazine is classified as negative at CCi and KFDA. The comprehensive test at Hiyoshi was positive, as was the confirmation test (on bottom row). Atrazine was classified as positive at Hiyoshi. The ICCVAM consensus result is negative.

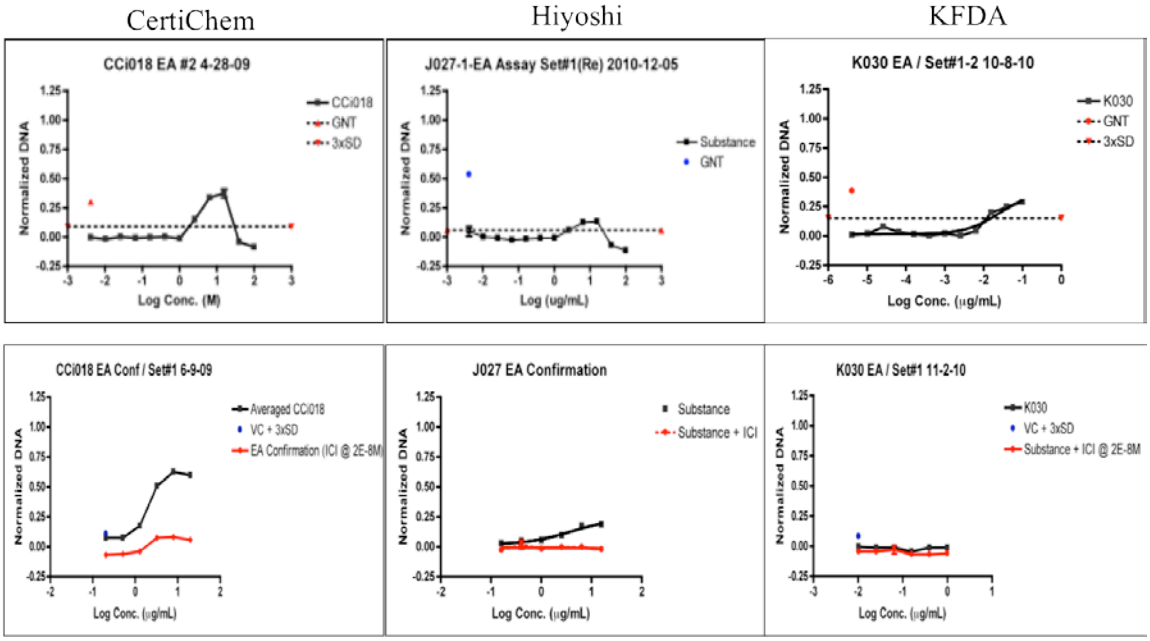
Figure 5-2 Clomiphene Agonist Graphs



Abbreviations: Conc. = concentration; EA = estrogen agonist; GNT = genistein; ICI = ICI 182,780; SD = standard deviation; VC = vehicle control.

Comprehensive tests at CCi and Hiyoshi were positive. Confirmation tests at CCi and Hiyoshi were positive and the classifications were positive. Comprehensive testing at KFDA was negative. No confirmation assay was performed. The ICCVAM consensus result is positive.

Figure 5-3 *p,p'*-DDE Agonist Graphs



Abbreviations: Conc. = concentration; EA = estrogen agonist; E2 = 17β-estradiol; GNT = genistein; ICI = ICI 182,780; SD = standard deviation; VC = vehicle control.

Comprehensive tests were positive at all three laboratories. Confirmation tests at CCi and Hiyoshi were positive. The confirmation test at KFDA was negative. The ICCVAM consensus result is positive.

5.3 Accuracy Analyses of the MCF-7 CP TM for ER Antagonists

Accuracy of the MCF-7 CP TM for ER antagonists was calculated for each laboratory. Separate accuracy results for CCi are shown for all 25 accuracy substances and for the subset of 13 accuracy substances that were tested by all three laboratories (**Table 5-5**). For the 13 accuracy substances, the accuracy at the individual laboratories ranged from 62% (8/13) to 92% (12/13).

