

TEST METHOD NOMINATION: MCF-7 CELL PROLIFERATION ASSAY OF ESTROGENIC ACTIVITY

This nomination application includes no confidential information.

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EXECUTIVE SUMMARY

Rational for the Proposed Test Method

On 6/4/04, in a letter to Dr. William Stokes, CertiChem, Inc. (CCi) nominated its MCF-7 Cell Proliferation Assay in manual and robotic format for validation studies of *in vitro* screens for estrogenic activity (EA). CCi nominated its test method for validation by NICEATM and ICCVAM because our assay is sensitive, reproducible, reliable and accurate in manual or robotic format when our results using this assay are compared to previously published *in vitro* data, such as those listed in Table 4 of ICCVAM, 2003. In brief, the need for a valid EA assay, especially in robotic format, is as follows:

- Recent scientific investigations have shown that many chemicals used to manufacture plastics, pharmaceuticals, pesticides, cosmetics, foodstuffs, etc. are endocrine disruptors (EDs) that interfere in various ways with the actions of estrogen, androgen or thyroid hormones (EDSTAC, 1998; NRC, 1999; EPA, 2000; ICCVAM, 2002a-c, 2003).
- The most common ED activity is EA that can have significant deleterious effects on many physiological processes. Most importantly, adverse EA effects sometimes occur at very low (picomolar to nanomolar) concentrations, especially on fetal or developing mammals, including humans (EDSTAC, 1998; NRC, 1999; NTP, 2001; ICCVAM, 2002a-c, 2003; Palanza, 2002).
- Since a large number of chemicals should be screened for possible EA, the EPA and ICCVAM have set a high priority on the development of high throughput, *in vitro*, robotic screening assays for EA (EDSTAC, 1998; ICCVAM 2002a-c, 2003).
- * Compared to *in vivo* assays, *in vitro* robotic assays are much cheaper and faster to run, and do not utilize live animals.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) was established in 1997 by the NIEHS and EPA (Public Law P.L.103-43), in part to develop and validate new ED test methods. ICCVAM was authorized in 2000 (P.L.106-545), as a 15 agency permanent committee, to co-ordinate the development, validation and acceptance of toxicological tests. As part of this mandate, ICCVAM and NICEATM [National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternate Toxicological Methods] formed an Endocrine Disruptor Working Group to assist ICCVAM in the evaluation status of assays for EDs. EDSTAC (1998) and ICCVAM (2002a,b) recommended that ER-dependent transcriptional activation (TA) assays (including cell proliferation assays) be developed because such "functional" assays are more sensitive than Relative Binding Affinity (RBA) assays, can distinguish agonists from antagonists, and can be conducted with and without exogenous metabolic activation. ICCVAM and NICEATM have concluded that "no validation studies have been completed on the [Transcriptional Activation (TA)] assays for EA being considered" (ICCVAM, 2002b, page 2) and have expressed a general preference (ICCVAM, 2002b, page 31) for the use of human ER subtypes in any *in vitro* TA screening assay. Given the large number of chemicals that should be screened for possible EA, the EPA, NIEHS and other congressionally-mandated interagency bodies (ICCVAM, NICEATM) for some years have also set a high priority on the development of high throughput, *in vitro*, robotic assays for EA (EDSTAC, 1998; ICCVAM 2002 a,b,c).

CCi's response to these needs expressed by ICCVAM and NICEATM

CCi, in a Phase I SBIR grant (R44 ESO11469-01), demonstrated the feasibility of developing a very sensitive, rapid, inexpensive *in vitro*, robotic assay for EA/anti-EA using proliferation of MCF-7 human breast cancer cells that meets published standards (ICCVAM, 2002a,b,c) for transcriptional activation (TA) assays of EA. CCi has started a Phase II SBIR grant (R44 ESO11469-02) to prepare this assay for commercialization in robotic format. Both Phase I&II grants received very favorable reviews from NIH Study Sections.

Overview of Test Method

CCi chose this assay because MCF-7 cells are perhaps the most widely used model of estrogen action *in vitro*. The MCF-7 cell line was derived from a human breast cancer (Soule et al., 1973). Estrogen-dependent cell proliferation by this cell line (Lippman et al., 1975, 1976) and the presence of estrogen receptors (ERs) were described simultaneously (Horwitz et al., 1975). ER- α and ER- β bind a large number of chemical ligands (Gorski et al. 1986; Kuiper et al., 1996, 1997, 1998). While binding affinities differ between estrogenic ligands (Kuiper, 1997), ER ligands typically bind to both receptors (ICCVAM, 2002a, 2003a; Routhledge et al., 2003). Both ERs bind to estrogen response elements, which are located upstream of the promoter regions of estrogen-activated genes (Paech et al., 1997; McDonnell and Norris, 2002). Chemicals with EA or anti-EA can bind to nuclear or extra-nuclear receptors (**Fig. 3, page 14**; Hewitt et al., 2005; Evinger and Levin, 2005). Our MCF-7 cell line contains both ER- α and ER- β , with ER- α dominating (V.C Jordan, personal communication). CCi's test method meets or exceeds all ICCVAM standards for TA assays of EA.

CCi's test method uses equipment and chemicals that are commercially available. Briefly, MCF-7 cells are maintained at 37°C in RMPI with Phenol Red for routine culture. An aliquot of cells maintained at 37°C are grown for two days in phenol-free media containing 5% charcoal stripped fetal bovine serum in a 25 cm² flask. Using a robotic dispenser such as an epMotion 5070 unit, MCF-7 cells are then seeded at 400 cells per well in 0.2 ml of this hormone-free medium in Corning 96-well plates. In its robotic format, the cells are adapted for 3 days in hormone-free culture medium prior to adding test chemicals. The media is changed daily for 6 days. At the end of the 7 day exposure, the media is removed, the wells are washed once with 0.2 ml of HBSS, and then assayed to quantify amounts of DNA/ well using a micro-plate modification of the Burton diphenylamine (DPA) assay (Burton, 1956; Natarajan et al., 1994). [The MCF-7 assay is more accurate, and less variable, when run in this robotic format.]

Substances Used for Validation of Proposed Test Method

CCi has tested about 45 chemicals to date from the list of 78 EA positive and EA negative reference chemicals recommended by ICCVAM (2003a) to assess the accuracy of TA assays for EA. Other chemicals tested are from a previous list of reference chemicals (ICCVAM, 2002c) to assess the accuracy of TA assays for EA. Yet other chemicals are from ICCVAM lists of reference chemicals recommended to assess the accuracy of *in vitro* assays for toxicity (ICCVAM) or chemicals that would not be expected to exhibit EA from QSAR studies and other considerations. This relatively large sample size was chosen to enable a rather accurate assessment of the rate of false negatives and false positives (the accuracy) of this MCF-7 cell proliferation assay to assess EA.

***In vivo* Reference Data**

This nomination application does not discuss similarities or differences in the endpoint measured in CCi's assay [MCF-7 cell proliferation] versus currently used *in vivo* test methods, other *in vitro* test methods, or human data *because no such validated data exist, to the best of our knowledge*. This nomination application includes no validation studies or references to other

validation studies, as such studies have not yet been performed or published by any laboratory or agency, to the best of our knowledge.

Accuracy and Reliability of the Proposed Test Method

Our results show that CCI's EA assay is very consistent (reliable, repeatable) when analyzing the same set of 9-10 chemicals in three different laboratories for a 3-12 month period (**Appendix A Figures 1-3 and Table 1, 4-5**). Furthermore, **Appendix A Figures 4-5 and Appendix A Tables 7** show that CCI's EA assay gives very similar data when run in robotic or manual format. **Table 6 in Appendix A** shows the comparison of the EC50s for 18 ICCVAM chemicals published by ICCVAM in a meta-study and from our assay in robotic format. CCI's robotic assay produces lower EC50s for most chemicals compared to the EC50s published by ICCVAM, indicating that CCI's robotic assay is relatively more sensitive than other assays referenced in ICCVAM's (2003a) meta-study.

Appendix A Tables 7-9 and Appendix A Figures 4-5 show *mean* EC50 values from CCI's assay and *median* EC50 values for the same set of chemicals compiled by ICCVAM (2003a) in a meta-analysis to provide a set of reference chemicals to be used to test the accuracy of EA TA assays. A comparison of CCI's data with ICCVAM's published data suggests that CCI's robotic EA assay is accurately measuring EA. For example, **Appendix A Table 10** shows that the rate of false negatives (4/29) and false positives (1/11) to date is very low when CCI's data are compared to ICCVAM's meta-study (2003a). The four false negatives are 4-hydroxytamoxifen, dexamethasone, 2,4,5-trichloro-phenoxyacetic acid, and clomiphene citrate. These chemicals were not conclusively analyzed in the ICCVAM meta-analysis. For example, ICCVAM (2003a) reported only one study as EA positive for dexamethasone and only one for 2,4,5-trichloro-phenoxyacetic acid. ICCVAM (2003a) reported 3/8 studies as EA positive for 4-hydroxytamoxifen, i.e. 5/8 reported the compound as EA negative. ICCVAM (2003a) anticipated that clomiphene citrate should be EA positive, but this chemical had not been tested for EA in any published TA study analyzed by ICCVAM (2003a). The one possible false positive (mifepristone) reported by CCI using its robotic EA assay has recently been reported to exhibit EA in other assays (ICCVAM, personal communications). Furthermore, CCI's robotic MCF-7 assay detected no false positives (0/34) for 34 other chemicals that have been reported to have no detectable EA in various *in vitro* or QSAR studies.[ICCVAM did not include these chemicals in its 2003a meta-study.]

The conclusion that CCI's proposed test method is accurate is further documented by **Appendix A Tables 11-12** that compare the rankings of CCI's mean EC50 values for a set of ICCVAM (2003a) reference chemicals to their median EC50 values (**Appendix A Table 11 as given by ICCVAM**. [ICCVAM gives no EC50 values for several reference chemicals declared to be EA positive; CCI *has* determined EC50 values for these same reference chemicals.]) **Appendix A Figure 6A** plots the CCI ranking (y axis) against the ICCVAM ranking for the chemicals listed in **Appendix A Table 11A**. **Appendix A Figure 6 B and C** plot the CCI ranking (y axis) against the MU and NWU rankings (x axes) for the chemicals listed in **Appendix A Table 11B**. **Appendix A Figure 6** also shows the least squares regression line that best fits these data. The least squares regression analysis shows that the rankings produced by CCI's proposed test method and the ICCVAM rankings (Figure 6A) do not differ significantly (null hypothesis, $p < 0.0001$). Figures 6B and 6C show that CCI's rankings do not differ significantly from those obtained at MU or NWU. That is, if one assumes that the ICCVAM rankings are reliable and accurate (valid), then **Figure 6A** suggests that CCI's test method is also valid. Furthermore, **Figures 6B and C** suggest that CCI's test method is repeatable (reliable) when run in different laboratories.

Animal Welfare and Practical Considerations of this Assay

- This *in vitro* assay can reduce animal use and does not use radioactive materials, in compliance with ICCVAM recommended protocols.
- This assay is highly sensitive, i.e. capable of detecting chemicals with high EA (e.g. beta-estradiol, diethylstilbesterol) at less than picomolar concentrations and chemicals with low EA (octylphenol, estriol) at less than micromolar concentrations.
- This assay can measure the EA of single chemicals, as well as complex mixtures of known and/or unknown chemicals.
- This assay requires only a small amount of a suspect chemical or chemical mixture.
- This assay cost is low to measure a suspect chemical or chemical mixture.
- The time (days) to measure a suspect chemical or chemical mixture is short.
- The manual and robotic protocols for this assay meet or exceed all published ICCVAM recommendations (ICCVAM, 2002a-c, 2003).
- The data obtained using this assay in manual or robotic format are reliable, i.e. reproducible within and between laboratories.
- The data obtained using this assay are accurate, i.e. the data contain very few, if any, false negatives or false positives when compared with data compiled and published by ICCVAM (2000a-c, 2003).
- This assay has excellent potential to be a good predictor of adverse health or environmental effects of chemicals having high or low EA.
- This assay should be applicable to the needs of various federal agencies (FDA, DOD, DHS, DOA) and commercial firms, especially since CCI has developed procedures to screen animal feeds, human foodstuffs, aqueous extracts from plastics, etc.

1: INTRODUCTION AND RATIONALE FOR THE PROPOSED TEST METHOD

MCF-7 Cell Proliferation Assay for Estrogenic Activity

1.1. INTRODUCTION

1.1.1. Historical Background

Description of Endocrine Disruptors

Many chemicals used in the manufacture of various products act as agonists or antagonists of androgenic or estrogenic hormones, while other chemicals interfere in multiple ways with the action of thyroid hormones (Brouwer, 1998; EDSTAC, 1998; NRC, 1999; ICCVAM, 2002a,b,c; Palanza et al., 2002; Singleton and Khan, 2003). Many studies of wildlife and laboratory animals exposed to such chemicals have shown that EDs adversely affect many physiological processes, such as: brain activity (behavior), reproduction, immune response, growth, development, and metabolic rate (Tyler, 1998; McLachlan, 2001; Guillette and Gunderson, 2001; Hayes et al., 2002; Markey et al., 2003). ED effects can be either gross or subtle when tested in animal model systems. Similar ED effects are almost certainly produced in humans, since basic endocrine mechanisms have been highly conserved across all classes of vertebrates (Kavlock et al., 1996; NRC, 1999; Thornton, 2001; Calafat et al., 2005; vom Saal et al. 2005). EDs can produce abnormal physical and/or behavioral effects ranging from increased risk of hypospadias, cryptorchidism, and vaginal carcinoma to impaired mental development, particularly when exposure occurs during critical stages of development, from early fetal stages through puberty (Juberg, 2000; Goldman, 2000; Baskin, 2001; Kawai et al., 2003; Markey et al., 2003).

Estrogenic activity (EA) is the most commonly known ED effect. In general, estrogenic EDs can produce fetal pathophysiology, abnormal brain maturation, reduced sperm count, prostate enlargement, ovarian and uterine dysfunction, learning disabilities, disorders of attention, motivation, emotion, and cognitive development, including changes in sexual orientation (Hines, 1992; EDSTAC, 1998; NRC, 1999; Bonde and Storgaard, 2002; Calafat et al. 2005). *In vivo* data from mice and rats have shown that exposure to estrogenic EDs at various developmental stages is associated with alterations in: the reproductive organs of infants and adults (vom Saal and Timms, 1999; Gray, 1998; Welshons et al., 1999; Baskin, 2001; Al Hiyasat and Elbetieha, 2004; Newbold et al., 2004), the rate of growth and time to sexual maturation (Howdeshell et al., 1999, 2000), and aggressive behavior (Palanza et al, 1999; Kawai et al., 2003).

History of Federal Regulation of EDs

Experimental data from *in vitro*, *in vivo*, ecological, and epidemiological studies showing that particular chemicals or chemical formulations possess varying degrees of ED activity have elicited concern from governmental bodies (EDSTAC, 1999; ICCVAM, 2002a,b,c), commercial entities (EDSTAC, 1998), non-profit organizations (Jordan et al., 2000), and scientific panels (NRC, 1999; Jordan et al., 2000; NTP,2001). In response to such concerns about ED effects of chemicals on humans and wildlife, the US Congress passed amendments to the Food Quality Protection Act (1996) and the Safe Drinking Water Act (1996) that require chemicals be tested for ED activity, with particular attention given to EA. To accomplish this goal, the EPA formed a committee, The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), to examine whether current toxicological testing procedures are adequate to determine ED activity.

The EDSTAC concluded that prior methods of testing for chemical toxicity had been inadequate to detect many types of ED (EDSTAC, 1998). This committee therefore recommended that many thousands of chemicals be tested in an Endocrine Disruptor Screening Program for various types of ED activity by a tiered set of *in vitro* and *in vivo* assays. As described in a report to the US Congress (EPA, 2000), the EDSTAC recommended a system consisting of two "Tiers" of ED testing. Tier 1 *in vitro* and *in vivo* tests are designed to identify substances that have the potential to interact with the endocrine system. Tier 1 robotic *in vitro* screening tests are especially desired as a way to more quickly identify ED chemicals –and at lower costs. To the best of our knowledge, CCI's recently developed robotic assay for EA is the only robotic assay commercially available. Tier 2 multigenerational tests are designed to confirm EDA and characterize the *in vivo* effects of EDs (EPA, 2000). The EPA (2000) also recommended the use of quantitative structure activity relationship (QSAR) modeling to predict whether a chemical would bind to ERs.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) was established in 1997 by the NIEHS and EPA (Public Law P.L.103-43) to develop and validate new ED test methods. ICCVAM was authorized in 2000 (P.L.106-545), as a 15 agency permanent committee, to co-ordinate the development, validation and acceptance of toxicological tests. As part of this mandate, ICCVAM and NICEATM [National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternate Toxicological Methods] formed an Endocrine Disruptor Working Group to assist ICCVAM in the evaluation status of assays for EDs. EDSTAC (1998) and ICCVAM (2002a,b) recommended that ER-dependent transcriptional activation (TA) assays (including cell proliferation assays) be developed because such "functional" assays are more sensitive than Relative Binding Affinity (RBA) assays, can distinguish agonists from antagonists, and can be conducted with and without exogenous metabolic activation. To assess the current validation status of *in vitro* TA assays for EA, ICCVAM prepared Background Review Documents (ICCVAM, 2002a), convened an independent expert panel to review the validation status of such assays (ICCVAM, 2002b,c), and published a final report stating their recommendations (ICCVAM, 2003a).

From all these evaluations, ICCVAM and NICEATM concluded that, "no validation studies have been completed on the [TA] assays being considered" (ICCVAM, 2002b, page 2). The panel expressed a general preference (ICCVAM, 2002b, page 31) for the use of human ER subtypes in any *in vitro* TA screening assay. CCI's MCF-7 cell proliferation assay uses a cell line derived from a Human ER subtype (see below). These panels developed minimum procedural standards for TA assays of EA. [CCI's manual or robotic EA assay nominated for validation is designed to meet or exceed all applicable ICCVAM standards and preferences.] Given the large number of chemicals that should be screened for possible EA, the EPA, NIEHS and other congressionally-mandated interagency bodies (ICCVAM, NICEATM) for some years have also set a high priority on the development of high throughput, *in vitro*, robotic assays for EA (EDSTAC, 1998; ICCVAM 2002 a,b,c). Hence, CertiChem Inc (CCI) submitted and successfully completed a Phase I SBIR showing it is feasible to develop a very sensitive, rapid, inexpensive *in vitro*, robotic assay for EA/anti-EA using proliferation of MCF-7 human breast cancer cells that meets published standards (ICCVAM, 2002a,b, c; 2003a) for TA assays of EA.

Historical Need for *In Vitro* Assays

EDSTAC (1998) recommended that two *in vitro* assays for estrogenic/ anti-estrogenic activity be performed: an ER binding assay, such as the Relative Binding Affinity (RBA) assays, and an ER transcriptional activation (TA) assay, including cell proliferation assays. EDSTAC (1998) recommended an ER binding assay because it is relatively simple, can be rapidly

performed, detects several specific mechanisms of xenobiotic activity, and has been well validated and replicated in manual formats. [For any assay, particular attention should be paid to issues of *Accuracy* (Does the test measure what it purports to measure? What is the rate of false negatives and false positives?) and *Reliability* (Does the test yield similar data when replicated in different laboratories?)]. The EDSTAC (1998) –and subsequently ICCVAM (2002a,b,c; 2003a)-- also recommended an ER-dependent TA assay (including cell proliferation assays) because such “functional” assays are more sensitive than RBA assays, can distinguish agonists from antagonists, and can detect some effects of xenobiotics that are dependent on metabolism. Such TA assays would preferentially use a stable cell line derived from mammalian tissues (ICCVAM 2002a,b,c; 2003a).

However, despite growing public and scientific concern about the possible health impact of environmental chemicals with EA as discussed above, most chemicals have not been tested for EA (EDSTAC, 1998; EDSTAC 2002a,b,c). Of about 80,000 chemicals produced commercially, 10,000-20,000 might be expected to exhibit EA (NRC, 1999; Hong et al., 2002). Due to the need to test many chemicals for EA, the NIEHS, EPA, and ICCVAM explicitly stated an interest in the development of a robotic high throughput assay for EA to begin to assess the ED effects of these chemicals on human health and wildlife (EDSTAC, 1998; ICCVAM, 2002a,b; 2003a). As part of this screening program, a high throughput pre-screening (HTPS) or "robotic" feasibility study for EA was conducted, but no success was achieved. The EPA concluded that the HTPS “needed more development before routine use” (Loder, 2000; EPA, 2000).

Given these considerations outlined above, in 1998 Dr. George Bittner, in collaboration with Dr. Fred vom Saal (a well-known investigator of ED effects on rodents), recognized that an emerging trend in endocrine research related to endocrine disruption had significant scientific interest, societal benefits, and commercial potential. Dr. Bittner incorporated CertiChem (CCi) in May of 2000 to specifically address such needs. In collaboration with Drs. Rottinghaus, vom Saal and Welshons at the University of Missouri (Columbia), Drs. Bittner and Yang at CCi submitted a Phase I SBIR to show it was feasible to develop a robotic assay using MCF-7 cell proliferation to reliably and accurately detect EA in 2002 (funded for 2003). CCi succeeded in showing that it should be feasible to develop this robotic *in vitro* EA assay because of considerable attention CCi gave to *many* details that individually might seem inconsequential. CCi also chose to use a stable human cell line that reliably responds to chemicals with EA and that has high sensitivity and specificity to detect EA in order to accurately measure EC50 of suspect chemicals. Furthermore, as shown in its Phase I grant, CCi’s EA assay can analyze very small amounts of mixtures of known and/or unknown chemicals, as well as single chemicals. Finally, in robotic format, this assay is rapid and cost effective.

CCi submitted a Phase II SBIR in 2003 (funded for 2004-2006) in collaboration with Dr. V.C. Jordan (formerly at Northwestern University Medical School in Chicago, now at Fox-Chase Cancer Center in Philadelphia) and Dr. W.J. Welsh (at Robert Wood Johnson Medical Center in Piscataway, NJ) to develop this robotic EA assay for commercialization. During this Phase II grant still in progress, CCi has further improved this assay (e.g. greatly increasing its sensitivity and reliability) and analyzed many ICCVAM (2003a) test chemicals, as reported elsewhere in this nomination document.

1.1.2. Summary of Peer Reviews Conducted to Date

Peer Reviews of this MCF-7 Cell Proliferation Assay in Robotic Format

This MCF-7 Cell Proliferation Assay in Robotic Format has been peer reviewed by NIH. The Resume and Summary of Discussion of each panel that reviewed our funded NIH SBIR

Phase I and Phase II grants are given verbatim [with notations] below. A copy of the complete text of each review is included in the **Appendix A**.

Phase I Review [June 2001]

“This application [An *in vitro* robotic assay for estrogenic activity] describes development of a high-throughput robotic cell proliferation assay to detect estrogenic activity by measuring *in vitro* proliferation of MCF-7 cells. The applicant and collaborators are well qualified to successfully complete the proposed studies. The proposal has many strengths, including the innovative use of robotics, good preliminary data, the straightforward experimental approach, the appropriate use of the assay for screening chemicals to identify potential endocrine disruptors, and the high commercialization potential of this system. There are several weaknesses in the proposed study, including the lack of detail regarding software [since resolved], the large amount of variation in estrogen responsiveness associated with MCF-7 cells [since shown to be greatly reduced by the robotic assay], the potential effects of estrogen-like activity from plastic associated with the experiments [CCi tests all plastics and media for EA and control for any background activity], and the applicants plan to continuously passage MCF-7 cells for one year (three months should be the maximum [since reduced to 2-3 months]. However, the study section felt these weaknesses were relatively minor given the qualifications and experience of the team of investigators. Overall, the study section enthusiastically supported the proposed development of a high-throughput robotic cell proliferation assay to detect estrogenic activity and identify potential endocrine disruptors.”

Phase II Review [June 2003]

“This Phase II SBIR application [An *in vitro* robotic assay for estrogenic activity] is from a team of experienced and well-qualified investigators. The Phase II research will extensively validate [reliability & accuracy] the *in vitro* robotic assay in their company facilities and demonstrate reliability and relevance [compared to published tables for 78 chemicals in ICCVAM 2003a. The relevance/accuracy of these data are debated by ICCVAM 2002a-c, 2003a]. Strengths of the application are the expertise of the investigative team; the completion of the goals of the proposed research in phase I with a robotic assay for estrogenic activity (EA) was successfully developed and initially characterized; the generally strong commercialization plans; the general feasibility of the high throughput assays; and the excellent research environment. These strengths and a few deficiencies were the focus of the discussion at the study section meeting. Although the development of a robotic assay for EA is exciting, some critical details of experimentation are not fully addressed. It is stated that their robotic assay is designed to meet or exceed all applicable agency standards and preferences. What is not clear is that [sic] if a standard solvent extraction step will be used for all varieties of test compounds or materials. [Standard water or ethanol extraction is used.] More attention to such details would be useful. All in all, the commercialization plans are appealing and well laid out and the project addresses an important area and hence a reasonably high priority score is recommended.”

1.1.3. Confidential Material in this Nomination Document

None

1.2. REGULATORY RATIONALE AND APPLICABILITY

1.2.1. Current Regulatory Testing Requirement

The EDSTAC concluded that prior methods of testing for chemical toxicity had been inadequate to detect many types of ED (EDSTAC 1998). This committee therefore recommended that many thousands of chemicals be tested in an Endocrine Disruptor Screening Program (EDSP) for various types of ED activity by a tiered set of *in vitro* and *in vivo* assays. As described in a report to the US Congress (EPA, 2000), the EDSTAC recommended a system consisting of two "Tiers" of ED testing. Tier 1 *in vitro* and *in vivo* tests are designed to identify substances that have the potential to interact with the endocrine system. Tier 1 robotic *in vitro* screening tests are especially desired as a way to more quickly identify ED chemicals at lesser costs. Tier 2 multigenerational tests are designed to confirm EDA and characterize the *in vivo* effects of EDs (EPA, 2000). The EPA (2000) also recommended the use of quantitative structure activity relationship (QSAR) modeling to predict whether a chemical would bind to ERs. [In collaboration with Dr. William Welsh at Robert Wood Johnson Medical School, CCI uses such a QSAR model program (Fang et al., 2003) to help identify EA and/or anti-EA in anti-oxidants (AOs) from foodstuffs or plastics.]

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) was established in 1997 by the NIEHS and EPA (Public Law P.L.103-43) to develop and validate new ED test methods. ICCVAM was authorized in 2000 (P.L.106-545), as a 15 agency permanent committee, to co-ordinate the development, validation and acceptance of toxicological tests. As part of this mandate, ICCVAM and NICEATM [National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternate Toxicological Methods] formed an Endocrine Disruptor Working Group (EDWG) to assist the EPA, NIEHS, NICEATM, and ICCVAM in the development and validation of assays for EDs. EDSTAC (1998) and ICCVAM (2002a,b) recommended that ER-dependent transcriptional activation (TA) assays (including cell proliferation assays) be developed because such "functional" assays are more sensitive than Relative Binding Affinity (RBA) assays for distinguishing agonists from antagonists and can be conducted with and without exogenous metabolic activation. To assess the current validation status of *in vitro* TA assays for EA, ICCVAM prepared Background Review Documents (ICCVAM, 2002a), further assessed by an expert panel (ICCVAM, 2002b,c).

The panel expressed a general preference (ICCVAM, 2002b, page 31) for the use of human ER subtypes in any *in vitro* TA screening assay. These panels then developed minimum procedural standards for TA assays of EA. Given the large number of chemicals that should be screened for possible EA, the EPA, NIEHS and other congressionally-mandated interagency bodies (ICCVAM, NICEATM) for some years have set a high priority on the development of high throughput, *in vitro*, robotic assays for EA (EDSTAC, 1998; ICCVAM 2002 a,b,c).

This regulatory environment is described by ICCVAM (2003b) as follows in italics:

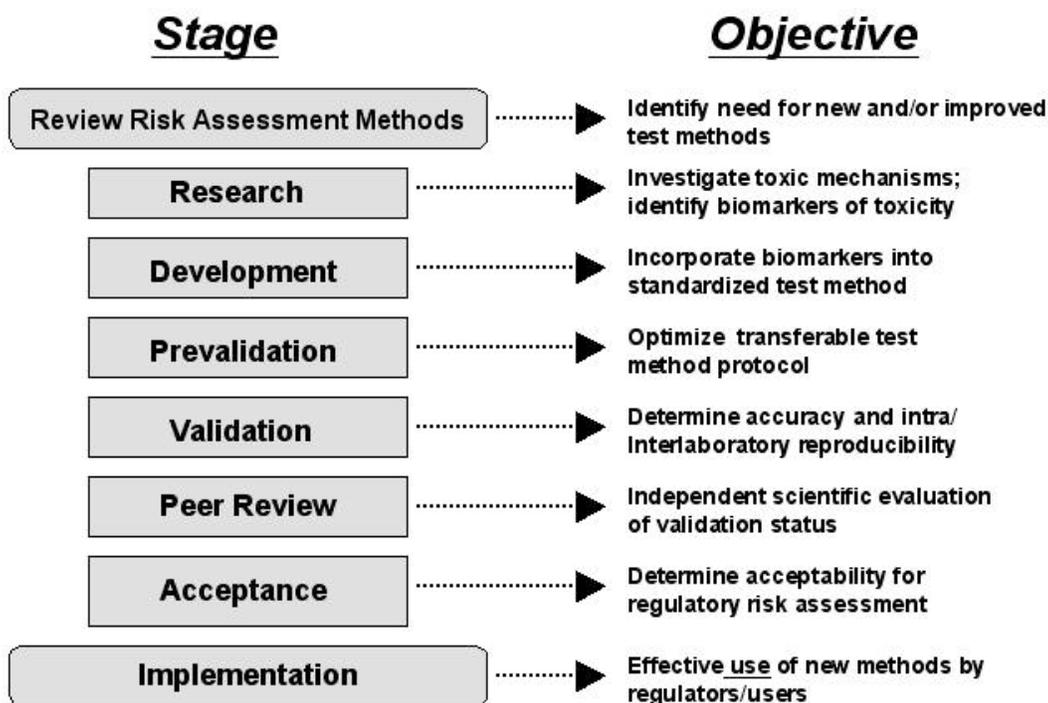
The National Institute of Environmental Health Sciences (NIEHS) established the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in 1997 to coordinate the interagency technical review of new, modified, and alternative test methods of interagency interest and to coordinate cross-agency issues relating to the validation, acceptance, and national and international harmonization of toxicological testing methods. ICCVAM was established as a permanent interagency committee of the NIEHS under the National Toxicology program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) on December 19, 2000, by the ICCVAM Authorization Act of 2000 (Public Law 106-545; Appendix E).

The Committee is comprised of representatives from the fifteen U.S. Federal regulatory and research agencies that use or generate toxicological information. ICCVAM promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately

assess the safety or hazards of chemicals and products and that refine (i.e., decrease or eliminate pain and distress), reduce, and replace animal use. NICEATM provides operational and scientific support for ICCVAM and ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal agencies.

ICCVAM is responsible for coordinating the interagency technical review of new or modified alternative test methods of interagency interest, and coordinating cross-agency issues relating to the validation, acceptance, and national and international harmonization of toxicological test methods throughout the U.S. Federal government. ICCVAM was established as a permanent interagency committee of the National Institute of Environmental Health Sciences (NIEHS) under NICEATM by the ICCVAM Authorization Act of 2000 (Public Law 106-545) (2; **Appendix E**). Priority is given to test methods that may provide for improved prediction of adverse human, animal, or ecological effects, and those that might reduce¹, refine², or replace³ animal use.

Figure 1. Test Method Validation Process



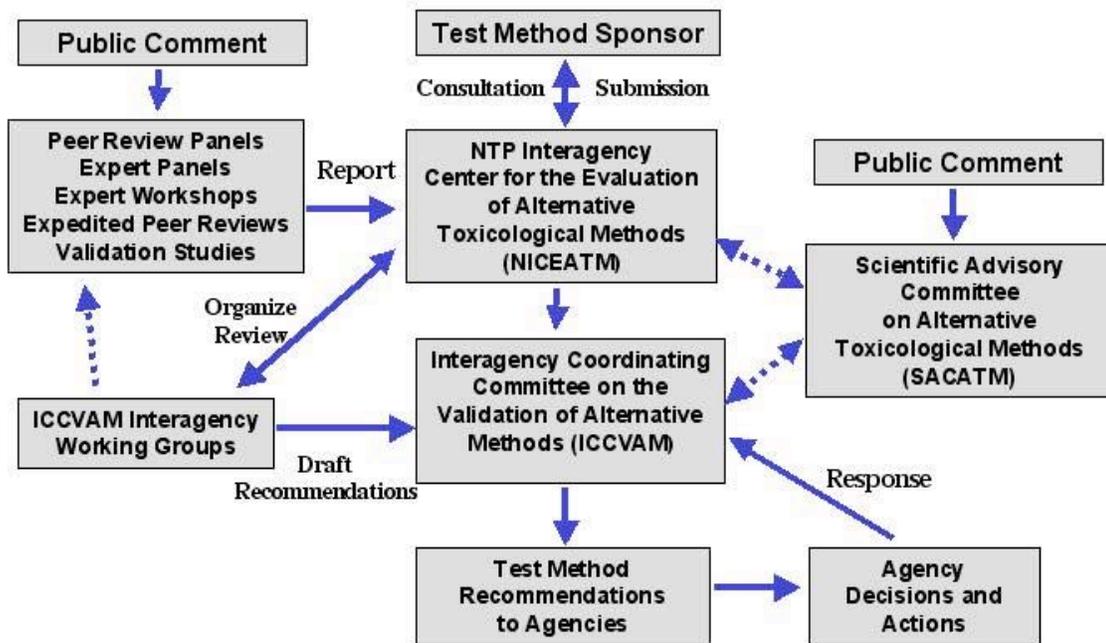
¹ Reduction alternative: A new or modified test method that reduces the number of animals required.

² Refinement alternative: A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being.

³ Replacement alternative: A new or modified test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one.

In the ad hoc ICCVAM report on the validation and regulatory acceptance of toxicological test methods (1), various stages were identified to move a proposed test method from concept to regulatory acceptance (**Figure 1**). A critical stage is the communication of a proposed test method by the sponsor or nominator to ICCVAM for consideration and review. NICEATM, on behalf of ICCVAM, receives proposed test method submissions or nominations and communicates with the submitting organization or individual. Typically, the ICCVAM evaluation process involves an initial assessment by NICEATM of the adequacy and completeness of the proposed test method submission or nomination, and a determination by ICCVAM of the priority that the proposed test method will have for technical evaluation.... Once a proposed test method has been accepted for evaluation, ICCVAM assembles an interagency working group of government scientists with scientific and regulatory expertise in the appropriate scientific disciplines to collaborate with NICEATM on the evaluation process. Depending on the validation status of the proposed test method, ICCVAM, in conjunction with NICEATM, develops recommendations and priorities for further efforts. Such efforts might include an expert workshop, an expert panel meeting, a peer review meeting, an expedited peer review process, or a validation study (**Figure 2**).

Figure 2. ICCVAM Test Method Evaluation Process



Following this review process, ICCVAM develops and forwards recommendations on the usefulness and limitations of the proposed test method for regulatory purposes to Federal agencies, in accordance with Public Law 106-545 (2). Based on their specific statutory mandates, each agency then makes a determination regarding the acceptability of the test

method. Agencies are required to respond to ICCVAM within 180 days of receipt of an ICCVAM test method recommendation. If the test method is accepted, appropriate actions (e.g., revision of existing regulations, publication of guidelines and/or guidance documents) are taken to inform the regulated community.

Sponsors and nominators should communicate with NICEATM/ICCVAM throughout the development, prevalidation, and validation process, and during the submission or nomination process. If requested and appropriate, ICCVAM may solicit interagency comments on proposed test method protocols and prevalidation or validation studies. Requests for comment on proposed prevalidation or validation study designs should include descriptions of the scientific basis and regulatory applicability of the proposed test method, the scientific rationale for the proposed prevalidation or validation studies, and responses to each section of the submission guidelines.

The NICEATM office is located at NIEHS, which is headquartered in Research Triangle Park, NC (NIEHS, P.O. Box 12233, MD EC-17, Research Triangle Park, NC 27709; telephone: 919-541-2384; fax: 919-541-0947; e-mail: iccvam@niehs.nih.gov). NICEATM serves as a communication link between test sponsors and Federal agencies during the development and validation process. In collaboration with ICCVAM, NICEATM convenes expert workshops, expert panel meetings, peer review meetings, and expedited peer reviews, and conducts validation studies when appropriate and recommended by ICCVAM.

1.2.2. Intended Regulatory Use

Initial screening of a chemical or chemical mixture for EA as part of a Tier 1 testing and screening program for EDA. Such screens will reduce the use of animals needed for *in vivo* testing.

1.2.3. Comparisons with Existing *in vivo* Reference Test Methods

No validated *in vivo* test methods yet established

1.2.4. How Proposed Test Method Fits into Overall Strategy of Hazard Assessment

This robotic MCF-7 *in vitro* assay for EA is intended as a Tier 1 screen for EA. That is, the EDSTAC recommended a system consisting of two "Tiers" of ED testing. Tier 1 *in vitro* and *in vivo* tests are designed to identify substances that have the potential to interact with the endocrine system. Tier 1 robotic *in vitro* screening tests are especially desired as a way to more quickly identify ED chemicals at lesser costs. Tier 2 multigenerational tests are designed to confirm EDA and characterize the *in vivo* effects of EDs (EPA, 2000).

1.3. SCIENTIFIC BASIS FOR PROPOSED TEST METHOD

1.3.1 Mechanistic Basis

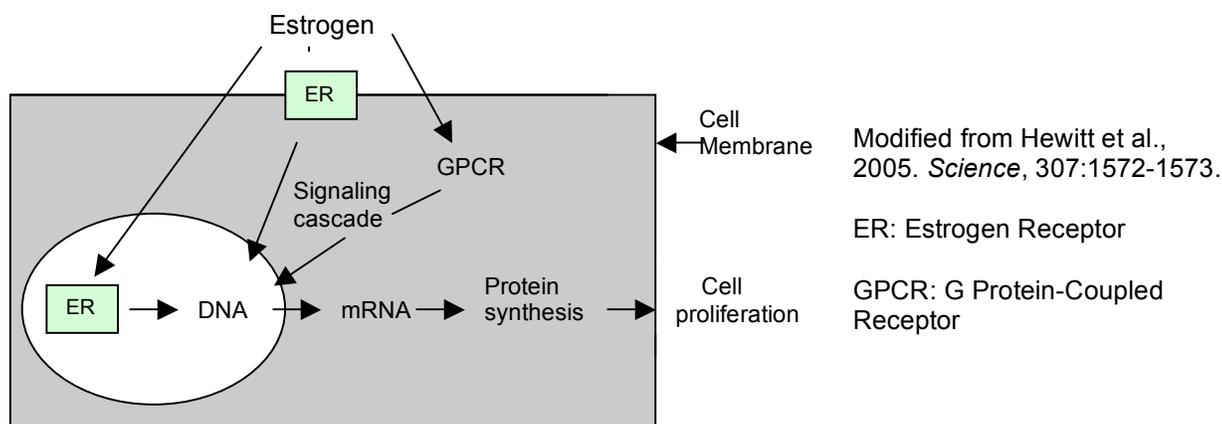
Mechanisms by which chemicals produce EA and Anti-EA

The mechanisms of estrogenic and anti-estrogenic EDs are shared with natural estrogens. Synthetic EDs present in the environment mimic endogenous hormonal activity by affecting the actions of the estrogen receptors (ERs) and other members of the nuclear receptor superfamily (Beato, 1989; Singleton and Khan, 2003; Hewitt et al., 2005). Estrogen Receptor- α (ER- α) and ER- β are promiscuous receptors, which bind a

wide variety of natural and synthetic ED chemicals and activate transcription of estrogen-responsive genes, leading to cell proliferation (**Fig. 3**). EDs bind ER- α and ER- β to induce conformational changes that allow ERs to proceed from inactive proteins to active transcriptional regulators that induce transcription of estrogen responsive genes (Matthews et al., 2002; Revankar et al., 2005). Anti-EA effects may be produced, in theory, by competitive inhibitors that bind to ER but do not activate them (e.g., ICI182,780 and ICI 164,384: Wakeling, 1993; Jordan and Murphy, 1990; Muller et al., 2002) or agonists that bind strongly to ER, but do not activate as strong an estrogenic response. Furthermore, selective ER modulators (SERMs) bind to ERs, but subsequently activate cellular responses that differ from those activated by the endogenous estrogen, 17 β -estradiol (E2) (Black et al., 1983, Yang et al., 1996; Shang and Brown, 2002; Leonard and Smith, 2003). It might also be possible for a chemical to bind directly to endogenous hormones and thereby reduce their effect. Most chemicals that bind to ERs produce some effect on ER activation, either estrogenic, anti-estrogenic, or as SERMs.

ER- α and ER- β bind a large number of chemical ligands (Gorski et al. 1986; Kuiper et al., 1996, 1997, 1998). While binding affinities differ between estrogenic ligands (Kuiper, 1997), ER ligands typically bind to both receptors (ICCVAM, 2002a, 2003a; Routhledge et al., 2003). Both ERs bind to estrogen response elements, which are located upstream of the promoter regions of estrogen-activated genes (Paech et al., 1997; McDonnell and Norris, 2002). Chemicals with EA or anti-EA can bind to nuclear or extra-nuclear receptors (**Fig. 3**; Hewitt et al., 2005; Evinger and Levin, 2005). Our MCF-7 cell line contains (mostly) ER- α and some ER- β (V.C Jordan, personal communication). Given all these data, CCI has developed cell proliferation assays first for EA and now for anti-EA linked (primarily) to ER- α and ER- β in human breast cancer-derived MCF-7 cells, a system with human relevance.

Figure 3: Mechanism of Estrogen Action



Scientific rationale for choosing MCF-7 cell proliferation to assay for EA

MCF-7 cells are perhaps the most widely used model of estrogen action *in vitro*. The MCF-7 cell line was derived from human breast cancer and described by Soule and colleagues in 1973 (Soule et al., 1973). Estrogen-dependent cell proliferation was described by Lippman (Lippman et al., 1975, 1976), and the presence of estrogen receptors was described simultaneously (Horwitz et al., 1975). The utility of the cell proliferative response in the study of estrogen action was enhanced by the observation that the phenol red indicator dye present in most media was estrogenic (Berthois et al., 1986); subsequent work indicated that the estrogenic chemicals were actually contaminants present in many commercial preparations of the dye (Welshons et al., 1988). Deletion of this indicator from culture media improved reliability of these cells to detect estrogenic

activity, many of our collaborators used these cells in many subsequent studies (Welshons et al., 1987, 1990; Grady et al., 1991; Nagel et al., 1997, 1998; in EDSTAC (1998) and ICCVAM (2000a-c, 2003) publications, such *in vitro* assays are intended as a Tier 1 screening tool.

Why develop an MCF-7 cell proliferation assay in robotic format?

Despite growing public and scientific concern about the possible health impact of environmental chemicals with EA, most chemicals have still not been tested for EA (EDSTAC, 1998). Of about 80,000 chemicals produced commercially, 10,000-20,000 might be expected to exhibit EA (NRC, 1999; Hong et al., 2002). Due to the need to test many chemicals for EA, the NIEHS, EPA, and ICCVAM explicitly stated an interest in the development of a robotic high throughput assay for EA to begin to assess the ED effects of these chemicals on human health and wildlife (EDSTAC, 1998; ICCVAM, 2002a,b). As part of this screening program, a high throughput pre-screening (HTPS) or "robotic" feasibility study for EA was conducted, but no success was achieved. The EPA concluded that the HPTS "needed more development before routine use" (Loder, 2000; EPA, 2000). In this context, CCI has shown that it is feasible to develop a robotic cell proliferation assay for EA, **because of CCI's choice of a superior model system (a stable MCF-7 cell line derived from human breast tissue) and the attention CCI has given to many details that individually may seem inconsequential** to characterize estrogenic activity of environmental estrogens. MCF-7 cell proliferation is also used by the E-Screen (Soto et al., 1995), although, rather than direct stimulation of growth, this assay uses release from inhibition of cell proliferation by the protein estrogenic inhibitor, a novel protein present in human serum. Estrogens release the inhibition of growth caused by human serum (Soto and Sonnenschein, 1985), and this forms the basis of the E-Screen patent (Sonnenschein and Soto, 1989, pat. no. 4,859,585). Estrogen-stimulated cell proliferation is a highly sensitive and widely-used bioassay, and may be the most widely published *in vitro* model of estrogen action. Furthermore, this assay can measure the estrogenic activity of single chemicals or mixtures of chemicals. In the latter case, the specific chemical(s) having estrogenic activity are not identified by the assay, but can often be uniquely identified by other means such as high-performance liquid chromatography (HPLC) fractionation combined with MCF-7 assays of the EA in each fraction.

1.3.2 Similarities and differences of modes and mechanisms of actions of proposed test method vs. various species of interest

This screen is oriented toward detecting EA effects in humans, and uses a human-derived cell line (MCF-7 cells) to perform this assay. Similar ED effects are almost certainly produced in many other mammals and "lower" vertebrates, since basic endocrine mechanisms have been highly conserved across all classes of vertebrates (Kavlock et al., 1996; NRC, 1999; Thornton, 2001; Calafat et al., 2005; vom Saal et al. 2005).

1.3.3. Range of substances amenable to the proposed test.

This MCF-7 cell proliferation assay is very versatile and has been used to detect EA not only in the 78 chemicals recommended as test chemicals by ICCVAM (2003a), but also in animal feeds, ingredients for animal feeds, and extracts from plastics and many other manufactured products. Chemicals or chemical mixtures are dissolved in water or ethanol. To date, CCI has not used DMSO to help dissolve any chemical or chemical mixture, as DMSO use has been discouraged by ICCVAM (2002a,b,c).

II. TEST METHOD PROTOCOL COMPONENTS

MCF-7 Cell Proliferation Assay for Estrogenic Activity

2.1. OVERVIEW OF TEST METHOD AND COMPARISON WITH ICCVAM (2003A) STANDARDS

2.1.1. Overview of the Test Method

Test chemicals with EA bind to ERs (either α or β) usually located in the nucleus and activate the transcription of estrogen-responsive genes, which leads to proliferation of MCF-7 cells (**Fig. 3, page 14**). EA is measured as an ability to induce MCF-7 cell proliferation by the natural estrogen, E2 (ICCVAM 2003a). MCF-7 cells are perhaps the most widely used *in vitro* model for studying EA / anti-EA and were derived from human breast cancer cell line (Soule et al., 1973). Shortly after the establishment of this MCF-7 cell line, estrogen-dependent cell proliferation (Lippmann et al., 1975, 1976) and the presence of ERs (Horwitz et al., 1975) was described. In the years since these landmark experiments, MCF-7 cell proliferation has been used as the *in vitro* benchmark for determining EA or anti-EA of many environmental chemicals (Welshons et al., 1987,1990; Grady et al, 1991; Nagel et al., 1997, 1998; Rivas, 2002).

This test method protocol outlines the procedures for assaying estrogenic activity and supports the *in vitro* validation study organized by ICCVAM and sponsored by NIEHS. The MCF-7 cell proliferation assay will be performed to determine EC50 values of test chemicals of varying estrogenic activity.

Most test method assays described in this document have been designed to rather accurately assess EC50 values. Accurate assays of EC50 generally require eleven sampling concentrations. This MCF-7 cell proliferation assay can also be used to detect the presence of EA of any chemical or chemical mixture. Such EA detection screening assays typically require six to eight concentrations of the test chemical. Such data can be acquired in less time and with less expense than accurate EC50 determination.