Table 5-5 Accuracy of the MCF-7 CP TM for ER Antagonists

Laboratory	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
CCi	25[a]	92% (23/25)	100% (3/3)	91% (20/22)	9% (2/22)	0% (0/3)
CCi	13[b]	100% (13/13)	100% (3/3)	100% (10/10)	0% (0/10)	0% (0/3)
Hiyoshi	13[b]	69% (9/13)	67% (2/3)	70% (7/10)	30% (3/10)	33% (1/3)
KFDA	13[b]	62% (8/13)	33% (1/3)	70% (7/10)	30% (3/10)	67% (2/3)

Abbreviations: MCF-7 CP TM = MCF-7 cellular proliferation test method; N = number of substances.

[a] The 25 accuracy substances were evaluated in the MCF-7 CP TM for ER antagonists at CCi.

[b] Of the 25 accuracy substances, 13 substances were evaluated in the MCF-7 CP TM for ER antagonists at all three laboratories.

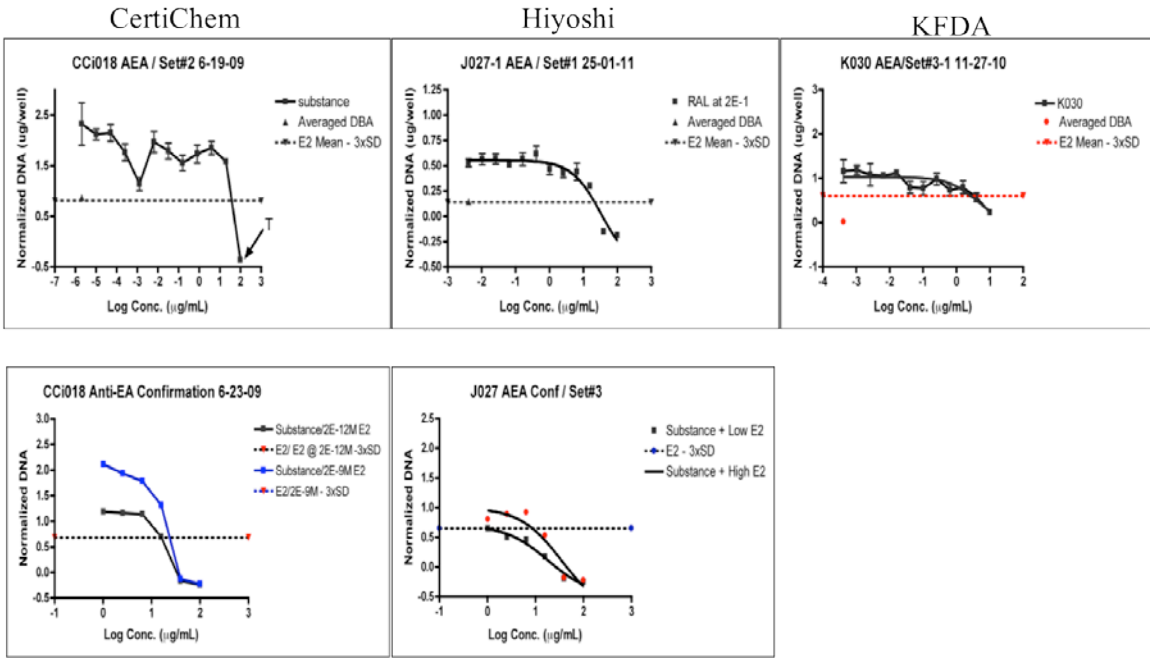
5.3.1 Discordant Substances for the Accuracy Analysis of the MCF-7 CP TM for ER Antagonists

Of the 13 accuracy substances that were tested at all three laboratories, five substances yielded results that were discordant with the ICCVAM reference result.

- *p,p'*-DDE was false positive at Hiyoshi and KFDA.
- Diethylhexyl phthalate was false positive at Hiyoshi and KFDA.
- Progesterone was false positive at Hiyoshi and KFDA.
- 4-Hydroxytamoxifen was false negative at Hiyoshi and KFDA.
- Raloxifene was false negative at KFDA.