2.1.2. Comparison With ICCVAM (2003a) Standards

The following 21 points compare CCI's EA assay standards (*in italics*) with 21 ICCVAM standards for transcriptional activation (TA) assays for EA (in normal typeface as per ICCVAM publication 03-4503, May 2003a, Appendix):

1). The reference estrogen should be 17 β -estradiol; the transcriptional activation [TA] response with this substance should be demonstrated by a full concentration response curve. *CCI uses 17 β -estradiol (E2) at eleven concentrations ranging from 10⁻¹⁶M to 10⁻⁹M to calculate EC50.*

2). Test substances must be prepared preferably in absolute ethanol or culture medium but DMSO could be used, if necessary. A set of solvent/vehicle-only controls (with the final solvent concentrations identical to those used in the reaction mixtures containing the test substance) must be included in each set of assays. *CCI typically dissolves test substances in absolute ethanol and then dilutes with estrogen-free culture medium.*

3). The solvent/vehicle volume must be the same as that in the reaction mixtures containing the test substances, and should remain constant for the concentration range tested. *Cci does this.*

4). A relatively active antagonist (e.g. ICI 182, 780) should be used as the positive control for antagonist studies. *CCI uses ICI 182,780.*

5). The limit concentration should be 1 mM, but the solubility characteristics of each test substance must be taken into consideration. (One panel member felt that this concentration was excessive and that 0.1 mM should be adequate). *CCi typically uses 0.5mM.*

6). The concentration range of test substances should consist of at least seven different concentrations spaced at one order of magnitude apart from each other (e.g. 1,10, 100 nM, 1, 10, 100 μM, 1 mM). However, if a lower maximum concentration is tested due to solubility constraints or excessive cytotoxicity, the number of concentrations tested can be reduced to account for the altered concentration range. *CCi uses **eight** concentrations spaced at one order of magnitude apart from each other for RANGE-FINDER experiments for each test chemical. CCi then uses **eleven** concentrations spaced at 1.87 fold to 5-fold apart from each other depending on the slope of concentration curve of each chemical (see test method 2.2.9.11). To simply detect whether estrogenic activity might be present, CCi typically uses **six** concentrations with a 3-fold dilution.*

7). Triplicate measurements should be performed at each test substance concentration level. *CCi always meets this standard, and sometimes performs quadruplicate measurements.*

8). Classification of a test substance as ‘positive’ for agonist or antagonist activity in transcriptional activation assays should be based on the generation of a concentration response curve. *CCi tests for both agonist activity (EA) and antagonist activity (anti-EA).*

9). Historical data should be used as part of the assay acceptance criteria (i.e., reference substances for agonism and antagonism must give appropriate responses). *CCi does this.*

10). The test report should include information on the test substance, the solvent used, the ER, the reporter plasmid, the cell line, the test conditions, the results, and a determination as to whether the substance is positive or negative. *CCi does this.*

11). Replicate studies are not mandated, but questionable data needs to be confirmed by retesting of the substance. However, one panel member recommended testing each substance at least three times in different experiments. *CCi tests each chemical at least three times in its current Phase II study and at least twice when screening samples for EA.*

12). The assays should be performed following Good Laboratory Practice guidelines. *CCi is adding this in its Phase II studies to develop a robotic EA assay for commercialization.*

Additional or modified minimum procedural standards recommended by the Panel:

13). Concentrations to be tested for the reference positive control, 17β-estradiol, should range from 1 pM to 1 μM, and clear guidance is needed about the expected response. *Because of the increased sensitivity of this assay, CCi uses 8 concentrations at 10-fold dilutions from 0.1fM to 1nM in its Range-Finder experiment, and 11 concentrations at 3-fold dilutions centered around the EC50 value acquired from the Range-Finder experiment in the experiments to accurately assess EC50.*

14). With regard to the preparation of test substances, the level of solvent that does not adversely affect assay response should be determined before testing by performing appropriate pre-validation studies using the reference estrogen. *CCi does this.*

15). A relatively weak estrogenic agonist (e.g., estriol) should be included as an additional control for agonist studies. *CCi does not do this because its assay has very few false positives and also because CCi’s experimental design contains several test chemicals, having at least one chemical serve as a weak estrogen agonist.*

16). A measure of cellular cytotoxicity should be incorporated into the assay to help define the upper limit for test material concentrations similar to the Maximum Tolerated Dose (MTD) approach used in vivo studies. *CCi measures the cytotoxicity of test chemicals by examining the cell morphology under microscopy. CCi is in the process of setting up a cytotoxicity assay which measures neutral red uptake.*

17). To measure that a positive agonist response reflects a receptor-mediated activity, the test substance could be re-tested with ICI 182, 780 (the candidate ER antagonist) present in the culture medium. *Not Applicable.*

18). For transient transfection assays, there is a need to include a constitutive reporter gene assay to control for the transfection efficiency. *Not Applicable.*

19). A standard definition for “relative activity” must be decided upon so that a positive/negative call for agonism and antagonism can be made. *CCi uses EC50 of E2 / EC50 of the test chemical.*

20). Suitable diagnostics must be performed on any statistical procedure to ensure that the model fits the data before it is finally chosen for analysis of the data. *CCi does this.*

21). The test report should also include the complete DNA sequences of constructs and vectors, the transfection methods used, the cell passage number (s) during the study, and the CO₂% level in the incubator. *Not Applicable, other than CO₂% levels and passage number, now included.*

2.2. DETAILED DESCRIPTION OF PROPOSED TEST METHOD

2.2.1. Materials, equipment, and supplies needed

A. Materials

1. Cell Line

MCF-7, clone WS8, obtained from Dr. V.C. Jordan formerly at Northwestern University School of Medicine, Chicago, IL, now at Fox Chase Cancer Center, Philadelphia, PA.

2. Technical Equipment

- a) 96-well Deep-well plates (e.g. Matrix, cat#: 4222) and Caps (e.g. Matrix cat#: 4422)
- b) 96-well plate spectrophotometer (i.e., plate reader) equipped with 590 nm and 700 ± 10 nm filter (e.g. Bio-Tek, Powerwave)
- c) 96-well [flat bottom] cell culture plates (e.g., Corning tissue culture-treated)
- d) Centrifuges
- e) Conical 15 ml and 50 ml tubes
- f) Cryotubes and liquid nitrogen container
- g) epMotion 5070 robotic station and accessories (tubs, racks, adaptors, tips, reservoirs, etc.)
- h) Eppendorf tubes
- i) Filters/filtration devices
- j) Hemocytometer
- k) Incubator: 37°C ± 1°C, 90 % ± 5 % humidity, 5.0 % ± 1 % CO₂/air
- l) Inverted phase contrast microscope and optical microscope
- m) Laboratory balances
- n) Laminar flow clean bench/cabinet (standard: "biological hazard")
- o) Parafilm to seal plates
- p) Pipettes, pipettors (multi-channel and single channel; multichannel repeater pipette)
- q) pH paper (wide and narrow range)
- r) Pipetting aid
- s) Sterile glass tubes with caps (e.g., 5 mL)
- t) Tissue culture flasks (e.g., 75 - 80 cm², 25 cm²)
- u) Vortex mixer

v) Water bath: 37°C ± 1°C

[Note: Tissue culture flasks and plates should be prescreened to ensure that they adequately support the growth of MCF-7 cells and that they have the least estrogenic activity compared to similar products.]

3. Chemicals, Media, and Sera

- a) Acetaldehyde (e.g. Fisher Cat. # 01004-250)
- b) Bovine insulin (e.g., Sigma Cat. # I-6634)
- c) Calf thymus DNA type I
- d) Dextran-coated charcoal (e.g. Sigma Cat. # C-6197)
- e) Dimethyl sulfoxide (DMSO), U.S.P. analytical grade (e.g. Sigma Cat. # D2438)
- f) Distilled H₂O or any purified water suitable for cell culture
- g) Diphenylamine (DPA) (e.g. Sigma Cat. # 242586)
- h) Ethanol (EtOH), U.S.P. analytical grade (100 %, non-denatured for test chemical preparation)
- i) Fetal Bovine Serum (FBS) (e.g., Gibco Cat. # 26140079)
- j) Glacial acetic acid, analytical grade
- k) Hanks' Balanced Salt Solution (HBSS) without Ca²⁺ and Mg²⁺ (CMF-HBSS)
- l) L-Glutamine 200 mM (e.g., Gibco Cat. # 25030081)
- m) Non-essential amino acids (NEAA) (e.g., Gibco Cat. # 1140050)
- n) Penicillin/streptomycin/antimycotic solution (e.g., Gibco Cat. # 15240062)
- o) Perchloric acid (e.g. Fisher Cat. # A228-7Lb)
- p) RPMI with L-Glutamine without Phenol Red (e.g., Gibco Cat. # 11835055)
- q) RPMI with L-Glutamine with Phenol Red (e.g., Gibco Cat. # 11875119)
- r) Trypan Blue Dye – tissue culture-grade, powder (e.g. Sigma Cat. # T-6146), liquid (e.g. Sigma Cat. # T-8154)
- s) 0.25 % Trypsin/0.02 % EDTA solution without phenol (e.g., (e.g., Gibco Cat. # 25200106)

[Note: Due to variability in different lots of fetal bovine serum, CCI pre-tests each serum lot for the validation study. Each lot of estrogen-stripped serum is also pre-tested for the ability of E2 to stimulate MCF-7 cell growth before use.]

B. Preparations of Media and Solutions

[Note: All solutions (except perchloric acid, DPA, and acetaldehyde stock solutions), glassware, pipettes, etc., are sterile and all procedures should be carried out under aseptic conditions in the sterile environment of a laminar flow cabinet (biological hazard standard).

1. Media

RPMI solutions containing Phenol Red supplemented with

a) Freeze Medium

40 %	FBS
20 %	DMSO

b) Routine Culture Medium

10 %	FBS
4 mM	L-Glutamine
6 ng/ml	Insulin
100 IU/mL	Penicillin (Gibco# 15240062)
200 µg/mL	Streptomycin (Gibco# 15240062)
100 µg/mL	Antimycotic (Gibco# 15240062)
1ml/100ml	NEAA (Gibco# 1140050)

RPMI without Phenol Red containing:

a) EA-free Medium

5 %	Dextran-Charcoal stripped FBS
4 mM	L-Glutamine
6 ng/ml	Insulin
100 IU/mL	Penicillin (Gibco# 15240062)
200 µg/mL	Streptomycin (Gibco# 15240062)
100 µg/mL	Antimycotic (Gibco# 15240062)
1ml/100ml	NEAA (Gibco# 1140050)

Note: 0.5% EtOH is typically added to EA-Free Medium when diluting test chemicals for concentration-response studies.

All the above solutions of RPMI media should be kept at 2-8°C and stored for no longer than two weeks.

2. Stock Solutions

50 ml of 0.16% Acetaldehyde Solution: 0.08 ml acetaldehyde (Fisher# 01004-250) + 49.92 ml double distilled H₂O (dd H₂O). The solution is stored at 4°C

255 ml of 20% Perchloric acid Solution: 85 ml 60% perchloric acid (Fisher Cat. # A228-7Lb) + 170 ml dd H₂O. The solution is stored at 4°C

100ml of DPA Solution: 4 g DPA + 100 ml glacial acetic acid. This solution needs to be prepared just before use.

3. Stripped Serum by Dextran-coated Charcoal

DAY ONE

1. Take serum out of freezer. Let bottle thaw and store at 4°C.
2. Prepare 250mL centrifuge tubes (e.g. VWR 21008-943). 12 bottles are needed for 1-500 ml bottle of serum.
3. Use a clean spatula. Fill 9 tubes (3 sets of 3 tubes) each with 1.11g of dextran-coated charcoal (Sigma C-6197). Leave last 3 tubes empty.

DAY TWO

4. Pipette ~167mL of serum into the first 3 tubes. (Split the entire bottle of serum between 3 tubes).
5. Make sure caps are tight and mix contents by shaking and inverting the tubes.
6. Lay tubes on their sides on ice. Shake constantly and invert the tubes every 2 minutes
7. Centrifuge at 2500rpm for 15 minutes. (Use a blank to balance the rotor, if needed.)
8. Pour supernatant into the second set of 3 tubes. Repeat steps 6 and 7.
9. Pour supernatant into third set of 3 tubes.
10. Repeat step 6, and then centrifuge at 3500rpm for 20 minutes.
11. Transfer supernatant to another set of 3 tubes. Do not shake tubes. Centrifuge at 4300rpm for 15 minutes.
12. During step 11, set up ~ten 50mL conical tubes in hood.
13. In the tissue culture hood, pour supernatants into sterile filtering units attached to a vacuum (e.g. 250mL filters from Millipore (SCGPU02RE), or Nalgene SFCA filters from Fisher (09-740-39A)).
14. Aliquot ~50 mL of sterile stripped serum into 50mL conical tubes.
15. Store 50 mL tubes in a -20° C freezer until ready to use.
16. Test stripped serum: each batch of stripped serum is tested before use for our experiments for estrogen-induced EA. If estrogen-induced EA with the stripped serum is less than four-fold, this batch of the serum needs to be re-stripped or discarded.

2.2.2 Selection of concentration range to examine a test chemical, including the need for any concentration range-finding studies or toxicity data prior to conducting a study, if applicable

A. Range-Finder Experiment

Eight concentrations of a test chemical shall be used to generate a concentration-response curve. First, the stock solution of the test chemical is diluted 200x with EA-free medium. The stock solution of the chemical is the maximum concentration (limit = 0.1M) of the chemical that will dissolve in 100% EtOH. This initial 200x dilution therefore has an EtOH concentration of 0.5%. The concentration of the test chemical after a 200x dilution depends upon its value in the stock solution. If the concentration of the test chemical in the stock solution is 0.1M, then the concentration of the test chemical after a 200x dilution is 5×10^{-4} M. The initial 200x dilution of the test chemical is then diluted 10x with EA free medium containing 0.5% EtOH; this 10x dilution step is then repeated six more times by diluting with EA free medium containing 0.5% EtOH. In the example given above, this seven step ten-fold serial dilution produces **eight** concentrations of a test chemical in EA-free medium all containing 0.5% EtOH as follows: 5×10^{-4} M, 5×10^{-5} M, 5×10^{-6} M, 5×10^{-7} M, 5×10^{-8} M, 5×10^{-9} M, 5×10^{-10} M, 5×10^{-12} M, 5×10^{-13} M, 5×10^{-14} M, 5×10^{-15} M. The concentration range for the strong EA chemicals could be 5×10^{-8} M, 5×10^{-9} M, 5×10^{-10} M, 5×10^{-12} M, 5×10^{-13} M, 5×10^{-14} M, 5×10^{-15} M. If the initial concentration of the test chemical in 100% EtOH is less than 0.1M due to solubility problem, then all subsequent 10-fold dilutions are affected accordingly.

This range-finder experiment for a test chemical should include the concentrations that produce minimal and maximal EA responses to the chemical. The range-finder experiment should also estimate the EC50 of the chemical in order to determine the concentration range to be used for the Main experiment described below. We run 4 replicates at each concentration for range-finder experiments.

B. Main Experiment

Briefly, from the range-finder experiment, determine which test chemical concentration is closest to the EC50 value (i.e., 50% of the maximal EA plateau response). Use the estimated EC50 value as a central concentration and adjust dilutions higher and lower in equal steps for the definitive concentration-response experiment (described in detail in **2.2.9.11. "Test Chemical Dilution in Main Experiment"**) to meet the following criteria: Each experiment should have at least one EA value between EC10 (10% of maximum plateau response) and EC50, and at least one data point between EC 50 and EC90. At least two data points must be within 10% of the maximal response (bottom plateau of a typical S curve). Any concentration-response plot must be repeated using different dilutions of the test chemical until all these conditions are met. Furthermore, as discussed elsewhere, only assays in which both positive and negative controls are acceptable on the same 96-well cell culture plate can be used. That is, E2 must adequately stimulate cell proliferation and the vehicle control must not have a standard deviation greater than 15% of its mean.

The main experiment should be performed at least in triplicate on 96-well cell culture plates and repeated at least two more times, each time on a different day.

2.2.3 Endpoint(s) measured

Cell proliferation. Total amount of DNA per well is measured using the DPA assay. Briefly, 60 µl of a 1:5 mixture of 0.16% acetaldehyde and 20% v/v perchloric acid, followed by 100 µl of 4% DPA in glacial acetic acid, are added to each well. A standard curve is prepared by adding samples of 0.0625 to 3 µg DNA/well in 10 µl of HBSS. After incubating the plate overnight at 37°C, the absorbance is measured as the difference between OD₅₉₅ and OD₇₀₀, e.g., using a Bio-Tek EL_x 808 plate reader. The output is converted to µg DNA/well by using a third degree polynomial curve fit of the standard curve; the correlation coefficient of the standard curve fit is typically greater than 0.99.

2.2.4 Duration of exposure

MCF-7 cells are exposed for 7 days in the test chemicals. Briefly, MCF-7 cells are maintained at 37°C in RPMI with Phenol Red for routine culture as described above. An aliquot of cells maintained at 37°C are grown for two days in phenol-free media containing 5% charcoal stripped fetal bovine serum (see above) in a 25 cm² flask. Using a robotic dispenser such as an epMotion 5070 unit, MCF-7 cells are then seeded at 400 cells per well in 0.2 ml of this hormone-free medium in Corning 96-well plates. The cells are adapted for 3 days in hormone-free culture medium prior to adding test chemicals. The media is changed daily for 6 days. At the end of the 7 day exposure, the media is removed, the wells are washed once with 0.2 ml of HBSS, and then assayed to quantify amounts of DNA/ well using a micro-plate modification of the Burton diphenylamine (DPA) assay (Burton, 1956; Natarajan et al., 1994), as described under Test Methods.

2.2.5 Known limits of use

Volatile chemicals can not be used for our current experimental design (8 chemicals on one plate). If a chemical is determined to be volatile, that particular test chemical is separated from other chemicals and tested in its own cell culture plate with a sealed cover to avoid contamination of the other test chemicals (see 2.2.9.12.6: Volatility of Test Chemicals)

2.2.6 Nature of the assessed response

This EA assay measures the ability of cells to respond to estrogens/estrogen-like substances by enhanced cell proliferation. The mechanisms of estrogenic EDs are shared with natural estrogens. Synthetic EDs present in the environment mimic endogenous hormonal activity by affecting the actions of the estrogen receptors (ERs) and other members of the nuclear receptor superfamily (Beato, 1989; Singleton and Khan, 2003; Hewitt et al., 2005). Estrogen Receptor- α (ER- α) and ER- β are promiscuous receptors, which bind a wide variety of natural and synthetic ED chemicals and activate transcription of estrogen-responsive genes, leading to cell proliferation. EDs bind ER- α and ER- β to induce conformational changes that allow ERs to proceed from inactive proteins to active transcriptional regulators that induce transcription of estrogen responsive genes (Matthews et al., 2002; Revankar et al., 2005). Anti-EA effects may be produced, in theory, by competitive inhibitors that bind to ER but do not activate them (e.g., ICI182, 780 and ICI

164,384; Wakeling, 1993; Jordan and Murphy, 1990; Muller et al., 2002) or agonists that bind strongly to ER, but do not activate as strong an estrogenic response. Furthermore, selective ER modulators (SERMs) bind to ERs, but subsequently activate cellular responses that differ from those activated by the endogenous estrogen, 17 β -estradiol (E2) (Black et al., 1983, Yang et al., 1996; Shang and Brown, 2002; Leonard and Smith, 2003). It might also be possible for a chemical to bind directly to endogenous hormones and thereby reduce their effect. Most chemicals that bind to ERs produce some effect on ER activation, either estrogenic, anti-estrogenic, or as SERMs.

ER- α and ER- β bind a large number of chemical ligands (Gorski et al. 1986; Kuiper et al., 1996, 1997, 1998). While binding affinities differ between estrogenic ligands (Kuiper, 1997), ER ligands typically bind to both receptors (ICCVAM, 2002a, 2003a; Routhledge et al., 2003). Both ERs bind to estrogen response elements, which are located upstream of the promoter regions of estrogen-activated genes (Paech et al., 1997; McDonnell and Norris, 2002). Chemicals with EA or anti-EA can bind to nuclear or extra-nuclear receptors (**Fig. 3, page 14**; Hewitt et al., 2005; Evinger and Levin, 2005). Our MCF-7 cell line contains both ER- α and ER- β , with ER- α dominating (V.C Jordan, personal communication). Given all these data, CCI developed cell proliferation assays for EA and anti-EA linked (primarily) to ER- α and ER- β in human breast cancer-derived MCF-7 cells, a system with human relevance.

2.2.7 Appropriate vehicle, positive and negative controls and the basis for their selection

A. Vehicle Control

The vehicle control is 0.5% EtOH in RPMI Phenol-free medium. [Note that test chemicals are dissolved in 100% EtOH and initially diluted 1:200 in EA-free Medium. (The final concentration of EtOH is therefore 0.5%.) Following its initial 1:200 dilution, test chemicals are then serially diluted in EA-free Medium containing 0.5% EtOH.]

B. Positive Control

For each set of test chemicals in an assay, E2 at 5×10^{-10} – 5×10^{-16} M should be used as a positive control on each set of 96-well cell culture plates run at the same time.

C. Anti-EA/Negative Control

ICI 182, 780 at 10-8M. For each set of test chemicals (usually 8 chemicals including positive control E2) in three cell culture plates typically used in an assay, ICI182, 780 at 10-8M is added to the first column of each 96-well cell culture plate.

Note: ICI 182, 780 at 10-8M is included as negative control in the cell culture plates, and CCI's target is for ICI to have about the same amount of DNA/well as the vehicle control (0.5% EtOH). However, a minimum difference between the negative control and the vehicle control *is not* a test acceptance criterion.

2.2.8 Nature of the data to be collected and the methods used for data collection

Acceptable range of vehicle, positive and negative control responses and the basis for the acceptable ranges

All of the following criteria must be met. EC50 of the positive control (E2) must be within 2.5 SD of the historical mean established by the test laboratory (2×10^{-13} M for CCI) and have an r^2 (coefficient of determination) value ≥ 0.9 calculated by Hill kinetics using GraphPad Prism. At least one data point on each EC50 plot of the positive control and the test chemical must be 10% - 50% of the maximum response and at least one data point must be 50% - 90% of the maximum response. At least two data points must be $< 10\%$ of the maximum response and these points constitute the bottom plateau of the concentration response curve. The standard deviation of all vehicle controls should not be more than 15% of the mean. The standard deviation of all negative controls should not be more than 15% of the mean. Any EC50 plot must be repeated using different dilutions of the appropriate positive control or test substance until all these conditions are met.

2.2.9 Nature of the data to be collected and the methods used for data collection

2.2.9.1. Nature of the data to be collected

The total cell number per well is based on the amount of DNA in a well (20 pg/cell) measured by the DPA Assay.

2.2.9.2. Cell Maintenance and Culture Procedures

MCF-7 cells are routinely grown as a monolayer in a 25 cm² flask (a flask with a 25 cm² surface for cells to grow) at 37°C \pm 1°C, 90 % \pm 5 % humidity, and 5.0 % \pm 1 % CO₂/air. The cells should preferably be examined on a daily basis under a phase contrast microscope, and any changes in morphology or their adhesive properties noted in a Study Workbook.

2.2.9.3. Receipt of Cryo-preserved MCF-7 Cells

Upon receipt, the vial(s) of cryo-preserved MCF-7 cells should be stored in a liquid nitrogen freezer until needed.

2.2.9.4. Thawing Cells

Thaw cells by putting vials into a water bath at 37°C \pm 1°C and use as soon as thawed.

- a) Re-suspend the cells in pre-warmed RPMI with Phenol Red Routine Culture Medium and centrifuge for 5 minutes at 1500 rpm. The cell pellet is re-suspended in 5 ml of pre-warmed RPMI with Phenol Red Routine Culture medium and transferred to a 25 cm² tissue-culture flask.
- b) Incubate at 37°C \pm 1°C, 90 % \pm 5 % humidity, and 5.0 % \pm 1 % CO₂/air.

- c) When the cells have attached to the bottom of the flask (within 4 to 24 h), decant the supernatant and replace with fresh pre-warmed (37°C) RPMI with Phenol Red Routine Culture Medium. Culture as described above.
- d) Passage at least two times before using the cells in an EA assay.

A fresh batch of frozen cells from the stock lot of cells should be thawed and cultured approximately every four months at a rate of one passage per week.

2.2.9.5. Routine Culture of MCF-7 Cells

MCF-7 cells should be passed once a week following the protocol described below. When cells exceed 50 % confluence (but less than 80 % confluence), they should be removed from the flask by trypsinization as follows:

- a) Decant medium, briefly rinse cultures with 5 mL HBSS (without Ca²⁺, Mg²⁺) in the 25 cm² flask(s). Wash cells by gentle agitation to remove any remaining serum that might inhibit the action of trypsin.
- b) Discard the washing solution.
- c) Add 0.6 mL trypsin-EDTA solution to the MCF-7 cell monolayer in the 25 cm² flask and incubate the cells at 37°C for 15-30 seconds.
- d) Lightly tap the flask to detach the cells into a single cell suspension.
- e) Transfer the cells into a 15 ml conical tube containing 10 ml of pre-warmed RMPI with Phenol Red for Routine Cell Culture Medium or 10 ml of pre-warmed RMPI without Phenol Red for EA Assay Medium.
- f) Centrifuge for 5 minutes at 1500 rpm (about 450xG) at room temperature.
- g) Discard the medium and gently tap the tube containing the cell pellet. Re-suspend the pellet in 4 ml of pre-warmed RPMI with Phenol Red for Routine Cell Culture Medium or 4 ml of pre-warmed EA-free Medium.
- h) Aspirate the re-suspended cells 4 times in a syringe with a 21 gauge needle so that an aliquot of thoroughly dissociated MCF-7 cells (single cell suspension) can be accurately counted.

2.2.9.6. Cell Counting

Place 0.1 mL of the cell suspension into a 0.5 ml Eppendorf tube and add 0.1 ml 0.4% trypan blue solution. Count the number of MCF-7 cells per ml using a hemocytometer.

2.2.9.7. Subculture of MCF-7 Cells.

After determining the cell number/ml, the culture can be sub-cultured into other flasks or seeded into 96-well microtiter plates. MCF-7 cells are routinely seeded at the cell densities listed in **Table 2-A**. Doubling time is 24-28h.

Table 2-A. Cell Density Guidelines for Subculturing

Days in Culture	Seeding Density (cells/cm ²)	Total Cells per 25 cm ² flask	Comments
2 (for EA assay)	40,000	10 ⁶	Cells grow slower in EA free medium
7 (for routine culture)	8,400	10 ⁵	The cell confluence is about 80% at the end of one week

[Note: Cells should be past their lag growth phase when used for any test.]

2.2.9.8. Freezing Cells (procedure required only if current stock of cells is depleted)

Stocks of MCF-7 cells can be stored in sterile, freezing tubes in a liquid nitrogen freezer. DMSO is used as a cryoprotective agent.

- a) Centrifuge trypsinized cells at approximately 450G.
- b) Suspend the cells in cold RPMI with Phenol Red for Routine Culture Medium (half the final freezing volume) to attain a final concentration of 1-2x10⁶ cells/mL.
- c) Slowly add cold Freeze Medium to the cells so that the solvent will equilibrate across the cell membranes. Bring the cell suspension to the final freezing volume. [The final cell suspension will be in 10 % DMSO.] Aliquot 1.2mL of the cell suspension into each cryo vial.
- d) Place the vials into an insulated container (e.g., styrofoam trays) and place in a freezer (-70 to -80°C) for 24 h. The vial contents should freeze at approximately 1°C/min.
- e) Place the frozen vials into liquid nitrogen for storage. After one week, thaw one vial to test cell viability. Cell viability should be greater than 90%.

2.2.9.9. Preparation of Cells in EA-free Media Prior to EA Assay:

- a) 10^6 MCF-7 cells are seeded in 25 cm² flasks in EA-free Medium as described above and should be fed with fresh EA-free Medium the next day.
- b) Two days after the cells are stripped of estrogen in a 25 cm² flask, trypsinize the cells and prepare a single cell suspension of 2×10^3 cells/ml in EA-free Medium. Using a robotic dispenser, e.g., an epMotion 5070, add 200 μ l of the single cell suspension of 2×10^3 cells/mL into each well of 96-well cell culture plates (400 cells/well). The seeding density should be noted to ensure that the cells in the E2-treated wells do not overgrow after ten days.
- c) Incubate cells for 3 days \pm 2 h at 37°C with 90 % humidity, 5 % CO₂. This incubation in EA-free media assures that cells are stripped of estrogen.

Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the plate and detect any cell seeding errors. Record observations in the Experimental Notebook.

2.2.9.10. Test Chemical Preparation

1. Test Chemicals in Solution

- a) Allow test chemicals to equilibrate to room temperature before dissolving and diluting.
- b) For chemicals dissolved in 0.5% EtOH in EA-free Medium (e.g. for chemicals having very low solubility in EtOH), prepare test chemical at 5×10^{-4} M (or the solubility limit) as the highest (initial) concentration applied directly to cells immediately prior to use. Test chemical solutions should not be prepared in bulk for use in subsequent tests. The solutions should not be cloudy nor have noticeable precipitate. Each preparation should have at least 10 ml total volume to ensure adequate volume of solution for one set of experiments (4 wells (quadruplicates) \times 6 (days of treatment) \times 0.2 ml/well = 4.8 mL, leaving 5.2 mL additional solution).
- c) For chemicals dissolved in 100% EtOH, store an aliquot (e.g., 1.5 mL) of this solution (e.g., for stable chemicals at 10⁻¹M) in a freezer at -70°C - 80°C for use in future chemical analyses.
- d) The final EtOH concentration to be applied to the cells must be 0.5 % (v/v) in the vehicle controls and in all the dilutions of the test chemical.
- e) The stock solution for each test chemical should be prepared at 10⁻¹M (or the solubility limit) in 100% EtOH. Thus, the highest (the initial) concentration of the test chemical applied to the cells in each range finding experiment is 5×10^{-4}

M (1/200 (initial dilution) x 10^{-1} M = 5×10^{-4} M), which is the same as chemicals prepared in EA-free medium containing 0.5% EtOH.

2. pH of Test Chemical Solutions

Measure the pH of the highest (or initial) concentration of each test chemical (e.g., 5×10^{-4} M or some other initial concentration determined by the solubility limit or some other consideration discussed herein). Use different pH papers to initially estimate the pH of the highest concentration of the test chemical and other pH papers to determine more precise values. The pH paper should be in contact with the solution for approximately one minute. Document the pH and note the color of the medium containing the highest concentration of the test chemical. The color of the medium for all dilutions of the test chemical should be noted in the experimental notebooks. Do *not* adjust the pH of the medium containing the highest concentration of the test chemical. If chemicals are toxic because of their high/low pH, any pH adjustment would eliminate the toxic effect.

3. Example of Deep-well Plate (DWP) Protocol

- 1) Label front, back, left, right sides of each 96-well DWP because the orientation of the DWP will be kept the same during the entire experimental procedure. Label with the date of the experiment. One DWP will be used for 8 chemicals (including E2 as positive control, PC).
- 2) Before beginning serial dilutions, the DWP should be prepared following the procedures below (see **Table 2-B**). The volume in each well will be determined by the volume needed for that particular experiment (the volume in each well is defined as the volume in each well after serial dilution is completed). The volume to be transferred from the wells in one column to the wells in the next column to the right is determined by the dilution factor (See description in **2.2.9.11. "Test Chemical Dilutions"** for how to calculate those volumes):
 - a. Dispense 0.5% EtOH in EA-free Medium into the upper 4 wells of column #12 (vehicle control, VC) and ICI 182,780 10^{-8} M into the lower 4 wells of columns #12 (negative control, NC).
 - b. Dispense 0.5% EtOH in EA free medium into all the wells of columns #2-11 (i.e. 11 concentrations will be tested for each chemical).
 - c. Dispense EA-free Medium into all wells of column #1.
 - d. Add test chemicals into different wells of column #1 (8 chemicals in 8 different wells) at 1:200 dilution (1 volume of the test chemicals dissolved in 100% EtOH and 199 volumes of EA-free medium, so the final concentration of EtOH in the wells of column #1 becomes 0.5%). The wells of column #1 contain the initial concentrations of the test chemicals.
- 3) Mix the solution in the wells of column #1 five times and then transfer an aliquot of the solution from the wells of column #1 to the wells of column #2.
- 4) Mix the solution in column #2 six times and transfer an aliquot of the solution of the wells in column #2 to the wells in column #3.

- 5) Repeat steps 3) and 4) until an aliquot of the solution in the wells of column #10 is transferred to the wells of column #11.
- 6) Mix the chemicals in the wells of column #11 five times and transfer the extra volume in the wells of column #11 to a waste reservoir, so that all wells have the same volume after the serial dilution is completed (Note: when the robotic transfers the solutions prepared in DWP into the 96-well cell culture plates, it needs to know the volume in each well of the DWP. It is much easier to program the robotic if the DWP has the same volume of solution in each well).
- 7) The chemicals in the wells of column #1 (initial concentration), the serially diluted chemicals in the wells of columns 2-11, and the VC and NC in column #12 will be transferred directly to 96-well cell culture plates in subsequent steps using the robotic epMotion 5070.

2.2.9.11. Test Chemical Dilutions in Main Experiment

Determination of Dilution Factor

An estimate of EC50 should have been obtained in the range-finder experiment described in Section 2.2.2.A) for “**Range-Finder Experiment**”. Depending upon the slope of the concentration-response curve estimated from the range-finder experiment, the dilution factor could be lower than 10-fold in the main experiment. The dilution factor for the test chemical should be chosen to produce at least one data point from EC10 to EC50 and at least one data point from EC 50 to EC 90. See Section 2.2.9.12.5 for “**Test Acceptance Criteria**”). Concentration–response experiments having less than one data point on each side of the EC₅₀ value should be repeated using a smaller dilution factor (see section 2.2.2.B) for “**Main Experiment**”).

Table 2-B shows an example of appropriate dilutions in a concentration-response experiment for a test chemical A whose EC50 is 10-13M and a test chemical B whose EC50 is 10-12M. Given that the Hill slope of a standard concentration-response curve is 1, then EC90 is nine times EC50 and EC10 is one ninth of EC50 ($EC_F = (F/(100-F))^{(1/H)} * EC_{50}$, where F is the percent and H is the Hill slope). In order to have at least one value between EC10 and EC50 and one between EC50 and EC90, a smaller dilution factor than 10-fold must be used to perform serial dilutions. Theoretically, a 3-fold serial dilution factor should produce one value between EC10 and EC50 at a concentration equal to EC50/3 and another value between EC50 and EC90 at the concentration equal to 3x EC50.

Table 2-B: Chemical Dilution Example -1

Chemical Name	Chemical concentrations above estimated EC50				Estimated EC50	Chemical concentrations below estimated EC50			
	3x higher	3x higher	3x higher	3x higher		3x lower	3x lower	3x lower	3x lower
Chemical A	8.1E-12	2.7E-12	9.0E-13	3.0E-13	1.0E-13	3.3E-14	1.1E-14	3.7E-15	1.2E-15
			Estimated EC90				Estimated EC10		
Chemical B	8.1E-11	2.7E-11	9.0E-12	3.0E-12	1.0E-12	3.3E-13	1.1E-13	3.7E-14	1.2E-14

If the rising phase of the Hill slope of a concentration-response curve is decreased from 1.0 to 0.5, then EC90 is eighty-one times EC50 and EC10 is one eighty-first of EC50. In this case, 3-fold serial dilutions would give three values between EC10 and EC50 and also three values between EC50 and EC90. Therefore, a 10-fold dilution factor could be used for a serial dilution to obtain one data point between EC10 and EC50 and another data point between EC50 and EC90 (see **Table 2-C**).

Table 2-C: Chemical Dilution Example - 2

Chemical Name	Chemical concentrations above estimated EC50				Estimated EC50	Chemical concentrations below estimated EC50			
	3x higher	3x higher	3x higher	3x higher		3x lower	3x lower	3x lower	3x lower
Chemical A	8.1E-12	2.7E-12	9.0E-13	3.0E-13	1.0E-13	3.3E-14	1.1E-14	3.7E-15	1.2E-15
			Estimated EC90				Estimated EC10		
Chemical B	8.1E-11	2.7E-11	9.0E-12	3.0E-12	1.0E-12	3.3E-13	1.1E-13	3.7E-14	1.2E-14

If the Hill slope of the concentration-response curve is greater than 1, a serial dilution factor less than 3-fold would be needed to obtain one data point between EC10 and EC50 and another data point between EC50 and EC90.

Calculation of Fluid Volumes in the Wells of a Deep-well Plate (DWP)

The fluid volume needed in the wells of a DWP is calculated as described below.

X is the volume in each of the wells in column #1 before serial dilutions begin.

Y is the volume in each of the wells in columns 2-11 before serial dilutions begin. **Y** is also equal to the final volume in each well after serial dilutions are completed in columns 1-11.

Z is the volume transferred from wells in one column to wells in the column to the right.

$$\mathbf{X} = (\mathbf{X}/\text{dilution factor}) + \mathbf{Y}$$

$$\mathbf{Z} = \mathbf{X}/\text{dilution factor}$$

The following example uses a **dilution factor of 3** for **800 μL final volume (Y)** remaining in all wells of a DWP after all serial dilutions are completed. This final volume in each well of a DWP is determined by the design of a particular experiment. For example, when quadruplicates of each concentration of a test chemical are required, then each well of a DWP should have 800 μL /well final volume because each DWP well must supply 200 μL to each of four wells of a 96-well cell culture plate in subsequent stages of this assay. For this set of conditions:

- 1) Volume (**X**) in column #1 of a 96-well DWP = $(\mathbf{X} / 3) + 800 \mu\text{L}$; **X** = 1200 μL , which consists of 6 μL (1200 $\mu\text{L}/200$) concentrated stock solution in 100% EtOH plus 1194 μL EA-free Medium. Note that the final EtOH concentration equals 0.5%.
- 2) Volume (**Y**): 800 μL . Each well in columns 2- 11 contains 0.5% EtOH in 800 μL of EA-free Medium.
- 3) Volume **Z** = $\mathbf{X}/\text{dilution factor} = 1200 \mu\text{L} / 3 = 400 \mu\text{L}$. Volume (**Z**) is the volume after mixing/equilibration transferred from column #1 to column #2, then column #2 to #3, etc.,

See columns 1-11 of **Figure 2-A** for an illustration of these conditions.

Note: The top four wells in column#12 contain vehicle control (VC) with the volume equal to Y. The lower four wells in column#12 contain negative control (NC) with the volume equal to Y.

Figure 2-A. 96-Well DWP Configuration for Positive Control (PC), Negative Control (NC), Vehicle (VC), and Test Chemicals

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	VC
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	VC
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	VC
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	VC
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	NC
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	NC
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	NC
H	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	NC

VC = Vehicle Control
 NC = Negative Control (10⁻⁸M ICI182, 780 in 0.5% EtOH in EA-free Medium)
 PC = Positive Control
 1 - 11 = Test Chemical or PC at eleven concentrations (1 = highest, 11 = lowest)
 A - G = Test chemicals A through G

2.2.9.12. Test Procedure

1. 96-Well DWP Configuration

Figure 2-A shows how 7 test chemicals and a positive control (E2) would be prepared in 11 concentrations before application to MCF-7 cells. The serial dilutions in the DWP are made in 0.5% EtOH in EA-free Medium. The vehicle control (VC) is EA-free medium containing 0.5% EtOH. The negative (NC) control is 10⁻⁸M ICI 182, 780. Both VC and NC are added to the appropriate wells in column #12 of the DWP.

2. Application of Test Chemicals

1) **Rotate DWP:** As described above, serial dilutions in the DWP were performed in such way that column #1 contains the highest concentration of test chemicals or E2, and column #11 contains the lowest concentration of test chemicals or E2 (11 concentrations will be tested for each chemical). Before the solutions are transferred from the DWP to cell culture plates, the DWP should be rotated 180 degrees, so that the column #12 with NC and VC is on the left side of the DWP and the column #1 with the highest concentration of chemicals is on the right side of the DWP (see **Figure 2-B** below).

Figure 2-B: 96-Well Deep-well plate configuration before and after 180 degree rotation before transferring solutions to test plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	VC
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	VC
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	VC
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	VC
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	NC
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	NC
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	NC
H	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	NC

Before Rotation

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	PC11	PC10	PC9	PC8	PC7	PC6	PC5	PC4	PC3	PC2	PC1
B	NC	G11	G10	G9	G8	G7	G6	G5	G4	G3	G2	G1
C	NC	F11	F10	F9	F8	F7	F6	F5	F4	F3	F2	F1
D	NC	E11	E10	E9	E8	E7	E6	E5	E4	E3	E2	E1
E	VC	D11	D10	D9	D8	D7	D6	D5	D4	D3	D2	D1
F	VC	C11	C10	C9	C8	C7	C6	C5	C4	C3	C2	C1
G	VC	B11	B10	B9	B8	B7	B6	B5	B4	B3	B2	B1
H	VC	A11	A10	A9	A8	A7	A6	A5	A4	A3	A2	A1

After Rotation

VC = Vehicle Control
 NC = Negative Control
 PC = Positive Control
 1 - 11 = Test Chemical or PC at eleven concentrations
 (1 = highest, 11 = lowest)

2) After 3 days incubation of the cells in 96-well cell culture plates with EA-free medium, the cells are ready for chemical tests (see 2.2.9.9. for “Preparation of Cells in EA-free medium Prior to EA Assay”).

3) **Configuration of cell culture plates:**

Configuration A: The chemical solutions in the DWP are transferred to 96-well cell culture plates so that the cell culture plates have the same configuration as the DWP after its rotation (See Figure 2-C). Triplicate determinations need 3 cell culture plates and quadruplicate determinations need 4 cell culture plates. Note that NC and VC are added to column# 1 of all cell culture plates, so that a systematic error between different cell culture plates can be easily detected.

Configuration B: The chemical solutions in the DWP are transferred to 96-well cell culture plates such that one cell culture plate contains only 3 (when using quadruplicates) or 4 (when using triplicates) concentrations of the 7 test chemicals and PC. Note that all NC and VC are located in the cell culture plate #1 (See Figure 2-D), so that a systematic error between different cell culture

plates can be detected by microscopic evaluation.

Note: Configuration A was used only at the beginning of the assay development because the method of transfer for configuration A uses many more tips than configuration B. CCI has been using configuration B for our robotic assay for over one and a half year.

Figure 2-C: Configuration A of 7 test chemicals and controls in cell culture plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	PC11	PC10	PC9	PC8	PC7	PC6	PC5	PC4	PC3	PC2	PC1
B	NC	G11	G10	G9	G8	G7	G6	G5	G4	G3	G2	G1
C	NC	F11	F10	F9	F8	F7	F6	F5	F4	F3	F2	F1
D	NC	E11	E10	E9	E8	E7	E6	E5	E4	E3	E2	E1
E	VC	D11	D10	D9	D8	D7	D6	D5	D4	D3	D2	D1
F	VC	C11	C10	C9	C8	C7	C6	C5	C4	C3	C2	C1
G	VC	B11	B10	B9	B8	B7	B6	B5	B4	B3	B2	B1
H	VC	A11	A10	A9	A8	A7	A6	A5	A4	A3	A2	A1

All the plates have the same configuration:
 Triplicates: 3 plates
 Quadruplicates: 4 plates

Figure 2-D: Configuration B of 7 test chemicals and controls in 3 cell culture plates (triplicates)

Col#	Plate #1				Plate #2				Plate #3			
	1-3	4-6	7-9	10-12	1-3	4-6	7-9	10-12	1-3	4-6	7-9	10-12
A	NC	PC11	PC10	PC9	PC8	PC7	PC6	PC5	PC4	PC3	PC2	PC1
B	NC	G11	G10	G9	G8	G7	G6	G5	G4	G3	G2	G1
C	NC	F11	F10	F9	F8	F7	F6	F5	F4	F3	F2	F1
D	NC	E11	E10	E9	E8	E7	E6	E5	E4	E3	E2	E1
E	VC	D11	D10	D9	D8	D7	D6	D5	D4	D3	D2	D1
F	VC	C11	C10	C9	C8	C7	C6	C5	C4	C3	C2	C1
G	VC	B11	B10	B9	B8	B7	B6	B5	B4	B3	B2	B1
H	VC	A11	A10	A9	A8	A7	A6	A5	A4	A3	A2	A1

Note: 4 cell culture plates are needed when quadruplicates are applied.

- VC = Vehicle Control
- NC = Negative Control
- PC = Positive Control
- 1 - 11 = Test Chemical or PC at eleven concentrations
(1 = highest, 11 = lowest)

The epMotion 5070 robotic with a multi-channel pipettor is used to transfer the solutions from the DWP to the appropriate positions on the cell culture plates, as described in step 3. These protocols ensure that the solutions of 7 test chemicals and PC can be transferred rapidly to the appropriate wells of the cell culture plates.

- 4) Remove 195 μ L EA-free Medium from the cell culture plates and immediately add back 195 μ L of freshly prepared solutions in the DWP to the appropriate positions of the cell culture plates using epMotion 5070 robotic with a multi-channel pipettor.
- 5) The serial dilutions in the DWP are performed every day for 6 days just before feeding the cells with fresh solution.

3. Microscopic Evaluation

Before beginning any treatment and after treating for 6 days, examine each plate under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Record any changes in morphology of the cells due to the cytotoxic effects of the test chemicals and any change in cell growth rate stimulated by test chemicals. Undesirable growth characteristics of control cells may indicate experimental error and cause rejection of the assay. Any cells having unusual properties should be documented in the Experimental Notebook.

4. Measurement of DNA

a) DNA standard preparation

- 1) Use calf thymus DNA, type 1, sodium salt (Sigma: D-1501).
- 2) Calibration of DNA is at 254: 1mg/ml=A254 of 20 or A254 of 1=50 μ g/ml
- 3) Make DNA Standards. Store in dark at -20C.
- 4) Use 16 Eppendorf tubes labeled as S1-16 as described in **Table 2-D**: S1 and S16 are blank tubes without DNA. Tubes S2-S15 have different concentrations of DNA (μ g/ 10 μ l) and are prepared from adding different volumes of DNA stock (1 mg/ml) and HBSS.

Table 2-D: DNA preparation schedule

	μ g/10 μ l	DNA Stock (μ l)	HBSS (μ l)		μ g/10 μ l	DNA Stock (μ l)	HBSS (μ l)
S1	0	-	1000	S9	1.5	150	850
S2	0.0625	6.25	993.75	S10	1.75	175	825
S3	0.125	12.5	987.5	S11	2.0	200	800
S4	0.25	25	975	S12	2.25	225	775
S5	0.5	50	950	S13	2.5	250	750
S6	0.75	75	925	S14	2.75	275	725
S7	1.0	100	900	S15	3.0	300	700
S8	1.25	125	875	S16	0	0	1000

- 5) Use one half of a 96 well plate to set up standard curve
- 6) Pipette 10 μ l to standard curve wells in triplicate
- 7) Pipette 10 μ l HBSS to blank wells (S1 and S16)

- b) **DPA Assay for test chemical plates**: Carefully remove the medium with the test chemicals and controls from the 96-well cell culture plates by gentle vacuum suction and rinse the cells very carefully with 200 μ L pre-warmed HBSS. Remove the rinsing solution by gentle vacuum suction.

Note: CCI has compared gentle vacuum suction versus dumping to remove medium and rinsing solution. No difference has been observed between those two methods. Therefore, we routinely use gentle vacuum suction to change media or any other solutions.

- 1) Combine chilled 0.16% acetaldehyde and 20% perchloric acid at a **1:5** ratio (1 ml + 5 ml per plate). Mix well. (About 3 mL extra volume is needed for robotic operations). Add 60 μ l the mix/well.
- 2) Add 100 μ l/well of 4% DPA.
- 3) Seal the sides of each plate with parafilm. Incubate at 37°C for 24 h.
- 4) Read plates with a reference wavelength of 690 nm and an absorption wavelength of 590 nm using the Bio-Tek El_x808_{iu} plate reader with KC junior software.
- 5) Calculate the optical density difference. Plot DNA amount (μ g) against optical density to obtain DNA standard curve (see **Figure 2-E**). The r^2 of CCI's standard curve is typically greater than 0.996.

Figure 2-E: DNA standard curve



- 6) Calculate the DNA concentrations of the test samples from the standard curve using KC junior software installed in the computer connected to the plate reader.

5. Quality Check of EA Assay

a) Test Acceptance Criteria

All acceptance criteria (i.e., criteria 1, 2, and 3) must be met for a test to be acceptable.

- 1) **PC**: The calculated EA values for PC must contain at least one EA value > 10 % and \leq 50.0 % maximal effect, at least one EA value > 50.0 % and < 90 % maximal effect, at least two EA values within 90% of maximal effect (top plateau), and at least two EA values within 10% of minimal effect (bottom

plateau). The PC EC₅₀ must be within \pm two and a half (2.5) standard deviations of its historical mean (for CCI, this is about 2×10^{-13} M) and must have an r^2 (coefficient of determination) value ≥ 0.9 calculated using the Hill model.