Comprehensive, and, where applicable, confirmation graphs of the discordant antagonist accuracy substances are presented in **Figures 5-4** to **5-8**. Where multiple comprehensive tests were conducted in a laboratory, a representative comprehensive graph has been selected.

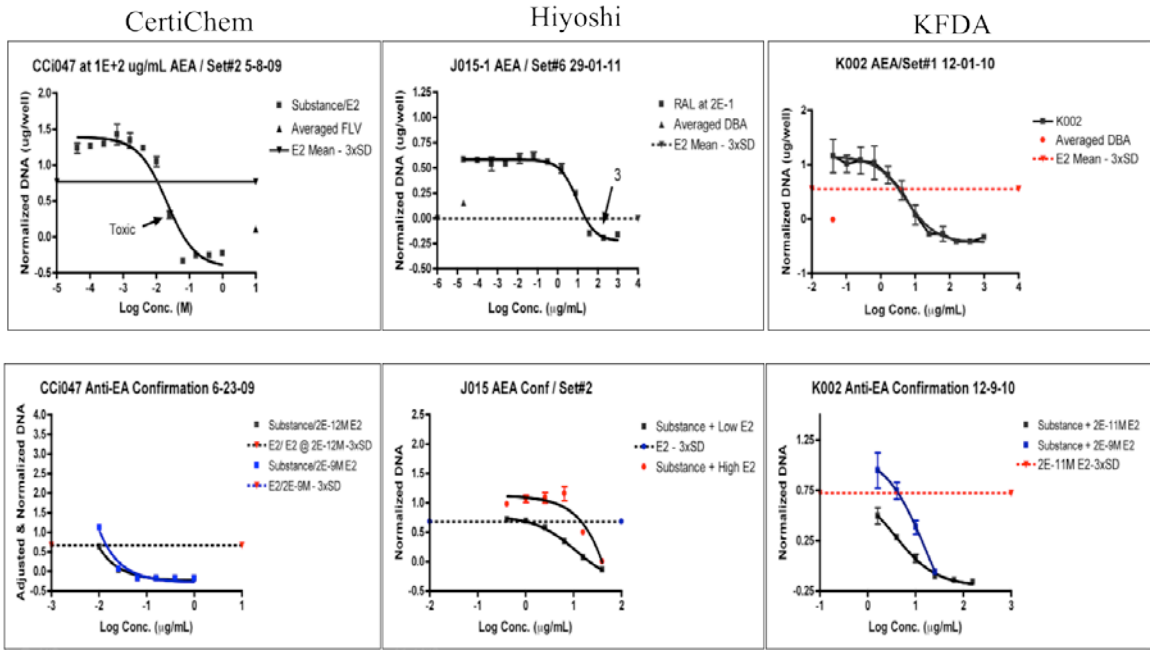
Figure 5-4 *p,p'*-DDE Antagonist Graphs



Abbreviations: AEA = estrogen antagonist; Conc. = concentration; DBA = dibenzo[a,h]anthracene; EA = estrogen agonist; E2 = 17β-estradiol; RAL = raloxifene; SD = standard deviation.

Comprehensive tests were positive at Hiyoshi and KFDA. Confirmation testing at Hiyoshi was positive. This substance was positive at Hiyoshi. Because no confirmation test was performed at KFDA, this substance was classified as unconfirmed positive. CCi confirmation testing, which should not have been conducted for this substance, was negative. This substance was therefore classified as negative at CCi. The ICCVAM consensus for the substance is negative.