- 2) **VC and NC:** The standard deviation of the mean of all VCs does not differ by more than 15% from the mean of all VCs. The standard deviation of the mean of all NCs does not differ by more than 15% from the mean of all NCs.
- 3) **Test Chemicals:** The calculated EA values must contain at least one EA value $> 10\%$ and $\leq 50.0\%$ maximal effect, at least one EA value $> 50.0\%$ and $< 90\%$ maximal effect, and at least two EA values within 10% of minimal effect (bottom plateau). The EC₅₀ must have an r^2 value ≥ 0.85 calculated using the Hill model.

Note: It is difficult to obtain two EA values within 90% of maximal effect (top plateau) for some toxic chemicals, so the requirement of top plateau has been set only for our positive control (E2), although most test chemicals meet this criterion.

Exception: If a test chemical has only one point between 10 and 90 % of the maximal effect **and** the smallest dilution factor (i.e., 1.87) was used **and** all other test acceptance criteria were met, then the test is considered acceptable.

[Note: A corrected Mean OD_{590 \pm 10nm} of 0.05 - 0.2 for the VCs and NCs, and 0.6 - 1.2 for PC are target OD values, but are not test acceptance criteria.]

b) Checks for Systematic Cell Seeding Errors

Cell seeding errors are detected by examining each plate under a phase contrast microscope to assure that the number of cells per well is consistent. Cell seeding errors are also determined by measuring the DNA concentration of the cells in the VC and NC wells from the cell culture plates.

6. Volatility of Test Chemicals

Highly volatile test chemicals can generate vapors from the treatment medium during the test chemical treatment incubation period. These vapors can be absorbed by the media in adjacent wells. In such cases, culture wells nearest the highest test chemical concentration can become contaminated by exposure to absorbed test chemical vapors.

If the volatility of test chemicals is a concern, the location of VC and NC adjacent to the highest concentrations of test chemicals can be changed (see **Figure 2-F**). If a test chemical is a strong estrogenic compound and is volatile, the cross contamination may produce a significant increase in cell growth in the vehicle controls (VC1 or NC1) adjacent to the highest test chemical concentrations, compared to the VCs further away.

Figure 2-F: Configuration of Cell Culture Plate Design for Volatile Test chemicals with High EA

Col#	Plate #1				Plate #2				Plate #3			
	1-3	4-6	7-9	10-12	1-3	4-6	7-9	10-12	1-3	4-6	7-9	10-12
A	PC11	PC10	PC9	PC8	PC7	PC6	PC5	PC4	PC3	PC2	PC1	NC
B	G11	G10	G9	G8	G7	G6	G5	G4	G3	G2	G1	NC
C	F11	F10	F9	F8	F7	F6	F5	F4	F3	F2	F1	NC
D	E11	E10	E9	E8	E7	E6	E5	E4	E3	E2	E1	NC
E	D11	D10	D9	D8	D7	D6	D5	D4	D3	D2	D1	VC
F	C11	C10	C9	C8	C7	C6	C5	C4	C3	C2	C1	VC
G	B11	B10	B9	B8	B7	B6	B5	B4	B3	B2	B1	VC
H	A11	A10	A9	A8	A7	A6	A5	A4	A3	A2	A1	VC

←

Col#	Plate #3					
	1-3	4-6	7-9	10	11	12
A	PC3	PC2	PC1	NC1	NC2	NC3
B	G3	G2	G1	NC1	NC2	NC3
C	F3	F2	F1	NC1	NC2	NC3
D	E3	E2	E1	NC1	NC2	NC3
E	D3	D2	D1	VC1	VC2	VC3
F	C3	C2	C1	VC1	VC2	VC3
G	B3	B2	B1	VC1	VC2	VC3
H	A3	A2	A1	VC1	VC2	VC3

VC = Vehicle Control
 NC = Negative Control
 PC = Positive Control
 1 - 11 = Test Chemical or PC at eleven concentrations
 (1 = highest, 11 = lowest)

If potential test chemical volatility is suspected or if the initial range finder test (in a non-sealed plate) results show evidence of EA effects in the control wells (i.e., > 15 % difference in DNA concentration between VC-1 in plate #1 and VC-3 in plate #3), then this particular test chemical should be separated from other chemicals and tested in its own cell culture plate with a sealed cover to avoid contamination of the other test chemicals.

2.2.9.13. Data Analysis

A calculation of cell number expressed as DNA/well is made for each test chemical concentration using the mean DNA/well of the four replicate values (minimum of three acceptable replicate wells). Blanks are subtracted from all wells without cells (D1 and H1 wells in the cell culture plates). The eleven concentrations of each test chemical should span a range from no detectable effect (bottom plateau) to maximal effect (top plateau). Data from the microtiter plate reader are transferred to the Excel® spreadsheet template. This template should automatically calculate the mean of the amount of DNA (µg/well) and plot 11 concentrations of each chemical. EC₅₀ and EC₈₀ values; statistical analyses (including r square and standard deviation) are calculated by Hill function analysis (e.g., by GraphPad PRISM® 4.0).

2.2.9.14. Data report

The experimenter performing the experiment should send an electronic copy of all raw data and analyzed data of each chemical to the Principal Investigator.

2.2.10. Type of media in which data are stored

Detailed procedures and any characteristic observations of experiments should be written in an experimental notebook. All data (including raw data and analyzed data) should be stored in computers and backed up daily on a CD.

2.2.11. Measures of variability

In brief, the EA assay variability is measured both within an experiment and between different experiments. Triplicate or quadruplicate samples are measured at each dilution for all test chemicals. The variability within an experiment is given on the submitted CD as the Standard Deviation (SD) of the amount of DNA ($\mu\text{g}/\text{well}$) at each concentration for each test or control chemical. The variability between experiments is given as the Standard Error of the Mean (SEM) of the EC50s for each chemical (see **Table 7** in **Appendix A**). The variability in EC50 data obtained by CCI's in its Austin facility and EC50 data published by ICCVAM or obtained at other laboratories is measured by ranking the EC50s (most active to least active) of a set of test chemicals obtained by each source and calculating the correlation coefficient (r^2) of the rank orders using GraphPad Prism software (see **Figure 6** in **Appendix A**).

2.2.12. Statistical or non-statistical methods used to analyze data.

- A.** The absorbance of OD₅₉₀ and the DNA concentrations of samples are calculated by using KC Junior software from Bio-Tek.
- B.** EC₅₀, EC₈₀, r square analyses are calculated using the Hill kinetic model provided by GraphPad Prism software.

2.2.13. Criteria used to classify a test chemical (e.g., positive, negative, or equivocal), as appropriate

- A. Negative Chemicals:** A chemical induces <15% of the maximal effect of the positive control (E2) at any concentration tested at least 3 times using the lowest dilution factor at 1.87, $r^2 < 0.85$.
- B. Positive Chemicals:** A chemical that meets the following criteria: The calculated EA values contain at least one EA value $> 10\%$ and $\leq 50.0\%$ maximal effect, at least one EA value $> 50.0\%$ and $< 90\%$ maximal effect, and at least two EA values within 10% of minimal effect (bottom plateau). The EC50 must have an r^2 value ≥ 0.85 calculated using the Hill model.

2.2.14 Information and data included in the study report

- a) Experiment Date
- b) Note Book ID
- c) Project
- d) Chemical Names
- e) Name of Experimenter performing assays

- f) Clearly described standard operating procedures (SOP)
- g) Any modifications made from the SOP
- h) Detailed observations obtained during any part of the experiment, e.g. solution color, precipitates, cell morphology, etc.

- i) Raw data obtained from the measurement made by Bio-Tek plate reader using KC Junior: e.g. absorbance of OD₅₉₀ and calculated DNA concentration, standard curve, etc

Table 2-E: An example of raw data

Plate: mm-dd-yy Experiment ID (e.g SET#1) Plate ID (#1) Date Created: mm/dd/yy 9:43:17 AM

Raw Data											
0.006	0.005	0.006	0.008	0.009	0.007	0.008	0.007	0.007	0.008	0.009	0.009
0.005	0.008	0.006	0.007	0.007	0.006	0.008	0.007	0.011	0.01	0.01	0.013
0.005	0.005	0.006	0.014	0.013	0.015	0.018	0.028	0.02	0.038	0.036	0.043
0.008	0.008	0.007	0.005	0.006	0.005	0.007	0.005	0.009	0.008	0.01	0.012
0.008	0.008	0.006	0.006	0.003	0.008	0.003	0.009	0.006	0.014	0.008	0.014
0.008	0.008	0.007	0.006	0.009	0.005	0.01	0.006	0.012	0.009	0.013	0.015
0.006	0.006	0.005	0.006	0.005	0.006	0.004	0.008	0.004	0.01	0.008	0.007
0.006	0.007	0.006	0.006	0.006	0.004	0.005	0.004	0.006	0.006	0.007	0.006

Calculated Concentrations											
0.075975	0.087735	0.079792	0.109683	0.124128	0.095238	0.109683	0.095238	0.095238	0.109683	0.124128	0.124128
0.089355	0.107735	0.106069	0.095238	0.095238	0.080792	0.109683	0.095238	0.153019	0.138574	0.138574	0.18191
0.070378	0.079347	0.070792	0.196355	0.18191	0.210801	0.254137	0.39859	0.283027	0.543044	0.514153	0.615271
0.108943	0.081902	0.066347	0.066347	0.080792	0.066347	0.095238	0.066347	0.124128	0.109683	0.138574	0.167465
0.109683	0.109683	0.080792	0.080792	0.037456	0.109683	0.037456	0.124128	0.080792	0.196355	0.109683	0.196355
0.109683	0.109683	0.095238	0.080792	0.124128	0.066347	0.138574	0.080792	0.167465	0.124128	0.18191	0.210801
0.080792	0.080792	0.066347	0.080792	0.066347	0.080792	0.051902	0.109683	0.051902	0.138574	0.109683	0.095238
0.080792	0.095238	0.080792	0.080792	0.080792	0.051902	0.066347	0.051902	0.080792	0.080792	0.095238	0.080792

Plate: mm-dd-yy Experiment ID (e.g. SET#1) Plate ID (#2) Date Created: mm/dd/yy 9:43:17 AM

Raw Data											
0.01	0.009	0.01	0.014	0.011	0.014	0.019	0.022	0.027	0.039	0.033	0.037
0.014	0.013	0.013	0.021	0.021	0.025	0.037	0.042	0.053	0.039	0.054	0.054
0.047	0.043	0.03	0.043	0.04	0.051	0.046	0.069	0.052	0.073	0.054	0.08
0.013	0.01	0.012	0.01	0.017	0.011	0.038	0.022	0.044	0.042	0.064	0.059
0.008	0.007	0.005	0.01	0.002	0.014	0.003	0.028	0.015	0.047	0.038	0.054
0.021	0.017	0.015	0.023	0.03	0.023	0.045	0.044	0.048	0.045	0.062	0.062
0.007	0.007	0.007	0.01	0.005	0.013	0.016	0.031	0.018	0.046	0.049	0.053
0.009	0.009	0.01	0.014	0.014	0.016	0.024	0.029	0.025	0.042	0.04	0.041

Calculated Concentrations											
0.138574	0.124128	0.138574	0.196355	0.153019	0.196355	0.268582	0.311918	0.384145	0.557489	0.470817	0.528599
0.196355	0.18191	0.18191	0.297473	0.297473	0.355254	0.528599	0.600825	0.759724	0.557489	0.77417	0.77417
0.673052	0.615271	0.427481	0.615271	0.571935	0.730834	0.658607	0.99085	0.745279	1.048631	0.77417	1.149749
0.18191	0.138574	0.167465	0.138574	0.239691	0.153019	0.543044	0.311918	0.629716	0.600825	0.918623	0.846396
0.109683	0.095238	0.066347	0.138574	0.023011	0.196355	0.037456	0.39859	0.210801	0.673052	0.543044	0.77417
0.297473	0.239691	0.210801	0.326363	0.427481	0.326363	0.644161	0.629716	0.687497	0.644161	0.889732	0.889732
0.095238	0.095238	0.095238	0.138574	0.066347	0.18191	0.225246	0.441926	0.254137	0.658607	0.701943	0.759724
0.124128	0.124128	0.138574	0.196355	0.196355	0.225246	0.340809	0.413036	0.355254	0.600825	0.571935	0.58638

Plate: mm-dd-yy Experiment ID (e.g. SET#1) Plate ID (#3) Date Created: mm/dd/yy 9:43:17 AM

Raw Data											
0.044	0.048	0.056	0.052	0.053	0.057	0.05	0.056	0.051	0.049	0.046	0.038
0.044	0.052	0.048	0.005	0.005	0.005	0.002	0.002	0.003	0.002	0.002	0.001
0.047	0.048	0.052	0.034	0.027	0.026	0.001	0.002	0.002	0.002	0.003	0
0.043	0.046	0.047	0.051	0.045	0.054	0.048	0.046	0.04	0.017	0.018	0.033
0.043	0.053	0.05	0.04	0.044	0.044	0.049	0.045	0.04	0.036	0.038	0.046
0.053	0.049	0.042	0.045	0.048	0.048	0.051	0.041	0.038	0.036	0.031	0.029
0.04	0.047	0.043	0.044	0.047	0.053	0.048	0.045	0.049	0.035	0.047	0.038
0.039	0.045	0.046	0.045	0.05	0.043	0.043	0.04	0.047	0.04	0.045	0.042

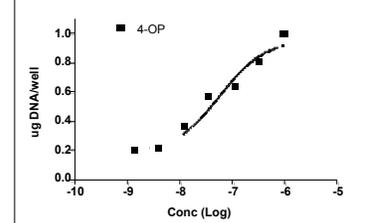
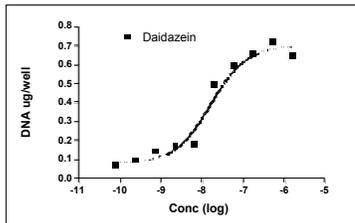
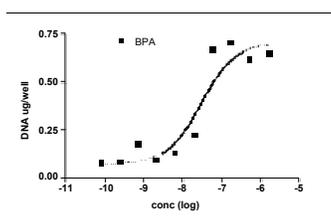
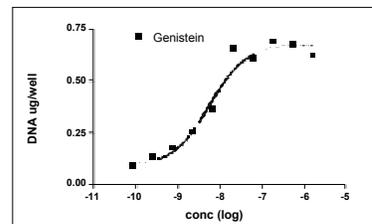
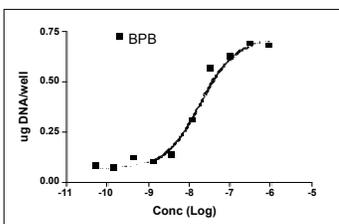
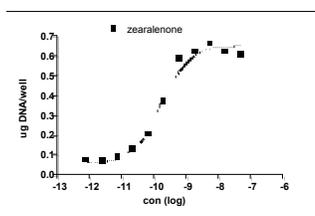
Calculated Concentrations											
0.629716	0.687497	0.80306	0.745279	0.759724	0.817506	0.716388	0.80306	0.730834	0.701943	0.658607	0.543044
0.629716	0.745279	0.687497	0.066347	0.066347	0.066347	0.023011	0.023011	0.037456	0.023011	0.023011	0.008566
0.673052	0.687497	0.745279	0.485262	0.384145	0.3697	0.008566	0.023011	0.023011	0.023011	0.037456	
0.615271	0.658607	0.673052	0.730834	0.644161	0.77417	0.687497	0.658607	0.571935	0.239691	0.254137	0.470817
0.615271	0.759724	0.716388	0.571935	0.629716	0.629716	0.701943	0.644161	0.571935	0.514153	0.543044	0.658607
0.759724	0.701943	0.600825	0.644161	0.687497	0.687497	0.730834	0.58638	0.543044	0.514153	0.441926	0.413036
0.571935	0.673052	0.615271	0.629716	0.673052	0.759724	0.687497	0.644161	0.701943	0.499708	0.673052	0.543044
0.557489	0.644161	0.658607	0.644161	0.716388	0.615271	0.615271	0.571935	0.673052	0.571935	0.644161	0.600825

j) Examples of data analyzed by Excel and GraphPad Prism: e.g. graphs, EC50, r^2 , etc.

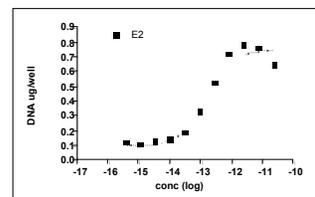
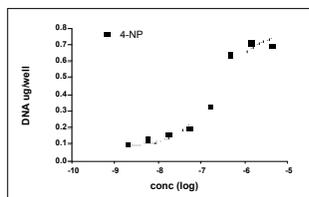
Table 2-F: An example of analyzed data

EA assay mm-dd-yy

Order on DWP	Concentrations used for test chemicals in deep-well plates (from High to Low)										ICI	0.5% EtoH	
Zearalenone	5.00E-08	1.67E-08	5.56E-09	1.85E-09	6.17E-10	2.06E-10	6.86E-11	2.29E-11	7.62E-12	2.54E-12	8.47E-13		
BPB	2.94E-06	9.79E-07	3.26E-07	1.09E-07	3.62E-08	1.21E-08	4.03E-09	1.34E-09	4.48E-10	1.49E-10	4.97E-11		
Genistein	5.00E-06	1.67E-06	5.56E-07	1.85E-07	6.17E-08	2.06E-08	6.86E-09	2.29E-09	7.62E-10	2.54E-10	8.47E-11		
BPA	5.00E-06	1.67E-06	5.56E-07	1.85E-07	6.17E-08	2.06E-08	6.86E-09	2.29E-09	7.62E-10	2.54E-10	8.47E-11		
Daidzein	5.00E-06	1.67E-06	5.56E-07	1.85E-07	6.17E-08	2.06E-08	6.86E-09	2.29E-09	7.62E-10	2.54E-10	8.47E-11		
4-OP	5.00E-05	2.68E-05	8.93E-06	2.98E-06	9.93E-07	3.31E-07	1.10E-07	3.68E-08	1.23E-08	4.09E-09	1.36E-09		
4-NP	5.00E-05	4.06E-05	1.35E-05	4.51E-06	1.50E-06	5.01E-07	1.67E-07	5.57E-08	1.86E-08	6.19E-09	2.06E-09		
E2	2.43E-11	8.10E-12	2.70E-12	9.00E-13	3.00E-13	1.00E-13	3.33E-14	1.11E-14	3.70E-15	1.23E-15	4.12E-16		
					Average								
Order on CCP													
E2	0.635	0.750	0.774	0.707	0.519	0.322	0.182	0.134	0.119	0.100	0.110		
4-NP	0.018	0.028	0.066	0.687	0.702	0.630	0.317	0.187	0.153	0.119	0.090		
4-OP	0.030	0.018	0.413	0.702	0.991	0.798	0.639	0.572	0.457	0.212	0.196		
Daidzein	0.322	0.639	0.716	0.649	0.589	0.495	0.177	0.163	0.139	0.095	0.071		
BPA	0.572	0.639	0.610	0.697	0.563	0.216	0.119	0.090	0.167	0.081	0.076		
Genistein	0.456	0.620	0.673	0.687	0.708	0.654	0.360	0.249	0.172	0.129	0.090		
BPB	0.572	0.678	0.687	0.620	0.560	0.307	0.129	0.095	0.114	0.071	0.076		
Zearalenone	0.606	0.620	0.659	0.620	0.586	0.370	0.206	0.129	0.086	0.066	0.071		
ICI												0.085	
0.5% EtoH													0.092



Chemicals	EC50	H slope	R2
Zearalenone	1.6E-10		0.99
BPB	1.2E-08		0.98
Genistein	5.4E-09		0.94
BPA	no mid. point		0.90
Daidzein	1.8E-08		0.99
4-OP	5.5E-08		0.96
4-NP	2.3E-07		0.97
E2	1.6E-13		0.97



Notes:

3. CHARACTERIZATION OF SUBSTANCES TESTED TO EVALUATE THE VALIDATION STATUS OF A PROPOSED TEST METHOD

MCF-7 Cell Proliferation Assay for Estrogenic Activity

3.1 RATIONALE FOR THE CHEMICALS SELECTED FOR USE IN THE VALIDATION PROCESS

Thirty seven chemicals with EA or no reported EA (see **Table 3-A**) tested to date are from the list of 78 EA positive and EA negative reference chemicals recommended by ICCVAM (2003a) to assess the accuracy of TA assays for EA. Three additional chemicals (chemicals with an asterisk in **Table 3-A**) are from a previous list of reference chemicals (ICCVAM, 2002c) to assess the accuracy of TA assays for EA. More specifically, 31 of the chemicals having the best-defined estrogenic activity (EA) in the ICCVAM tables were chosen, as were 9 chemicals in the ICCVAM tables having no reported EA (to serve as negative control, see **Table 10 in Appendix A**). Of the 31 chemicals having the best-defined estrogenic activity (EA) in the ICCVAM tables, 18 test chemicals with an EC50 estimated by ICCVAM from its meta-analysis and 13 chemicals with EA determined only from Qualitative Data by ICCVAM from its meta-analysis (see Table 4-1 of ICCVAM 2003a) were chosen for analysis by CCI in its Austin facility (see **Table 5 and 6 in Appendix A**). Methyl testosterone was the only test chemical with an EC50 estimated by ICCVAM from its 2003a meta-analysis that was *not* tested by CCI because purchase of those substances requires DEA licensing. Finally, prior to any published ICCVAM list of recommended test chemicals, CCI began to study the accuracy of its proposed assay by examining 10 test chemicals, some with strong EA (17 β -estradiol (E2), diethylstilbestrol (DES), 17 α -ethinyl estradiol EE), some with moderate EA (GEN, BPA), some with weak EA (MC, NP, OP) – as well as a negative control (PG), and a standard anti-estrogen (ICI, (see Table 4-2 of ICCVAM 2003a)). These chemicals were typically tested 5-7 times each at UM, NWU, and CCI in Austin.

3.2 RATIONALE FOR THE NUMBER OF SUBSTANCES TESTED

In total, CCI has tested 31 chemicals reported to exhibit EA in ICCVAM meta-studies (ICCVAM 2002c, 2003a) and 30-50 chemicals with no reported EA by ICCVAM and/or QSAR analyses. This relatively large sample size was chosen to provide a rather accurate assessment of the rate of false negatives and false positives, i.e. the accuracy, of this MCF-7 cell proliferation assay to assess EA.