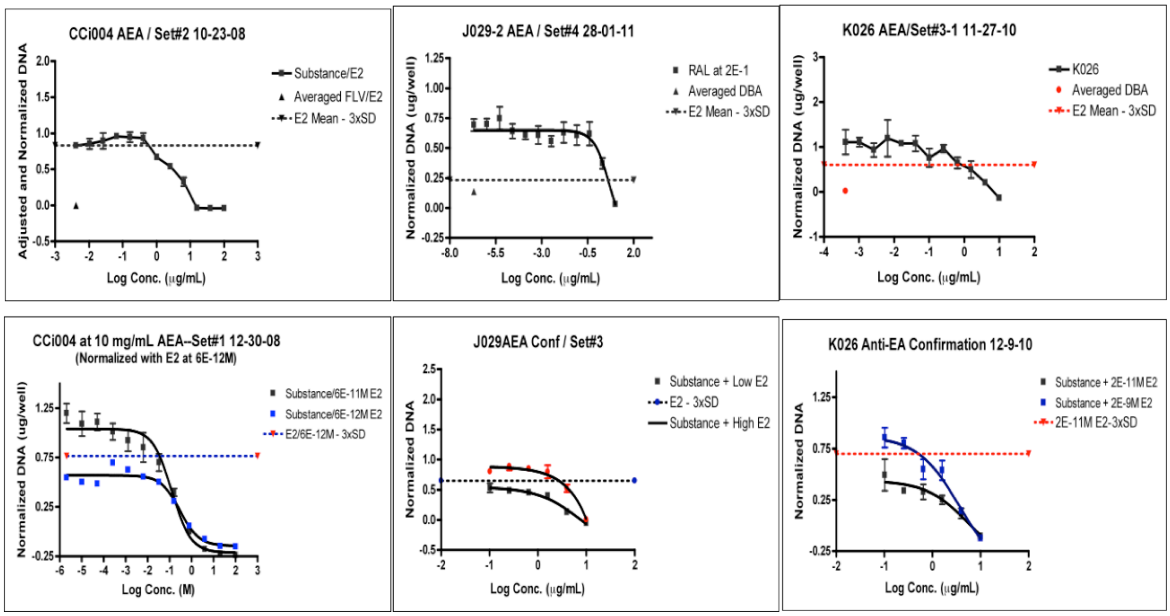
Figure 5-5 Diethylhexyl Phthalate Antagonist Graphs



Abbreviations: AEA = estrogen antagonist; Conc. = concentration; DBA = dibenzo[a,h]anthracene; EA = estrogen agonist; E2 = 17β-estradiol; FLV = flavone; RAL = raloxifene; SD = standard deviation.

Comprehensive tests were positive at Hiyoshi and KFDA. Confirmation testing at Hiyoshi and KFDA was positive. This substance was positive at Hiyoshi and KFDA. CCi confirmation testing, which should not have been conducted for this substance, was negative. This substance was negative at CCi. The ICCVAM consensus result is negative.

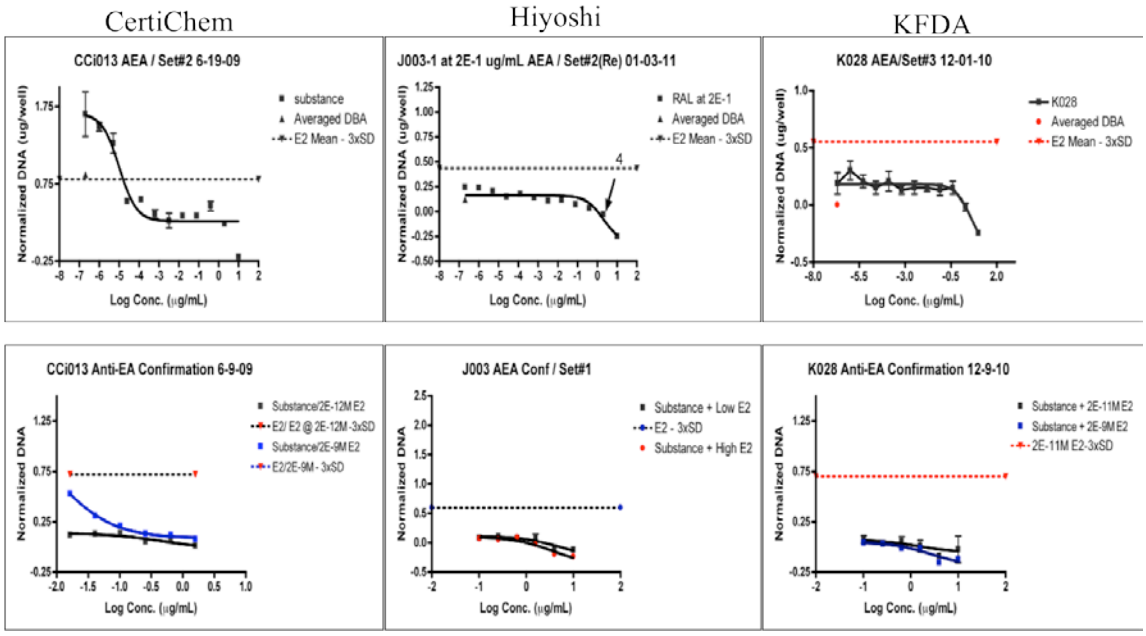
Figure 5-6 Progesterone Antagonist Graphs