3.3 DESCRIPTION OF CHEMICALS EVALUATED

See **Table 3-A FOR 3.3.1 – 3.3.8**

Table 3-A: Description of ICCVAM Reference Chemicals Tested by CCI

Substances	CASRN	Chemical Class	Concentrations tested (M)	Purity	Source of Substance
17α-Estradiol	57-91-0	Steroid, phenolic; Estrene	1E-4 - 1E-16	98%	Sigma
17α-Ethinyl estradiol	57-63-6	Steroid, phenolic	1E-4 - 1E-16	98%	Sigma
17β-Estradiol	50-28-2	Steroid, phenolic; Estrene	1E-4 - 1E-16	99%	Sigma
2,4,5-Trichlorophenoxyacetic acid	93-76-5	Organochlorine; Chlorinated aromatic hydrocarbon	1E-5 - 1E-12	99%	ChemService
4 - tert -Octylphenol	140-66-9	Alkylphenol; Phenol	1E-4 - 1E-12	97%	TCI
4-Cumylphenol	599-64-4	Phenol	1E-4 - 1E-12	99%	Acros
4-Hydroxytamoxifen	68047-06-3	Triphenylethylene; Benzylidene; Stilbene; Phenol	1E-5 - 1E-12	99%	Sigma
Apigenin	520-36-5	Flavonoid; Flavone; Phenol	5E-6 - 5E-13	>99%	Calbiochem
Atrazine	1912-24-9	Aromatic amine; Triazine; Arylamine	5E-5 - 5E-12	99.2%	Supelco
Bisphenol A	80-05-7	Diphenylalkane; Bisphenol; Phenol	1E-4 - 1E-13	99%	Sigma
Bisphenol B	77-40-7	Diphenylalkane; Bisphenol; Phenol	1E-4 - 1E-13	>99%	Aldrich
Butylbenzyl phthalate	85-68-7	Phthalate	1E-4 - 1E-13	98%	TCI
Clomiphene citrate	50-41-9	Chlorinated triphenylethylene; Benzylidene; Stilbene	5E-6 - 5E-13	99%	MP Biomedical
Corticosterone	50-22-6	Steroid, nonphenolic	5E-5 - 5E-12	>99%	TCI
Coumestrol	479-13-0	Coumestan; Benzopyranone; Coumarin; Ketone	1E-4 - 1E-16	97.5%	Fluka
Cycloheximide*	66-81-9	Piperidine; Glutaramide	1E-4 - 1E-12	98.0%	Sigma
Cyproterone acetate	427-51-0	Nitrile; Diphenyl ether; Organochlorine	5E-5 - 5E-12	98%	Sigma
Daidzein	486-66-8	Flavonoid; Isoflavone; Phenol	5E-6 - 5E-13	98%	Acros
Dexamethasone	50-02-2	Steroid, nonphenolic	5E-5 - 5E-12	97%	Sigma
Diethylstilbestrol	56-53-1	Stilbene; Benzylidene; Diphenylalkane	1E-4 - 1E-16	99%	Sigma
Estriol*	50-27-1	Resorcylic acid lactone; Phenol	1E-4 - 1E-16	99%	Sigma
Estrone	53-16-7	Steroid, nonphenolic; Androstene	1E-4 - 1E-16	99%	Acros
Fenarimol	60168-88-9	Heterocycle; Pyrimidine	5E-6 - 5E-13	99%	Protocol
Flavone	525-82-6	Flavonoid; Flavone	1E-4 - 1E-11	99%	Acros
Flutamide	13311-84-7	Amide; Anilide; Nitrobenzene	1E-4 - 1E-11	99%	Sigma
Genistein	446-72-0	Flavonoid; Isoflavone; Phenol	1E-4 - 1E-12	99%	Sigma
Haloperidol*	52-86-8	Butyrophenone; Ketone; Piperazine	1E-4 - 1E-12	99%	Sigma
ICI 182,780	129453-61-8	Steroid, phenolic	1E-4 - 1E-16	>98%	Sigma
Kaempferol	520-18-3	Flavonoid; Flavone; Phenol	1E-4 - 1E-12	>96%	Fluka
Kepone	143-50-0	Organochlorine; Chlorinated bridged cycloalkane	1E-4 - 1E-12	99.9%	Supelco
Linuron	330-55-2	Urea	5E-5 - 5E-12	99%	ChemService

<i>meso</i> -Hexestrol	84-16-2	Diphenylalkane; Bisphenol; Phenol	1E-4 - 1E-16	99%	TCI
Mifepristone	84371-65-3	Steroid, nonphenolic; Estrene	5E-5 - 5E-12	98%	Tocris
<i>o,p'</i> -DDT	789-02-6	Organochlorine; Diphenylalkene	5E-5 - 5E-12	98.2%	ChemService
<i>p-n</i> -Nonylphenol	104-40-5	Alkylphenol; Phenol	1E-4 - 1E-12	98.5%	Supelco
<i>p,p'</i> -DDE	72-55-9	Organochlorine; Diphenylalkene	5E-5 - 5E-12	99%	Acros
<i>p,p'</i> -Methoxychlor	72-43-5	Organochlorine; Chlorinated hydrocarbon	5E-5 - 5E-12	99.3%	Supelco
Procymidone	32809-16-8	Organochlorine; Cyclic imide	5E-6 - 5E-13	99%	Protocol
Progesterone	57-83-0	Steroid, nonphenolic; Pregnenedione	1E-4 - 1E-11	98%	Acros
Zearalenone	17924-92-4	Steroid, phenolic; Estrene	1E-5 - 1E-12	>99%	MP Biomedial

3.3.1 Chemical or product name, or if a mixture, provide information on all components

3.3.2 CASRN

3.3.3 Chemical and product class

3.3.4 Physical/chemical characteristics (e.g., water and lipid solubility, pH, pK_a, etc.). Any characteristics thought or known to impact test method accuracy and/or reliability should be clearly described

3.3.5 Stability of the test substance in test medium

3.3.6 Concentrations tested

3.3.7 Purity, including the presence and identity of contaminants and stabilizing additives

3.3.8 Supplier or source

3.4 CODING PROCEDURES USED IN THE VALIDATION STUDIES.

None

3.5 COMPARISON WITH VALIDATED TEST METHODS

No validated test methods yet available to make such comparisons.

4.0 IN VIVO REFERENCE DATA USED FOR AN ASSESSMENT OF THE ACCURACY OF THE PROPOSED METHOD

MCF-7 Cell Proliferation Assay for Estrogenic Activity

No *in vivo* reference data yet available.

5.0 TEST METHOD DATA AND RESULTS

MCF-7 Cell Proliferation Assay for Estrogenic Activity

5.1 DESCRIPTION OF TEST METHOD PROTOCOL USED TO GENERATE EACH SUBMITTED SET OF DATA

5.1.1. Protocols at U Missouri (Columbia) for MCF-7 Robotics Cell Proliferation Assay to Analyze EA

A. Equipment: A Tomtec Quadra 96 robotic workstation with an optional serial dilution unit, a device that attaches to the workstation, was used to perform the assays at the University of Missouri (Columbia). This equipment was used for seeding cells in 96-well plates and preparing serial dilutions of the test chemicals in tissue-culture medium. The workstation was also used to change media and prepare 96-well plates for DNA quantification assays. The assay for DNA quantification, based on the diphenylamine reaction, was conducted on a Bio-Tek PowerWave_x 96-well plate reader spectrophotometer. The data (DNA/well) was automatically plotted on spreadsheets.

B. Materials: Minimum essential medium with nonessential amino acids (powdered), HEPES, bovine insulin, calf thymus DNA type I, Hoechst dye 33258, streptomycin sulfate, penicillin-G, EDTA, Hanks' balanced salt solution (HBSS), bovine serum albumin (BSA), E2, EE, DES, and MC were obtained from Sigma Chemical Company (St. Louis, MO). Bovine calf serum, phenol red (sodium salt) and lyophilized trypsin were obtained from Gibco BRL (Grand Island, NY). BPA (research grade) was obtained from Aldrich Chemical Company (St. Louis, MO). OP (technical grade) and NP (technical grade) were purchased from Chem Service (West Chester, Pennsylvania), ICI was from Tocris, Inc. (St. Louis, MO), GEN from Indofine Chemical Co (Sommerville NJ), and PG from Eastman Kodak (Rochester, NY). 1,2,6,7-³H 17 β -estradiol, 100 Ci/mol, was purchased from DuPont New England Nuclear (Boston, MA). All chemicals were reagent grade, unless otherwise specified. The 96-well plates, culture dishes, etc were made of glass or polyethylene plastics that do not release chemicals with detectable EA [In contrast, polystyrene and polycarbonate plastics often release EA].

C. Cell Culture: MCF-7 cells were obtained from Dr. V. Craig Jordan, Northwestern University Medical School, and a CCi consultant. MCF-7 cells were maintained in MEM with nonessential amino acids, 10 μ g/ml phenol red, 10 mM HEPES, 6 ng/ml insulin, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 5% charcoal-stripped calf serum (maintenance medium) (Lippman et al., 1976; Welshons et al., 1987). Because the responsiveness of MCF-7 cells can drift, cells were propagated for 2-4 months and then replaced with cells derived from a primary source in liquid N₂ storage.

D. Cell preparation: Estrogen-responsive MCF-7 cell proliferation assays were performed in a 96-well robotic format. Using the Tomtec unit, MCF-7 cells were seeded at 2200 cells per well in 0.2 ml hormone-free medium in 96-well plates that contained 0.1 ml hormone-free medium per well. The cells were adapted for 3 days in hormone-free culture medium prior to adding test chemicals.

E. Preparation of estrogen-free cell culture medium: Calf serum was stripped by charcoal and added to estrogen-free culture medium that contained no phenol red. Each new batch of charcoal-stripped serum was routinely tested for estrogen-stimulated MCF-7 cell growth in experiments including negative, positive, and anti-estrogen controls. If E2-stimulated cell proliferation was less than two-fold, we did not use that batch of serum.

F. Preparation of test chemicals: All test chemicals were dissolved in ethanol. The stock chemicals were diluted 100 times with estrogen-free medium for the starting concentration and were serially diluted by the robotic workstation, as indicated below. Each chemical at each concentration was added to 4 wells of 96-well plates and incubated for 4 days with estrogen standards (E2 or DES, in serial dilution from 0.1pM to 1nM) on each plate. The test chemical was serially diluted from 0.1nM to 10 μ M (or to the solubility limit). [NP and OP were accidentally contaminated during weighing in one assay. To avoid this problem, test chemicals were subsequently weighed on a different scale than that used to weigh potent estrogens, e.g. E2, DES and EE.] All media was changed daily. At the end of the 4 day exposure, the medium was removed, the wells were washed once with 0.2 ml of HBSS, and then assayed to quantify amounts of DNA/ well using a micro-plate modification of the Burton diphenylamine DNA assay (Burton, 1956; Natarajan et al., 1994), described below.

G. DNA Quantification: Briefly, 60 μ l of a 1:5 ratio mixture of 0.16% acetaldehyde and 20% v/v perchloric acid, and then 100 μ l of 4% diphenylamine in glacial acetic acid, were added to each well. A standard curve was prepared by adding samples of 0.125 to 5 μ g DNA/well in 10 μ l of HBSS. After incubating the plate overnight at 37°C, the absorbance was measured as the difference between OD₅₉₅ and OD₇₀₀ in a Bio-Tek PowerWave_x plate reader. The output was converted to μ g DNA/well by using a third degree polynomial curve fit of the standard curve; the correlation coefficient of the standard curve fit was typically greater than 99.99%.

H. Calculation of EA: Estrogenic stimulation (as opposed to nonspecific stimulation) of MCF-7 proliferation (measured as increased amount of DNA per well) induced by the test chemical was confirmed as estrogenic in a second assay if the stimulation was suppressed by co-incubation with an anti-estrogen (ICI 182,780 at 1 μ M). If the test chemical was estrogenic, then its half-maximum stimulation of cell proliferation (EC₅₀, in M) was obtained from the dose-response data by Michaelis-Menton kinetics (Sakai and Gorski, 1984). The EA of a test chemical was expressed relative to the EA of 17 β -estradiol (E2, positive control) and was calculated as: **EC₅₀ of the test chemical / EC₅₀ of E2**. EC₅₀ of a test chemical was given in M or as a percentage, with E2 = 100%. If the test chemical did not induce MCF-7 cell proliferation and could not be inhibited by an anti-estrogen, then the test chemical was determined to have no detectable EA. All data for EA, anti-EA, and control chemicals were calculated as the mean of three or more independent assays. In order to minimize errors of manual entry of EA data, CCI developed a more-automated data handling system. Only assays in which both positive and negative controls were acceptable were considered for our studies. That is, E2 adequately stimulated cell proliferation, the negative control proliferation was not lowered by ICI, and the stimulation of cell proliferation by the test chemical was inhibited by ICI.

5.1.2. Protocols at U Missouri (Columbia) for MCF-7 Manual Assay to Measure Relative Binding Affinity (RBA) for ER.

A. Cell Culture: Growth medium and cell maintenance were the same as described for the robotics EA assay. The RBA assay was performed in a standard 24-well "manual" (non-robotic) format, as previously reported (Winneker et al, 1981; Nagel et al, 1998). MCF-7 cells were seeded in 24-well cell culture plates in *estrogen-free* culture medium for 3 days prior to adding test chemicals, as described below.

B. Parameters measured to calculate RBA:

1) *The total amount of tritiated E2 bound to ER in the presence of a test chemical* was calculated as follows:

Incubate cells for 18 hours in estrogen-free culture medium containing 1 nM tritiated E2 alone or in the presence of 0.1 nM to 10 μ M (or the solubility limit) of the test chemical. Wash cells with 6 x 2 ml HBSS. Dissolve the tritium-labeled cells in 1 ml of 10 mM EDTA pH 12.5, then neutralize to pH 7.2. Measure the amount of tritiated E2 /well that remains bound to the intracellular ER in aliquots taken for scintillation counting, protein, or DNA determinations (Nagel et al., 1998).

2) *The amount of nonspecific binding of tritiated E2* was calculated as follows: **(a)** Incubate cells for 18 hours in estrogen-free culture medium containing 1 nM tritiated E2 *AND* 100 nM non-radioactive E2 (a 100-fold excess of the non-radioactive ligand). **(b)** Wash and dissolve cells as described above, and measure the remaining amount of tritiated E2 /well.

3) *The amount of specific binding of E2 to ER* was calculated by subtracting the nonspecific binding of E2 from the total amount of E2 binding.

4) *The reduction of specific binding of E2 in the presence of a test chemical* was obtained by subtracting the specific binding of E2 from the total amount of E2 bound to ER in the presence of a test chemical.

C. RBA Calculation: The IC₅₀ of the test chemical and the IC₅₀ of non-radioactive E2 were defined as those concentrations of either the test chemical or the non-radioactive E2 that produced 50% inhibition of the specific binding of radiolabeled E2, respectively (see **Appendix A Table 2**). The IC₅₀ of the test chemical and that of the non-radioactive E2 reference were then used to calculate the relative binding affinity (RBA) as: IC₅₀ of non-radioactive E2 / IC₅₀ of the test chemical (see **Appendix A Table 3**). If the test chemical was not able to reduce the binding of radiolabeled E2, then the test chemical was determined to have no binding to ER in the concentration range tested. Positive controls for EA included non-radioactive E2, DES and ICI. All data were calculated as the mean of 2-5 independent assays.

5.1.3. Protocols at Northwestern U Medical School for MCF-7 Cell Manual Assay for EA

Protocols used by Northwestern University are similar to the ones that have been used by CertiChem, except the following changes:

- 1) All the procedures were performed manually using a multi-channel pipette
- 2) Estrogen-free medium contained 10% stripped FBS

- 3) MCF-7 cells were stripped on a 75 cm² flask for four days before the cells were seeded into 96-well cell culture plates
- 4) MCF-7 cells were seeded at 500 cells/well into 96-well cell culture plates
- 5) The next day, cells were treated with test compounds
- 6) Fresh medium containing test chemicals was changed every other day.
- 7) At the end of 7th day, the DNA concentration in cell culture plates was measured using CyQuant Cell Proliferation Assay Kit (Molecular Probes) as described below (NWU used the CyQuant method to measure DNA concentration instead of the Burton Diphenylamine assay used by UM and CCI, in large part because NWU had been using the CyQuant method for many years and the CyQuant method was faster. The Burton Diphenylamine Assay takes a longer time, but it involves fewer steps).
 - a. When cells are ready to be harvested, discard medium and leave plates in -70°C for at least 30 min.
 - b. Take plate out of freezer and leave at RT.
 - c. Make DNA Standards. Keep in dark.
 - i. Get 9 Eppendorf tubes. Label with final concentrations as follows (all in ng/mL):

1000	500	250	125	62.5	31.25	15.63	7.815	0 (blank)
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 - ii. Add 400 μ l of the lysis/dye solution to all but the first tube.
 - iii. In the first tube dilute the stock DNA (100 μ g/ml). Take 792 μ l of ddH₂O and add 8 μ l of DNA.
 - iv. From the first tube (1000), take 400 μ l and add to the next tube (500). Mix well. Take 400 μ l from the 500 tube and put into the next tube (250). Mix. Continue until you get to the 7.815 tube. No DNA in the last tube (blank)
 - d. Add 150 μ l of lysis/dye buffer (Molecular Probe) into each well. Incubate in the dark for 5 minutes.
 - e. Transfer DNA standards onto a black-bottom 96-well plate. (120 μ l per well, each dilution in triplicates)
 - f. Pipette cell lysis 3 times to mix and transfer 120 μ l of the cell lysis into black-bottom 96 well plate.
 - g. Read the black-bottom plate containing cell lysis and DNA standards by a plate reader (Mikrowin) using the CyQuant program at 485-530 nm.
 - h. DNA concentration is then calculated as ng/mL
- 8) EC50s are obtained from the dose-response data by Hill Equation using GraphPad Prism software that is more accurate and easier to use than Michaelis-Menton.
- 9) Statistical Analyses: The variability within an experiment for replicate samples is given on the submitted CD as the Standard Deviation (SD) of the amount of DNA (μ g/well) at each concentration for each test or control chemical. The variability between experiments for

different experimental runs of the same chemical (labeled “experiments” in Figures and Tables) is given as the Standard Error of the Mean (SEM) of the EC50s for each chemical (see **Table 7** in **Appendix A**). The variability in EC50 data obtained by CCI in its Austin facility and EC50 data published by ICCVAM or obtained at other laboratories is measured by ranking the EC50s (most active to least active) of a set of test chemicals obtained by each source and calculating the correlation coefficient (r^2) of the rank orders using GraphPad Prism software (see **Figure 6** in **Appendix A**). That is, a least squares regression analysis is calculated to examine whether the rankings produced by CCI’s proposed test method differ significantly (null hypothesis) from ICCVAM rankings in their published meta-analysis of the same 18 test-chemicals, or rankings obtained at UM or NWU for the same 9 or 10 test chemicals.

5.1.4. Protocols at CCI for MCF-7 Cell Proliferation Robotic Assay to Measure EA

A. Equipment: CCI uses an epMotion 5070 robotic workstation to perform these assays. This workstation is used for seeding cells in 96-well plates and preparing serial dilutions of the test chemicals in tissue-culture medium. The workstation is also used to change media and prepare 96-well plates for DNA quantification assays. CCI’s assay for DNA quantification, based on the diphenylamine reaction, is conducted on a Bio-Tek Elx808_{iu} 96-well plate reader spectrophotometer. The data (DNA/well) is automatically plotted on spreadsheets.

B. Materials: HEPES, bovine insulin, calf thymus DNA type I, Hoechst dye 33258, EDTA, Hanks' balanced salt solution (HBSS), and E2 were obtained from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum to maintain the cells, RPMI Medium 1640 with phenol for cell maintenance and RPMI medium 1640 without phenol red for EA and anti-EA assays, L-glutamine, Minimum Essential Medium (MEM) non-essential amino acids, HEPES, antibiotics, antimycotics, and lyophilized trypsin were obtained from Gibco.

CCI’s polypropylene deep-well plates are from Matrix Technologies. CCI’s 96-well cell culture plates and cell culture flasks are from Corning and are made of a polystyrene plastic that does not release as much EA as those made by other firms. CCI has tested many brands of culture dishes and other labware so as to use those that release the least EA. CCI carefully checks each newly-purchased batch of labware for EA. CCI knows of no manufacturer of glass 96-well plates for cell culture or deep well plates for chemical serial dilution, which presumably would release *no* EA.

C. Cell preparation: MCF-7 cells were obtained from Dr. V. Craig Jordan, Northwestern University Medical School. Dr. Jordan is a CCI consultant. Estrogen-responsive MCF-7 cells are stored in liquid nitrogen. MCF-7 cells are also maintained at 37°C in RMPI with nonessential amino acids, 10 µg/ml phenol red, 10 mM HEPES, 6 ng/ml insulin, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5% charcoal-stripped fetal bovine serum (maintenance medium) (Lippman et al., 1976; Welshons et al., 1987). Because the responsiveness of MCF-7 cells can drift, cells are propagated for 2-3 months and then replaced with cells derived from the primary source in liquid N₂ storage. An aliquot of cells maintained at 37°C are grown for two days in phenol-free media containing 5% charcoal stripped fetal bovine serum in a 25 cc flask. Using the epMotion 5070 unit, MCF-7 cells are seeded at 300 cells per well in 0.2 ml of this hormone-free medium in Corning 96-well plates (Other plates, e.g. BD plates, leach more EA). The cells are adapted for 3 days in hormone-free culture medium prior to adding test chemicals.

CCi routinely tests each new batch of charcoal-stripped serum for estrogen-stimulated MCF-7 cell growth in experiments including negative (1% EtOH), positive (previously tested striped serum), and anti-estrogen (ICI 182,780). Unless E2-stimulated cell growth is ≥ 4 times the negative control growth in seven days, we do not use that batch of serum.

D. Preparation of test chemicals: All test chemicals are dissolved in ethanol and/or water for a stock concentration of 10^{-1} M (or to the highest concentration that the solubility allows). These stock chemicals are then diluted 100 times in estrogen-free medium for a starting concentration of 10^{-3} M (or to the solubility limit) and are serially diluted by our robotic workstation to a concentration of 10^{-11} M. Each test chemical at each concentration, as well as an estrogen positive control (E2 in serial dilution from 10^{-9} M to 10^{-16} M), is added in quadruplicate to MCF-7 cells in a 96-well plate. The media is changed daily for 7 days. At the end of the 7 day exposure, the media is removed, the wells are washed once with 0.2 ml of HBSS, and then assayed to quantify amounts of DNA/ well using a micro-plate modification of the Burton diphenylamine (DPA) assay (Burton, 1956; Natarajan et al., 1994), described below.

E. DNA Quantification: Briefly, 60 μ l of a 1:5 ratio mixture of 0.16% acetaldehyde and 20% v/v perchloric acid, and then 100 μ l of 4% diphenylamine in glacial acetic acid, are added to each well. A standard curve is prepared by adding samples of 0.125 to 5 μ g DNA/well in 10 μ l of HBSS. After incubating the plate overnight at 37°C, the absorbance is measured as the difference between OD₅₉₅ and OD₇₀₀ in a Bio-Tek EL_x 808 plate reader. The output is converted to μ g DNA/well by using a third degree polynomial curve fit of the standard curve; the correlation coefficient of the standard curve fit is typically greater than 0.99.

F. Calculation of EA: Stimulation of MCF-7 proliferation (measured as an increased amount of DNA per well) induced by the test chemical is confirmed as estrogenic (as opposed to nonspecific stimulation) in a second assay to examine whether any stimulation is suppressed by co-incubation with an anti-estrogen (ICI 182,780 at 1 μ M). If the test chemical is estrogenic, then its half-maximum stimulation of cell proliferation (EC₅₀, in M) is obtained from the dose-response data by Hill kinetics (Sakai and Gorski, 1984) using Graph Pad Prism. The EA of a test chemical is expressed as an EC₅₀ or is expressed relative to the EA of 17 β -estradiol (E2, positive control), calculated as: REA = EC₅₀ of E2 / EC₅₀ of the test chemical. If the test chemical does *not* induce MCF-7 cell proliferation, then this test chemical is stated to have no detectable EA. All data for EA, anti-EA, and control chemicals are calculated as the mean of four independent assays. Only assays in which both positive and negative controls are acceptable are used in our studies. That is, E2 must adequately stimulate cell proliferation, cell proliferation of the negative control lacking E2 must not be lowered by ICI 182,780, and the stimulation of cell proliferation by the test chemical must be inhibited by ICI.

5.2 DATA OBTAINED WITH EACH TEST METHOD

See **Appendix A** Note that the MCF-7 assay is more accurate, and less variable, when run in the robotic format developed by CCi in Austin, TX.

5.3 STATISTICAL APPROACH USED TO EVALUATE DATA

See analyses for each data set. Different data require different statistical approaches.

5.4 SUMMARY OF RESULTS

See Figures and Tables in **Appendix A** for this Section

5.4.1. Summary of Data from University of Missouri (Columbia)

Appendix A Table 1 gives the EC50 values obtained by the MCF-7 robotic assay for the ten chemicals sampled at The University of Missouri (UM) on the dates given at the top of each column. [All but PG (propyl gallate) are on the ICCVAM (2003a) list of 78 reference chemicals.] These EC50 data are also plotted in **Appendix A Figure 1**, in part to show the consistency (reproducibility in the same laboratory) of our EA assay and in part to demonstrate the range of EC50 values that our initial EA assay was able to detect. Note that assays of each chemical give very consistent data when repeated 5-7 times. These data obtained by CCI at the University of Missouri (UM) suggest that our robotics EA assay is reliable when repeated at different times in the same lab.

EC50 values for ten test chemicals in **Appendix A Table 1** and **Appendix A Figure 1** were obtained from concentration-response data fitted by Michaelis-Menton kinetics. A concentration-response curve was plotted for each chemical every time it was tested; each point on the curve represents an average of four wells. Over 70 such concentration-response curves were generated in this study (data not shown). OP and NP obtained from ChemService were consistently weakly estrogenic each time they were tested. However, OP and NP ordered from Sigma exhibited no detectable EA the first two times tested, and sometimes showed weak EA in subsequent tests (data not shown). CCI cannot explain the differences in EA activity for OP and NP obtained from Sigma vs. ChemService. This inconsistency of EA in chemicals obtained from different sources (or from the same source at different times) illustrates a problem noted by ICCVAM (2002a,c; 2003a), namely that there is no agreed-upon source or standard for the 78 reference chemicals (ICCVAM, 2003a) recommended for validation of EA.