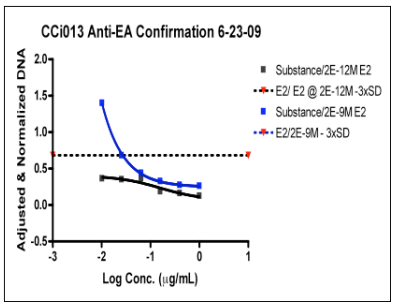


Abbreviations: AEA = estrogen antagonist; Conc. = concentration; DBA = dibenzo[a,h]anthracene; EA = estrogen agonist; E2 = 17β-estradiol; FLV = flavone; RAL = raloxifene; SD = standard deviation.

Comprehensive tests were positive at CCi, Hiyoshi, and KFDA. The confirmation test at CCi was negative. Confirmation tests at Hiyoshi and KFDA were positive. This substance was therefore classified as positive at Hiyoshi and KFDA. The ICCVAM consensus for this substance is negative.

Figure 5-7 4-Hydroxytamoxifen Antagonist Graphs

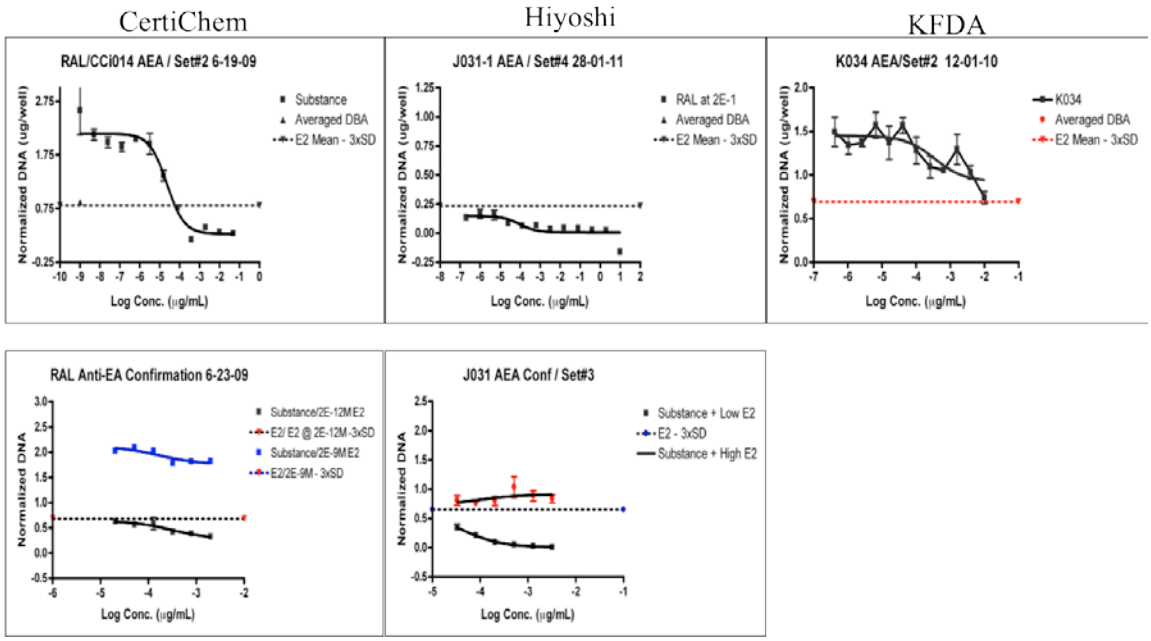




Abbreviations: AEA = estrogen antagonist; Conc. = concentration; DBA = dibenzo[a,h]anthracene; EA = estrogen agonist; E2 = 17β-estradiol; RAL = raloxifene; SD = standard deviation.

Comprehensive tests were positive at CCi, Hiyoshi, and KFDA. The first confirmation test at CCi was negative. The second confirmation test at CCi was positive. This substance was classified as positive at CCi. Confirmation tests at Hiyoshi and KFDA were negative. This substance was therefore classified as negative at Hiyoshi and KFDA. The ICCVAM consensus for this substance is positive.

Figure 5-8 Raloxifene HCl Antagonist Graphs



Abbreviations: AEA = estrogen antagonist; Conc. = concentration; DBA = dibenzo[a,h]anthracene; EA = estrogen agonist; E2 = 17β-estradiol; RAL = raloxifene; SD = standard deviation.

Comprehensive tests were positive at CCi and Hiyoshi. Confirmation tests were positive at CCi and Hiyoshi. This substance was positive at CCi and Hiyoshi. The comprehensive test at KFDA for this substance was negative. It was not confirmation tested. This substance was therefore classified as negative at KFDA. The ICCVAM consensus for this substance is positive.

6.0 Test Method Reliability

An assessment of test method reliability (intra- and interlaboratory reproducibility) is an essential element of any performance evaluation of an alternative test method (ICCVAM 2003b, 2003a). Intralaboratory reproducibility refers to the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol.

Interlaboratory reproducibility refers to the extent to which different laboratories can replicate results using the same protocol and test substances; it is indicative of successful test method transferability

among laboratories. Interlaboratory reproducibility was evaluated using overall results (comprehensive and confirmation) for each test substance.

This section describes the reliability assessment for the MCF-7 CP TM.

6.1 Intralaboratory Reproducibility

As described in **Section 2.0**, test substances were initially classified as positive or negative for ER agonist or antagonist activity based on a specific set of criteria that were applied to comprehensive tests. Substances that tested positive in comprehensive tests were tested in confirmation tests. During Phase 2, comprehensive tests were conducted a minimum of three times at each laboratory. Intralaboratory reproducibility was evaluated by comparing the results of repeated comprehensive testing within each laboratory. The confirmation test results were not used because only one confirmation test per positive substance was performed at each laboratory.

6.1.1 Intralaboratory Reproducibility of Phase 2 Agonist Reference Substances

Two Phase 2 agonist substances tested by CCi were different from those tested by Hiyoshi and KFDA. CCi tested *p*-*n*-nonylphenol and *o,p'*-DDT during Phase 2. Hiyoshi and KFDA did not test these substances; they tested *p,p'*-DDE and progesterone in Phase 2. However, CCi tested *p,p'*-DDE and progesterone during Phase 3. Therefore, the 12 agonist substances used to evaluate intra- and interlaboratory reproducibility included *p,p'*-DDE and progesterone since they were tested multiple times by all laboratories, albeit in Phase 3 by CCi.

The comprehensive classifications for the 12 substances that were tested at least three times at each laboratory (except for bisphenol A, bisphenol B, and vinclozolin which were tested twice at CCi) were used to evaluate intralaboratory reproducibility (see **Table 6-1**). Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the repeat tests for all 12 substances.