This original version of CCI's robotic EA assay was highly sensitive, albeit not as sensitive as the current assay. For example the mean EC50 of E2 of CCI's original assay at UM was 3.4×10^{-12} M (0.2 pg/ml). CCI's assay as currently nominated has an EC50 of about 2.6×10^{-13} M. This high sensitivity facilitates detection of chemicals with weak EA (such as exhibited by NP, OP, MC, BPA or BHT). Such weak EA is often missed in less sensitive assays, including most or all published transient transfection assays (TAs) using estrogen-responsive reporter genes or RBA assays (ICCVAM, 2002a; 2003a). Detection sensitivity is critical for assays of EA because less sensitive assays will miss "low dose" estrogenic effects (NTP, 2001; vom Saal, 2005; Welshons et al. 2005).

The data presented in **Appendix A Table 1** demonstrate that CCI's robotics assay for EA at UM is accurate, as well as reliable. **Appendix A Table 1** shows that the *mean* EC50 values obtained in CCI's robotics assay are similar to *median* EC50 values compiled and published by ICCVAM (2003a). CCI's mean EC50 values using this robotic assay are also very similar to published EC50 values obtained for some of these chemicals using an MCF-7 cell proliferation assay in manual format (see Nagel et al., 1998 for E2, EE, BPA, and GEN).

To further validate the initial robotic EA assay at UM, CCI tested the Relative Binding Affinity (RBA) of the ten chemicals in **Appendix A Table 2** using an ER-binding assay recommended by EDSTAC (1998) and ICCVAM (2002a,b,c; 2003a). **Appendix A Table 2** presents our IC50 data for these same 10 chemicals. Note that these IC50 data appear to be reliable, i.e. RBA IC50 (**Appendix A Table 2**) values are reproducible when repeated 2-5 times over two months.

Appendix A Table 3 compares data from the manual RBA assay with CCI's robotic cell proliferation assay for EA, as originally run at UM. CCI's robotic TA assay and manual RBA assay both show EA for those 5 chemicals (E2, DES, EE, GEN, BPA) that have been reported to have moderate to strong EA. Both assays show no detectable EA for the one chemical (PG) having no reported EA. As expected, the results of these two assays *do not* agree for the anti-estrogen ICI because ICI binds to ER, but does not activate estrogenic responses such as MCF-7 cell proliferation. As expected, ICI always showed strong anti-EA activity in the robotic assay. In contrast, the results of CCI's RBA and cell proliferation assays did *not* agree for three chemicals (NP, OP, and MC) reported to have weak EA in our robotic MCF-7 assay, in other TA assays and *in vivo* assays (ICCVAM, 2002a,c). For each of these three chemicals, the robotic MCF-7 assay at UM showed weak EA, whereas the RBA assay showed no detectable ER binding activity. Therefore, based on these data, CCI's robotic TA (cell proliferation) assay for EA appears to be more relevant and more sensitive compared to a standard ER RBA assay for EA. This conclusion is consistent with that of ICCVAM (2002a,b; 2003a) that TA assays are generally more relevant and sensitive compared to RBA assays to determine the EA of a chemical. [As noted by ICCVAM (2002a,b,c; 2003a) and EDSTAC (1998), any conclusion from *in vitro* data must be confirmed by *in vivo* tests.]

In brief, these data from the UM study strongly suggest that CCI's initial robotics assay for EA was valid, i.e., produces EA data that were reliable and relevant.

5.4.2. Summary of Data from Northwestern University Medical School (Chicago)

Nine of the ten chemicals sampled at UM were also sampled repetitively at NWU with CCI's EA assay run in manual format (Genistein was not tested in NWU). The EC50s were obtained from dose-response curves according to the Hill Equation using GraphPad Prism software (data not shown). **Appendix A Figure 2** gives the EC50 values obtained by CCI's EA assay for nine chemicals sampled at Northwestern University Medical School (NWU) in manual format for different experimental runs of each chemical. These EC50 data are also summarized in **Appendix A Table 4**. Note that the mean values and sensitivity are similar at NWU and CCI (see **Appendix A Table 5** and **Appendix A Figure 3**), but the variation in the data for different experimental runs of the same chemical are usually greater in manual format at NWU than in robotic format at CCI. Also note that the EC50 of NP is much lower at NWU than the EC50 from UM or CCI – or than the EC50 reported by ICCVAM (2003a) in its meta-analysis. We suspect that this low EC50 for NP at NWU is due to contamination by a strong estrogen.

5.4.3. Summary of Data from CCI's Laboratory (Austin)

CCI has modified its robotic MCF-7 cell proliferation assay originally developed at UM so as to increase its sensitivity and decrease its variability. For example, CCI optimized assay sensitivity by exposing MCF-7 cells to test chemicals for 7 days, rather than 3-5 days. **Appendix A Figure 3** and **Appendix A Table 5** show that E2 EC50 at CCI is now about 2×10^{-13} M. **Appendix A Figure 3** and **Appendix A Table 5** also give the EC 50 values obtained by CCI's robotic assay for ten chemicals sampled at CCI's Laboratory in Austin, TX, in robotic format from five different experimental runs. Note that for each chemical, the EC50 is very repeatable for different experimental runs in CCI's facility. **Appendix A Table 6** gives EC50 data for other reference chemicals on the ICCVAM (2003a) list of 78 Chemicals.

5.4.4. Comparison of Data from ICCVAM, UM, NWU, and CCI to show reliability and accuracy of CCI's MCF-7 robotic assay for EA

Appendix A Figures 1-5 and **Appendix A Tables 1 and 4-6** show that CCI's robotic EA assay is very consistent (reliable, repeatable) when analyzing the same set of 9-10 chemicals in the same laboratory or in different laboratories (CCI, UM, NWU). [GEN was *not* sampled at NWU.] Furthermore, **Appendix A Figures 4-5** and **Appendix A Tables 7-8** show that CCI's EA assay gives very similar data when run in robotic (CCI, UM) or manual (NWU) format. These tables and figures in the **Appendix A** also show that the data from the assay run in robotic format at CCI are *less* variable than data from the assay run in manual format run at NWU.

Appendix A Tables 7-9 and **Appendix A Figures 4-5** show *mean* EC50 values from CCI's assay and *median* EC50 values for the same set of chemicals compiled by ICCVAM (2003a) in a meta-analysis to provide a set of reference chemicals to be used to test the accuracy of EA TA assays. A comparison of CCI's data with ICCVAM's published data suggests that CCI's robotic EA assay is accurately measuring EA. For example, **Appendix A Table 10** shows that the rate of false negatives (4/29) and false positives (1/11) to date is very low when CCI's data are compared to ICCVAM's meta-study (2003a). The four false negatives are 4-hydroxytamoxifen, dexamethasone, 2,4,5-trichloro-phenoxyacetic acid, and clomiphene citrate. These chemicals were not conclusively analyzed in the ICCVAM meta-analysis. For example, ICCVAM (2003a) reported only one study as EA positive for dexamethasone and only one for 2,4,5-trichloro-phenoxyacetic acid. ICCVAM (2003a) reported 3/8 studies as EA positive for 4-hydroxytamoxifen, i.e. 5/8 reported the compound as EA negative. ICCVAM (2003a) anticipated that clomiphene citrate should be EA positive, but this chemical had not been tested for EA in any published TA study analyzed by ICCVAM (2003a). The one possible false positive (mifepristone) reported by CCI using its robotic EA assay has recently been reported to exhibit EA in other assays (ICCVAM, personal communications). Furthermore, CCI's robotic MCF-7 assay detected no false positives (0/34) for 34 other chemicals that have been reported to have no detectable EA in various *in vitro* or QSAR studies. [ICCVAM did not include these chemicals in its 2003a meta-study.]

The conclusion that the proposed test method is accurate is further documented by **Appendix A Tables 11-12** that compare the ranking a set of ICCVAM (2003) reference chemicals according to ICCVAM's meta-analysis of median EC50 values (**Appendix A Table 11**) with the ranking of CCI's mean EC50 values using the proposed test method (**Appendix A Table 12**). [ICCVAM gives no EC50 values for several reference chemicals declared to be EA positive; CCI *has* determined EC50 values for these same reference chemicals.] **Appendix A Figure 6** plots the CCI ranking (y axis) against the ICCVAM ranking (x axis) for the chemicals listed in **Appendix A Table 11**. **Appendix A Figure 6** also shows the least squares regression line that best fits these data. The least squares regression analysis shows that the rankings produced by CCI's proposed test method and the ICCVAM rankings do not differ significantly (null hypothesis, $p < 0.001$).

5.4.5. Data demonstrating versatility of this MCF-7 assay

Appendix A Figures 8-12 and **Appendix A Table 13** show the high versatility of this assay to measure EA in complex mixtures of chemicals, e.g., plastics and foodstuffs. These and other data also give examples of how CCI has helped several well-known firms solve problems with undesired levels of EA in foodstuffs, plastic containers, baby bottles, labware, etc. As one example, from data such as that shown in **Appendix A Figures 8-12 & Appendix A Table 13**, we have identified the following possible sources of EA in some animal feeds:

- (1) endogenous EA from some ingredients such as yeast residues obtained from brewer's yeast [many other ingredients like oats are routinely free of detectable EA],
- (2) exogenous EA leaching from plastic storage containers holding oily or moist ingredients such as casein and gluten, but not dry ingredients such as oats,
- (3) antioxidant (AO) additives.

5.5 Coded chemicals tested using GLP guidelines.

None

5.6 "Lot-to-lot" consistency of the test substances.

Not explicitly examined. The lots used by UM, NWU, and CCI for test chemicals are different. Nevertheless, these different lots gave very similar data, with the exception of 4-NP used by NWU which produced a higher variability and a lower EC50 than 4-NP used by CCI and UM. We strongly suspect that this chemical tested at NWU was contaminated with a strong estrogen.

5.7 Availability of any data not submitted for external audit, if requested.

All data available unless proprietary to a commercial customer.

6.0 TEST METHOD ACCURACY

MCF-7 Cell Proliferation Assay for Estrogenic Activity

6.1 ACCURACY OF MCF –7 TEST METHOD COMPARED WITH THE REFERENCE TEST METHOD

At UM, we tested the Relative Binding Affinity (RBA) of ten chemicals (**Appendix A Table 3**) using an ER-binding assay recommended by EDSTAC (1998) and ICCVAM (2002a,b,c; 2003a). **Appendix A Table 2** presents our IC50 data for these same 10 chemicals. Note that these IC50 data appear to be reliable, i.e. RBA IC50 (**Appendix A Table 2**) values are reproducible when repeated 2-5 times over a month.

The data presented in **Appendix A Table 1** provide evidence that CCI's robotics assay for EA at UM is accurate. Table 1 shows that the *mean* EC50 values obtained in this robotics assay are similar to *median* EC50 values compiled and published by ICCVAM (2003a). The mean EC50 values using this robotic assay are also very similar to published EC50 values obtained for some of these chemicals using an MCF-7 cell proliferation assay in manual format (see Nagel et al., 1998 for E2, EE, BPA, and GEN).

Appendix A Table 3 compares data from CCI's manual RBA assay with CCI's robotic cell proliferation assay for EA, as originally run at UM. CCI's robotic TA assay and manual RBA assay both show EA for those 5 chemicals (E2, DES, EE, GEN, BPA) that have been reported to have moderate to strong EA. Both assays show no detectable EA for the one chemical (PG) having no reported EA. As expected, the results of these two assays do *not* agree for the anti-estrogen ICI because ICI binds to ER, but does not activate estrogenic responses such as cell MCF-7 proliferation. As expected, ICI always showed strong anti-EA activity in CCI's robotic assay. In contrast, the results of CCI's RBA and cell proliferation assays did *not* agree for three chemicals (NP, OP, and MC) reported to have weak EA using CCI's robotic MCF-7 assay, or in other TA assays and *in vivo* assays (ICCVAM, 2002a,c). For each of these three chemicals, CCI's robotic MCF-7 assay at UM showed weak EA, whereas the RBA assay showed no detectable ER binding activity. Therefore, based on these data, CCI's robotic TA (cell proliferation) assay for EA appears to be more relevant and more sensitive compared to a standard ER RBA assay for EA. This conclusion is consistent with that of ICCVAM (2002a,b; 2003a) that TA assays are generally more relevant and sensitive compared to RBA assays to determine the EA of a chemical. [As noted by ICCVAM (2002a,b,c; 2003a) and EDSTAC (1998), any conclusion from *in vitro* data must be confirmed by *in vivo* tests.]

Appendix A Tables 7-9 and **Appendix A Figures 4-5** show *mean* EC50 values from CCI's assay performed at UM, NWU and CCI's Austin facility and *median* EC50 values for the same set of chemicals compiled by ICCVAM (2003a) in a meta-analysis to provide a set of reference chemicals to be used to test the accuracy of EA TA assays. A comparison of CCI's data with ICCVAM's published data suggests that CCI's robotic EA assay is accurately measuring EA. For example, **Appendix A Table 10** shows that the rate of false negatives (4/29) and false positives (1/11) to date is very low when CCI's data are compared to ICCVAM's meta-study (2003a). The four false negatives are 4-hydroxytamoxifen, dexamethasone, 2,4,5-trichloro-phenoxyacetic acid, and clomiphene citrate. These chemicals were not conclusively analyzed in the ICCVAM meta-analysis. For example, ICCVAM

(2003a) reported only one study as EA positive for dexamethasone and only one for 2,4,5-trichloro-phenoxyacetic acid. ICCVAM (2003a) reported 3/8 studies as EA positive for 4-hydroxytamoxifen, i.e. 5/8 reported the compound as EA negative. ICCVAM (2003a) anticipated that clomiphene citrate should be EA positive, but this chemical had not been tested for EA in any published TA study analyzed by ICCVAM (2003a). The one possible false positive (mifepristone) reported by CCI using its robotic EA assay has recently been reported to exhibit EA in other assays (ICCVAM, personal communications). Furthermore, CCI's robotic MCF-7 assay detected no false positives (0/34) for 34 other chemicals that have been reported to have no detectable EA in various *in vitro* or QSAR studies. [ICCVAM did not include these chemicals in its 2003a meta-study.]

The conclusion that CCI's proposed test method is accurate is further documented by **Appendix A Tables 11-12** that compare the ranking a set of ICCVAM (2003) reference chemicals according to ICCVAM's meta-analysis by ranking ICCVAM's median EC50 values (**Appendix A Table 11**) with the ranking of CCI's mean EC50 values using CCI's proposed test method (**Appendix A Table 12**). [ICCVAM gives no EC50 values for several reference chemicals declared to be EA positive; CCI has determined EC50 values for these same reference chemicals.] **Appendix A Figure 6** plots the CCI ranking (y axis) against the ICCVAM ranking (x axis) for the chemicals listed in **Appendix A Table 11**. **Appendix A Figure 6** also shows the least squares regression line that best fits these data. This least squares regression analysis shows that the rankings produced by CCI's proposed test method and the ICCVAM rankings do not differ significantly (null hypothesis, $p < 0.001$). The data from Table 6 are plotted in Appendix A Figure 7 that shows that the Mean EC50s of most chemicals obtained by CCI's facility in Austin are very similar to the median EC50 obtained by ICCVAM's meta-analysis. We should note that our assay is more sensitive so that the EC50 for the positive control is much lower, as are the EC50s for meso-Hexestrol, Estrone, coumestrol and several other test chemicals (see **Figure 7** in **Appendix A**).

6.2 Discordant results with respect to the *in vivo* reference method.

No *in vivo* reference method currently exists.

6.3 Accuracy of the proposed test method compared to data from humans.

No such data exist.

6.4 Strengths and limitations of the proposed test method.

6.4.1. Strengths

- CCI's *in vitro* assay can reduce animal use and does not use radioactive materials, in compliance with ICCVAM recommended protocols.
- CCI's assay is highly sensitive, i.e. capable of detecting chemicals with high EA (e.g. beta-estradiol, diethylstilbesterol) at less than picomolar concentrations and chemicals with low EA (octylphenol, estriol) at less than micromolar concentrations.
- CCI's assay can measure the EA of single chemicals, as well as complex mixtures of known and/or unknown chemicals.
- CCI's assay requires only a small amount of a suspect chemical or chemical mixture.
- CCI's assay cost is low to measure a suspect chemical or chemical mixture.

- CCI's time (days) to measure a suspect chemical or chemical mixture is short.
- CCI's robotic protocols meet or exceed all published ICCVAM recommendations (ICCVAM, 2002a-c, 2003).
- CCI's data obtained using this assay in manual or robotic format are reliable, i.e. reproducible within and between laboratories.
- CCI's data obtained using this assay are accurate, i.e., the data contain very few, if any, false negatives or false positives when compared with data compiled and published by ICCVAM (2000a-c, 2003).
- CCI's assay has excellent potential to be a good predictor of adverse health or environmental effects of chemicals having high or low EA.
- CCI's assay should be applicable to the needs of various federal agencies (FDA, DOD, DHS, DOA) and commercial firms, especially since CCI has developed procedures to screen animal feeds, human foodstuffs, aqueous extracts from plastics, etc.

6.4.2. Weaknesses

A general weakness is that this robotic MCF-7 *in vitro* assay for EA is intended as a Tier 1 screen for EA, not a definitive final test for EA. That is, the EDSTAC recommended a system consisting of two "Tiers" of ED testing. Tier 1 *in vitro* and *in vivo* tests are designed to identify substances that have the potential to interact with the endocrine system. Tier 1 robotic *in vitro* screening tests are especially desired as a way to more quickly identify ED chemicals at lesser costs. Tier 2 multigenerational tests are designed to confirm EDA and characterize the *in vivo* effects of EDs (EPA, 2000).

Two specific weaknesses are:

1) Test chemicals may not be pure and may have contaminants having various levels of EA or Anti-EA. We have observed that a particular chemical obtained from different sources consistently exhibit different levels of EA or anti-EA. This observation confirms a problem noted by ICCVAM (2002a,c; 2003a) that there is no agreed-upon source or standard for the 78 reference chemicals recommended for validation of EA in TA or RBA assays – much less non-reference chemicals or chemical mixtures used for other purposes.

2) Proper Anti-EA assessment requires appropriate interpretation of each of three assays for EA, anti-EA and cell toxicity (ICCVAM 2003a). CCI is developing a robotic assay for EA that we believe will pass all tests for validity and are now developing a robotic anti-EA assay that should eventually pass all tests for validity. CCI has also begun to develop a robotic assay using dye uptake to measure cell toxicity. Only by carefully assessing data from all three assays can one properly distinguish anti-EA from toxic effects. Once such analyses are completed, CCI's data for chemicals exhibiting EA and/or anti-EA should be one of the most extensive and accurate of any scientific, commercial, or governmental laboratory.

Although some potential problems as listed above may occur, nevertheless CCI expects this proposed MCF-7 assay for EA to be validated in short order.

6.5 Salient issues of data interpretation.

6.5.1. A few test chemicals at high concentrations, such as corticosterone, dexamethasone, procymidone, stimulated cell growth at about 10-15% of E2-stimulate in the **Range-Finder Experiment**. However, these chemicals produce no detectable stimulation in **Concentration-Response Experiments** that have been repeated at least three times with a dilution factor of 1.87. Hence, CCI considers those chemicals to be EA negative.

6.5.2. CCI ranks the EA of test chemicals in THREE ways:

- A. By EC50 from lowest to highest value
- B. Relative EC50: that is, the ratio of E2 EC50/test chemical EC50
- C. Ranking from chemical with the highest EA potential to the chemical with the lowest EA potential. Among the chemicals tested, 27 chemicals are EA positive. E2 has the highest EA potential (EC50 is $2 \times 10^{-13} \text{M}$), E2 is ranked as 1; DDE has the lowest EA potential (EC50 is 7.30×10^{-6}), p,p' -DDE is ranked as 27. (See Table 12 in Appendix A).

6.6 Comparison with mechanistically and functionally similar validated test method.

No such validated test exists.

7. TEST METHOD RELIABILITY (REPEATABILITY/REPRODUCIBILITY)

MCF-7 Cell Proliferation Assay for Estrogenic Activity

7.1. Rationale for the substances used to evaluate the reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) of the proposed test method.

Most substances tested were chosen from the 78 reference chemicals published by ICCVAM (2003a) for use in validating TA assays of EA [CCi's robotic assay using MCF-7 cells is classified as a TA assay by ICCVAM (2002a,b,c; 2003a).] Specifically, many chemicals (40, see **Table 3-A**) tested to date are from the list of 78 EA positive and EA negative reference chemicals recommended by ICCVAM (2003a) to assess the accuracy of TA assays for EA. Other chemicals (chemicals with an asterisk in **Table 3-A**) are from a previous list of reference chemicals (ICCVAM, 2002c) to assess the accuracy of TA assays for EA.

7.2. Repeatability and reproducibility of the proposed test method.

The reliability of our test method is summarized in **Appendix A Figure 6**. The repeatability is given as the correlation of variation using GraphPad Prism software. The P value less than 0.001 for the Null Hypothesis in comparing the data obtained from either ICCVAM, UM, NWU indicates that CCi rankings do **not** significantly differ from ICCVAM UM or NWU rankings.

Appendix A Table 1 gives the EC 50 values obtained by our robotic assay for ten chemicals sampled at The University of Missouri (UM) on the dates given at the top of each column. [All but PG (propyl gallate) are on the ICCVAM (2003a) list of 78 reference chemicals.] These EC50 data are also plotted in **Appendix A Figure 1**, in part to show the consistency (reproducibility in the same laboratory) of CCi's EA assay and in part to demonstrate the range of EC50 values that CCi's initial EA assay was able to detect. Note that assays of each chemical give very consistent data when repeated 3-7 times in different experimental runs over a 2-3 month period. These data obtained by CCi at the University of Missouri (UM) suggest that this robotics EA assay is reliable when repeated at different times in the same lab.

EC50 values for ten test chemicals in **Appendix A Table 1** and **Appendix A Figure 1** were obtained from concentration-response data fitted by Michaelis-Menton kinetics. A concentration-response curve was plotted for each chemical every time it was tested; each point on the curve represents an average of four wells. Over 70 such concentration-response curves were generated in this study. OP and NP obtained from ChemService were consistently weakly estrogenic each time they were tested. However, OP and NP ordered from Sigma exhibited no detectable EA the first two times tested, and sometimes showed weak EA in subsequent tests (data not shown). CCi cannot explain the differences in EA activity for OP and NP obtained from Sigma vs. ChemService. This inconsistency of EA in chemicals obtained from different sources (or from the same source at different times) illustrates a problem noted by ICCVAM (2002a,c; 2003a). That is, there is no agreed-upon source or standard for the 78 reference chemicals (ICCVAM, 2003a) recommended for validation of EA.

As noted above, this original version of CCI's robotic EA assay was highly sensitive, albeit not as sensitive as CCI's current assay. For example the mean EC50 of E2 of CCI's original assay at UM was $3.4 \times 10^{-12} \text{M}$ (0.2 pg/ml). CCI's assay as currently nominated has an EC50 of about $2 \times 10^{-13} \text{M}$. This high sensitivity facilitates detection of chemicals with weak EA (such as exhibited by NP, OP, MC, BPA or BHT). Such weak EA is often missed in less sensitive assays, including most or all published TA assays using estrogen-responsive reporter genes or RBA assays (ICCVAM, 2002a; 2003a). Detection sensitivity is critical for assays of EA because less sensitive assays will miss "low dose" estrogenic effects (NTP, 2001; vom Saal, 2005; Welshons et al. 2005).

The data presented in **Appendix A Table 1** demonstrate that CCI's initial robotics assay for EA at UM is accurate, as well as reliable. **Appendix A Table 1** shows that the *mean* EC50 values obtained by CCI's initial robotics assay were similar to *median* EC50 values compiled and published by ICCVAM (2003a). CCI's mean EC50 values using this robotic assay were also very similar to published EC50 values obtained for some of these chemicals using an MCF-7 cell proliferation assay in manual format (see Nagel et al., 1998 for E2, EE, BPA, and GEN).