Table 6-1 Intralaboratory Reproducibility for 12 Phase 2 Agonist Substances

	CCi	Hiyoshi	KFDA
Concordant Substances /Total (Percentage)	12/12 (100%)	12/12 (100%)	12/12 (100%)

Abbreviations: CCi = CertiChem; KFDA = Korean Food and Drug Administration

6.1.2 Intralaboratory Reproducibility of Phase 2 Antagonist Reference Substances

Two Phase 2 antagonist substances tested by CCi were different from those tested by Hiyoshi and KFDA. CCi tested *p*-*n*-nonylphenol and *o,p'*-DDT during Phase 2. Hiyoshi and KFDA did not test these substances; they tested *p,p'*-DDE and bisphenol B in Phase 2. However, CCi tested *p,p'*-DDE and bisphenol B during Phase 3. Therefore, the 12 antagonist substances used to evaluate intra- and interlaboratory reproducibility included *p,p'*-DDE and bisphenol B since they were tested by all laboratories, albeit in Phase 3 by CCi.

The comprehensive classifications for the 12 substances that were tested at least three times at each laboratory were used to evaluate intralaboratory reproducibility (see **Table 6-2**). Agreement of the repeat tests ranged from 33% (4/12) at Hiyoshi to 92% (11/12) at KFDA.

Table 6-2 Intralaboratory Reproducibility for 12 Phase 2 Antagonist Substances

	CCi	Hiyoshi	KFDA
Concordant Substances /Total (Percentage)	8/12 (67%)	4/12 (33%)	11/12 (92%)

Abbreviations: CCi = CertiChem; KFDA = Korean Food and Drug Administration

6.2 Interlaboratory Reproducibility

The classifications for the 26 substances that were tested by all three laboratories for agonist and antagonist activity during Phases 2 and 3 were used to evaluate interlaboratory reproducibility.

6.2.1 Interlaboratory Reproducibility of Phase 2 and 3 Substances

For the 12 Phase 2 substances and 14 Phase 3 substances that were tested by all three laboratories, agreement among the laboratories was determined based on the overall classification (comprehensive and confirmation) assigned by each laboratory (see **Tables 4-1** through **4-4** for agonist and antagonist results). For agonist testing, there was concordance for 19 of 26 (73%) substances, while for antagonist testing, 14/26 (54%) of substances were concordant between labs (**Table 6-3**). Of the concordant agonist test substances, 14 were classified as positive (agonist) while 5 were negative. For the antagonist results, all 14 substances were classified by the three labs as negative (non-antagonist).

Table 6-3 Interlaboratory Reproducibility for 26 Phase 2 and 3 Substances

	Agonist Test	Antagonist Test
Concordant Substances /Total (Percentage)	19/26 (73%)	14/26 (54%)

6.3 Confirmation Tests

Confirmation testing was performed if a substance was positive in the comprehensive test, as described in **Section 2.10**. For a number of test substances, particularly in the antagonist assay, a single confirmation test result changed the overall classification of a substance that had been tested multiple times on multiple days.

6.3.1 Agonist Confirmation Tests

At CCi, 47 test substances underwent confirmation testing; one overall classification (2%) for fenarimol was changed from positive to negative. Hiyoshi performed 18 confirmation tests resulting in three test substances (17%) with overall classifications opposite from the comprehensive test result (corticosterone, kepone, and progesterone). KFDA performed confirmation tests on 14 substances; only the overall classification for *p,p'*-DDE (7%) was changed from positive to negative by the confirmation test result. Progesterone comprehensive test results were negative at Hiyoshi, and although not necessary under the protocol, a confirmation test was performed, resulting in a positive result and overall positive classification.

6.3.2 Antagonist Confirmation Tests

At CCi, 65 out of 78 total test substances underwent antagonist confirmation testing. A total of 38 (55%) had confirmation test results different from comprehensive test results. At Hiyoshi, out of the 26 substances tested, 22 underwent confirmation testing; 10 of these (45%) had results different from the comprehensive tests. KFDA also tested 26 total substances. Of these, 21 of them underwent confirmation testing, with 16 substances (76%) having different comprehensive and confirmation results.

6.3.3 Unconfirmed Positive Overall Classification

Only the MCF-7 CP TM for antagonists had any results classified as unconfirmed positive, which occurred when a positive comprehensive test result was not followed with a confirmation test, as described in **Section 2**. KFDA had two unconfirmed positive overall classifications for this reason (*p,p'*-DDE and ethyl paraben). For the purposes of accuracy evaluation, these two substances were treated as positive.

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