To further validate the initial robotic EA assay at UM, CCI tested the Relative Binding Affinity (RBA) of the ten chemicals in **Appendix A Table 2** using an ER-binding assay recommended by EDSTAC (1998) and ICCVAM (2002a,b,c; 2003a). **Appendix A Table 2** presents our IC50 data for these same 10 chemicals. Note that these IC50 data appear to be reliable, i.e. RBA IC50 (**Appendix A Table 2**) values are reproducible when repeated 2-5 times over a month.

Appendix A Table 3 compares data from CCI's manual RBA assay with CCI's robotic cell proliferation assay for EA, as originally run at UM. CCI's robotic TA assay and manual RBA assay both show EA for those 5 chemicals (E2, DES, EE, GEN, BPA) that have been reported to have moderate-to-strong EA. Both assays show no detectable EA for the one chemical (PG) having no reported EA. As expected, the results of these two assays do *not* agree for the anti-estrogen ICI because ICI binds to ER, but does not activate estrogenic responses such as cell MCF-7 proliferation. As expected, ICI always showed strong anti-EA activity in the robotic assay. In contrast, the results of CCI's RBA and cell proliferation assays did *not* agree for three test chemicals (NP, OP, and MC) reported to have weak EA in CCI's robotic MCF-7 assay, in other TA assays and *in vivo* assays (ICCVAM, 2002a,c). For each of these three chemicals, our robotic MCF-7 assay at UM showed weak EA, whereas the RBA assay showed no detectable ER binding activity. Therefore, based on these data, CCI's robotic TA (cell proliferation) assay for EA appears to be more relevant and more sensitive compared to a standard ER RBA assay for EA. This conclusion is consistent with that of ICCVAM (2002a,b; 2003a) that TA assays are generally more relevant and sensitive compared to RBA assays to determine the EA of a chemical. [As noted by ICCVAM (2002a,b,c; 2003a) and EDSTAC (1998), any conclusion from *in vitro* data must be confirmed by *in vivo* tests.]

In brief, these data from UM study strongly suggest that CCI's robotics assay for EA is valid, i.e., produces EA data that are reliable and relevant.

Appendix A Figure 2 and **Appendix A Table 4** give examples of CCI's sensitive EA assay run in NWU in manual format. **Appendix A Table 4** gives the EC50 values obtained by CCI's robotic assay for nine chemicals sampled at Northwestern University Medical School (NWU) in manual format from different trials. All these chemicals were sampled repetitively at

UM and CCI; GEN (genistein was also sampled repetitively at UM and CCI, but not NWU.) These EC50 data are also plotted in **Appendix A Figure 2**. Note that the mean values and sensitivity are similar at NWU and CCI (see **Appendix A Table 5** and **Appendix A Figure 3**), but the variation in the data are greater in manual format at NWU than in robotic format at CCI. In general, the MCF-7 assay is more accurate, and less variable, when run in the robotic format developed by CCI in Austin, TX.

Appendix A Figures 1-5 and **Appendix A Tables 1 and 4-6** show that CCI's robotic EA assay is very consistent (reliable, repeatable) when analyzing the same set of 9-10 chemicals in the same laboratory or in different laboratories (CCI, UM, NWU). [NP has a much lower EC50 in trials at NWU perhaps due to a strong EA contaminant; GEN was *not* sampled at NWU.] Furthermore, **Appendix A Figures 4-5** and **Appendix A Tables 7-8** show that CCI's EA assay gives very similar data when run in robotic (CCI, UM) or manual (NWU) format. These tables and figures in **Appendix A** also show that the data from the assay run in robotic format at CCI are *less* variable than data from the assay run in manual format run at NWU.

7.3. SUMMARY OF POSITIVE AND NEGATIVE CONTROL DATA

Appendix A Tables 7-9 and **Appendix A Figures 4-5** show *mean* EC50 values from CCI's assay and *median* EC50 values for the same set of chemicals compiled by ICCVAM (2003a) in a meta-analysis to provide a set of reference chemicals to be used to test the accuracy of EA TA assays. A comparison of CCI's data with ICCVAM's published data suggests that CCI's robotic EA assay is accurately measuring EA. For example, **Appendix A Table 10** shows that the rate of false negatives (4/29) and false positives (1/11) to date is very low when CCI's data are compared to ICCVAM's meta-study (2003a). The four false negatives are 4-hydroxytamoxifen, dexamethasone, 2,4,5-trichloro-phenoxyacetic acid, and clomiphene citrate. These chemicals were not conclusively analyzed in the ICCVAM meta-analysis. For example, ICCVAM (2003a) reported only one study as EA positive for dexamethasone and only one for 2,4,5-trichloro-phenoxyacetic acid. ICCVAM (2003a) reported 3/8 studies as EA positive for 4-hydroxytamoxifen, i.e. 5/8 reported the compound as EA negative. ICCVAM (2003a) anticipated that clomiphene citrate should be EA positive, but this chemical had not been tested for EA in any published TA study analyzed by ICCVAM (2003a). The one possible false positive (mifepristone) reported by CCI using its robotic EA assay has recently been reported to exhibit EA in other assays (ICCVAM, personal communications). Furthermore, CCI's robotic MCF-7 assay detected no false positives (0/34) for 34 other chemicals that have been reported to have no detectable EA in various *in vitro* or QSAR studies.[ICCVAM did not include these chemicals in its 2003a meta-study.]

The conclusion that the proposed test method is accurate is further documented by **Appendix A Tables 11-12** that compare the ranking of reference chemicals according to ICCVAM's meta-analysis median EC50 values (**Appendix A Table 11**) with the ranking of CCI's mean EC50 values using the proposed test method (**Appendix A Table 12**). [ICCVAM gives no EC50 values for several reference chemicals declared to be EA positive; CCI *has* determined EC50 values for these same reference chemicals.] **Appendix A Figure 6** plots the ICCVAM ranking (y axis) against the CCI ranking for the chemicals listed in **Appendix A Table 11**. **Appendix A Figure 6** also shows the least squares regression line that best fits these data. The least squares regression analysis shows that the rankings produced by CCI's

proposed test method and the ICCVAM rankings do not differ significantly (null hypothesis, $p < 0.0001$).

7.4. Reliability compared to mechanistically and functionally similar validated test method.

No such validated test exists.

8. TEST METHOD DATA QUALITY

MCF-7 Cell Proliferation Assay for Estrogenic Activity

8.1 ADHERANCE TO NATIONAL AND INTERNATIONAL GLP GUIDELINES (7-12) FOR SUBMITTED DATA.

8.1.1. Standard Operating Procedures (SOP's)

A set of reference test chemicals have been assayed for EA by protocols described in **Section 2**. CCI is writing these Standard Operating Procedures (SOPs).

8.1.2. Data Recording

All the experimental procedures should be well documented in the experimenters' notebook. Raw data obtained by 96-well plate reader are kept in the computer connected to the plate reader, in the experimenter's computer and in the PI's computer. Analyzed data (EC50, STDEV, Slope, etc.) are stored in the experimenter's computer as well as in the PI's computer. A hard copy of all the analyzed data is also kept in the experimenter's notebook as well as in PI's notebook. All data stored in CCI's computers are backed up every day.

8.1.3. Instrumentation validation

The equipment in CCI's facility is calibrated on a pre-determined schedule, such equipment including balances, pH meters, water bath, pipettes, CO₂ content and temperature of the CO₂ incubator. CCI's autoclave has an automatic printout showing autoclave cycle information. The biological hood is certified annually by ENV Services.

8.1.4. Reagent/materials certification

Reagent and materials used for CCI's EA assay should meet the following criteria:

- A. Reagent or analytical grade
- B. Purity more than 97% or the purest ones that are available from suppliers

8.1.5. Statistical procedures for data evaluation

- A. Mean of replicates: Excel
- B. EC50: GraphPad Prism
- C. r^2 : GraphPad Prism

8.1.6. Lab facilities

CCi's testing facility is well designed to conduct the proposed MCF-7 cell proliferation assays or other assays for detecting EA.

8.1.7. Chemical/Sample tracking

Tracking of all test chemicals/samples should contain the following information:

- A. Sources, purity and molecular weight
- B. Received date and date the container was opened
- C. Chemical name, concentration, and preparation date on any test tubes containing the chemical to be assayed
- D. Material Safety Data Sheet (MSDS)

8.2 DATA QUALITY AUDITS

None.

8.3 IMPACT OF DEVIATIONS FROM GLP GUIDELINES OR ANY NONCOMPLIANCE DETECTED IN THE DATA QUALITY AUDITS

Not Applicable.

8.4 Availability of laboratory notebooks or other records for an independent audit.

Available

9.0 OTHER SCIENTIFIC REPORTS AND REVIEWS

MCF-7 Cell Proliferation Assay for Estrogenic Activity

9.1 Available and relevant data from other published or unpublished studies conducted using the proposed test method.

No such studies exist.

9.2 Comparison with independent peer-reviewed reports of the proposed test method.

CCi have modified its original assay so as to satisfy critiques received for a Phase I SBIR application submitted 4/01 and funded beginning 3/02 as follows:

Phase I Review [June 2001]

“This application [An *in vitro* robotic assay for estrogenic activity] describes development of a high-throughput robotic cell proliferation assay to detect estrogenic activity by measuring *in vitro* proliferation of MCF-7 cells. The applicant and collaborators are well qualified to successfully complete the proposed studies. The proposal has many strengths, including the innovative use of robotics, good preliminary data, the straightforward experimental approach, the appropriate use of the assay for screening chemicals to identify potential endocrine disruptors, and the high commercialization potential of this system. There are several weaknesses in the proposed study, including the lack of detail regarding software [since resolved], the large amount of variation in estrogen responsiveness associated with MCF-7 cells [since shown to be greatly reduced by the robotic assay], the potential effects of estrogen-like activity from plastic associated with the experiments [CCi tests all plastics and media for EA and control for any background activity], and the applicants plan to continuously passage MCF-7 cells for one year (three months should be the maximum [since reduced to 2-3 months]). However, the study section felt these weaknesses were relatively minor given the qualifications and experience of the team of investigators. Overall, the study section enthusiastically supported the proposed development of a high-throughput robotic cell proliferation assay to detect estrogenic activity and identify potential endocrine disruptors.”

The critique for our (funded) Phase II application had no suggestions for modifying the assay, other than to clarify the solvent(s) used to dissolve chemicals.

Phase II Review [June 2003]

“This Phase II SBIR application [An *in vitro* robotic assay for estrogenic activity] is from a team of experienced and well-qualified investigators. The phase II research will extensively validate [reliability & accuracy] the *in vitro* robotic assay in their company facilities and demonstrate reliability and relevance [compared to published tables for 78 chemicals in ICCVAM 2003a. The relevance/accuracy of these data are debated by ICCVAM 2002a-c, 2003a]. Strengths of the application are the expertise of the investigative team; the completion of the goals of the proposed research in phase I with a robotic assay for estrogenic activity (EA) was successfully developed and initially characterized; the generally strong commercialization plans; the general feasibility of the high throughput assays; and the excellent research environment. These strengths and a few deficiencies were the focus of the discussion at the study section meeting. Although the development of a robotic assay for EA is exciting, some critical details of experimentation are not fully addressed. It is stated that their robotic assay is designed to meet or exceed all applicable agency standards and preferences. What is not clear is that [sic] if a standard

solvent extraction step will be used for all varieties of test compounds or materials. [Standard water or ethanol extraction is used.] More attention to such details would be useful. All in all, the commercialization plans are appealing and well laid out and the project addresses an important area and hence a reasonably high priority score is recommended.”

9.3 Studies conducted with the validated test method subsequent to the ICCVAM evaluation.

Not applicable

**10.0 ANIMAL WELFARE CONSIDERATIONS (Refinement, Reduction,
and Replacement)**

**MCF-7 Cell Proliferation Assay
for Estrogenic Activity**

**10.1 How the proposed test method will refine (reduce or eliminate pain or distress),
reduce, or replace animal use compared to the reference test method.**

This *in vitro* test method uses a modified human cell line, so no animals are needed.
At present, a reference test method does not exist.

11.0 PRATICAL CONSEDERATIONS

MCF-7 Cell Proliferation Assay for Estrogenic Activity

11.1 Proposed test method transferability.

A. Facilities and major fixed equipment needed to conduct a study using the proposed test method.

1. 96-well Deep-well plates (e.g. Matrix, cat#: 4222) and Caps (e.g. Matrix cat#: 4422)
2. 96-well plate spectrophotometer (i.e., plate reader) equipped with 590 nm and 700 ± 10 nm filter (e.g. Bio-Tek, Powerwave)
3. 96-well [flat bottom] cell culture plates (e.g., Corning tissue culture-treated)
4. Centrifuges
5. Conical 15 ml and 50 ml tubes
6. Cryotubes and liquid nitrogen container
7. epMotion 5070 robotic station and accessories (tubs, racks, adaptors, tips, reservoirs, etc.)
8. Eppendorf tubes
9. Filters/filtration devices
10. Hemocytometer
11. Incubator: $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90\% \pm 5\%$ humidity, $5.0\% \pm 1\%$ CO_2 /air
12. Inverted phase contrast microscope
13. Laboratory balance
14. Laminar flow clean bench/cabinet (standard: "biological hazard")
15. Parafilm to seal plates
16. Pipettes, pipettors (multi-channel and single channel; multichannel repeater pipette)
17. pH paper (wide and narrow range)
18. Pipetting aid
19. Sterile glass tubes with caps (e.g., 5 mL)
20. Tissue culture flasks (e.g., $75 - 80 \text{ cm}^2$, 25 cm^2)
21. Vortex mixer
22. Water bath: $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$

B. General availability of other necessary equipment and supplies.

Generally Available.

11.2 Level of proposed test method training.

A. Discuss the required level of training and expertise needed for personnel to conduct the proposed test method.

The personnel to run the proposed test methods need to be generally trained in cell culture techniques, sterilization procedures, and precise adherence to experimental procedures. Specific training is needed for robotic programming and manipulation.

B. Training requirements needed for personnel to demonstrate proficiency and describe any laboratory proficiency criteria that should be met

None

11.3 Cost Considerations

Cost of materials to run a range-finder or final test experiment: \$40 – 50/sample.
Labor cost: \$100-300/sample, depending on salaries, number of samples run in batch, number of replicates, and number of concentrations/sample. Cost for final analysis and report: \$300 - \$1500/sample, depending on above costs, overhead costs, number of samples run/week, extent of desired analysis and/or GLP documentation, and sample type (known or unknown test chemical).

11.4 Time Considerations

8-14 days from start of test run to completion of final report depending on extent of desired analysis and/or GLP documentation, and sample type (known or unknown test chemical).

12. REFERNECS

12.1. List all publications referenced in the submission.

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13. SUPPORTING MATERIALS (APPENDICES)

MCF-7 Cell Proliferation Assay for Estrogenic Activity

13.1 Provide the complete, detailed protocol for the proposed test method.

See Section 3

13.2 Provide the detailed protocol(s) used to generate reference data for this submission and any protocols used to generate validation data that differ from the proposed protocol.

See Section 5

13.3 Provide copies of all relevant publications, including those containing data from the proposed test method, the in vivo reference test method, and if applicable, a comparable validated test method with established performance standards.

NA.

13.4 Provide copies of all relevant publications, including those containing data from the proposed test method, the in vivo reference test method, and if applicable, a comparable validated test method with established performance standards.

NA

13.5 Include all available nontransformed original data for both the proposed test method, the in vivo reference test method, and if applicable, a comparable validated test method with established performance standards.

NA.

13.6 If appropriate performance standards for the proposed test method do not exist, performance standards for consideration by NICEATM and ICCVAM may be proposed. Examples of established performance standards can be located on the ICCVAM/NICEATM web site at <http://iccvam.niehs.nih.gov>.

We have cited ICCVAM meta-studies.

Table 1: EC50s of 10 Chemicals Tested Repetitively at UM in Robotic Format

Chemical	5/2/2002	5/31/2002	6/28/2002	7/5/2002	7/12/2002	8/2/2002	8/10/2002
E2	1.1E-12	3.1E-12	3.3E-12	3.5E-12	3.9E-12	3.6E-12	5.0E-12
DES	2.1E-11	8.6E-12	1.1E-11	1.5E-11	2.0E-11	9.5E-12	1.4E-11
EE	1.2E-12	1.8E-12	2.3E-12	4.5E-12	4.6E-12	3.5E-12	1.9E-12
GEN	2.4E-08	5.3E-08	5.2E-08	6.2E-08	4.8E-08	2.2E-07	3.0E-07
BPA	2.0E-07	4.9E-07	5.9E-07	2.7E-07	1.9E-07	3.0E-08	7.6E-08
MC	7.9E-06	4.4E-05	8.3E-06	1.2E-05	5.3E-06	9.7E-06	6.2E-06
ICI	NG	NG	NG	NG	NG	NG	NG
PG	NG	NG	NG	X	NG	NG	NG
NP	NA	NA	2.7E-07	2.5E-07	1.4E-07	1.7E-06	1.8E-07
OP	NA	NA	1.0E-07	1.6E-07	1.0E-07	1.8E-06	1.7E-07

NG: negative (no detectable EA); NA: not analyzed; n: number of experiments for that chemical;
 X: contaminated sample for that experiment; The EC50 for each experiment is the mean of three replicates.
 EC50s are given in molar units.

E2: 17 β -Estradiol; DES: Diethylstilbestrol; EE: 17 α -Ethyl estradiol; GEN: Genistein; BPA: Bisphenol A;
 MC: p,p'-methoxychlor; ICI: ICI 182,780; PG: Propyl gallate; NP: p-n-Nonylphenol; OP: 4-tert-
 Octylphenol.

Note that NP and OP assayed at UM were obtained from ChemService, and were obtained from Sigma for
 assays at CCI and at NWU.

Table 2: IC50s (M) for 10 Chemicals Assessed by Radiolabel ER Binding Assay at UM

Chemical	Mean IC50 (M)	SEM	n	7/24/2002	7/31/2002	8/7/2002	8/9/2002	8/22/2002
E2	7.4E-10	2.8E-10	5	1.9E-09	4.8E-10	4.5E-10	4.2E-10	4.8E-10
DES	2.3E-09	1.1E-09	4	5.7E-09	1.3E-09	9.3E-10	1.2E-09	NA
EE	9.2E-10	4.0E-10	2	1.3E-09	NA	NA	NA	5.3E-10
GEN	7.2E-06	4.3E-06	4	2.0E-05	3.0E-06	NA	2.7E-06	3.1E-06
BPA	1.2E-05	2.9E-06	4	2.0E-05	1.1E-05	8.0E-06	7.4E-06	NA
NP	NG		2	NA	NG	NG	NA	NA
OP	NG		2	NG	NA	NG	NA	NA
MC	NG		3	NA	NG	NA	NG	NG
ICI	8.1E-09	6.2E-10	4	NA	9.9E-09	7.5E-09	7.6E-09	7.2E-09
PG	NG		2	NG	NA	NG	NA	NA

NG: negative (no detectable ER binding); NA: not analyzed; n: number of experiments for that chemical; The IC50 for each experiment is the mean of three replicates. IC50s are given in molar units.

E2: 17 β -Estradiol; DES: Diethylstilbestrol; EE: 17 α -Ethyl estradiol; GEN: Genistein; BPA: Bisphenol A; NP: p-n-Nonylphenol; OP: 4-tert-Octylphenol; MC: p,p'-methoxychlor; ICI: ICI 182, 780; PG: Propyl gallate; .

Table 3: Comparison of REAs and RBAs for 10 Chemicals Tested Repetitively at UM

	E2	DES	EE	GEN	BPA	NP	OP	MC	ICI	PG
RBA	1.0E0	3.2E-1	8.0E-1	1.0E-4	6.0E-5	NG	NG	NG	9.2E-2	NG
n	5	4	2	4	4	2	2	3	4	2
REA	1.0E0	2.4E-1	1.2E0	3.1E-5	1.3E-5	6.7E-6	7.3E-6	2.5E-7	NG	NG
n	7	7	7	7	7	5	5	7	7	7

RBA: Relative Binding Affinity calculated as IC50 of E2 / IC50 of test chemical, RBA is expressed as the mean RBA for multiple experiments.

REA: Relative Estrogenic Activity calculated as EC50 of E2 / EC50 of test chemical. REA is expressed as the mean REA for multiple experiments.

n: number of experiments for that chemical; NG: Negative (No detectable ER binding).

E2: 17 β -Estradiol; DES: Diethylstilbestrol; EE: 17 α -Ethyl estradiol
 GEN: Genistein; BPA: Bisphenol A; NP: p-n-Nonylphenol; OP: 4-tert-Octylphenol; MC: p,p'-methoxychlor;
 ICI: ICI 182, 780; PG: Propyl gallate.

Table 4: EC50s of 9 chemicals tested repetitively at NWU in manual format

Chemical	Exp. #1	Exp. #2	Exp. #3	Exp. #4
E2	2.1E-13	6.3E-13	4.1E-13	1.1E-13
DES	2.7E-11	3.9E-12	7.6E-12	NA
EE	1.2E-11	3.1E-12	1.1E-12	NA
BPA	3.3E-08	1.0E-08	1.2E-08	1.9E-09
MC	7.3E-06	4.0E-07	3.1E-06	1.2E-06
NP	1.4E-11	2.3E-10	NMAC	NMAC
OP	5.0E-07	3.5E-07	5.1E-07	5.2E-07
ICI	NG	NG	NG	NG
PG	NG	NG	NG	NG

NG: negative (no detectable EA); NA: not analyzed; NMAC: does Not Meet Acceptance Criteria, that is, the r square of the dose-response curve for that experiment is less than 0.85. The EC50 for each experiment is the mean of three-six replicates. EC50s are given in molar units.

E2: 17 β -Estradiol; DES: Diethylstilbestrol; EE: 17 α -Ethyl estradiol; BPA: Bisphenol A; MC: p,p'-methoxychlor; NP: p-n-Nonylphenol; OP: 4-tert-Octylphenol; ICI: ICI 182, 780; PG: Propyl gallate.

Note that NP EC50 at NWU is much lower than EC50s from ICCVAM, UM and CCI. NP at NWU may have been contaminated by a strong estrogen.

Table 5: EC50s of 10 chemicals tested repetitively at CCI in robotic format

Chemical	Exp.#1	Exp. #2	Exp. #3	Exp. #4	Exp. #5	Exp. #6
E2	5.7E-14	5.3E-14	1.2E-13	1.7E-13	1.6E-13	3.8E-13
DES	3.7E-11	4.3E-12	1.6E-11	1.4E-11	1.1E-11	2.3E-11
EE	3.7E-11	4.9E-12	1.6E-11	1.4E-11	1.1E-11	1.0E-12
GEN	1.7E-08	2.9E-08	2.4E-08	5.4E-09	5.4E-09	3.9E-09
BPA	2.8E-08	7.8E-09	2.0E-08	2.1E-08	4.9E-08	2.9E-08
MC	1.3E-05	1.1E-06	2.9E-07	1.5E-07	4.1E-07	NA
ICI	NG	NG	NG	NG	NG	NG
PG	NG	NG	NG	NG	NG	NG
NP	9.4E-07	4.8E-08	5.1E-07	1.3E-06	2.3E-07	NA
OP	9.4E-07	2.7E-07	1.1E-08	1.0E-07	1.3E-08	3.5E-08

NG: negative (no detectable EA); NA: not analyzed; n: number of experiments for that chemical. The EC50 for each experiment is the mean of three-four replicates. EC50s are given in molar units.

E2: 17 β -Estradiol; DES: Diethylstilbestrol; EE: 17 α -Ethyl estradiol; GEN: Genistein; BPA: Bisphenol A; MC: p,p'-methoxychlor; ICI: ICI 182,780; PG: Propyl gallate; NP: p-n-Nonylphenol; OP: 4-tert-Octylphenol.

The EC50 for each experiment is the mean of three - four replicates. EC50s are given in molar units.

Table 6: EC50s for Other ICCVAM Chemicals Tested at CCI

Chemical	ICCVA	CCI		
	M (Median) (M)	Mean (M)	SEM	n
<i>meso</i> -Hexestrol	2.0E-10	1.6E-11	7.9E-12	5
Estrone	3.2E-09	2.3E-11	1.1E-11	5
17 α -Estradiol	4.6E-11	8.0E-11	3.7E-11	5
<i>Estriol</i>	7.1E-10	2.8E-10	1.2E-10	4
Zearalenone	2.0E-09	1.9E-10	5.9E-11	8
Coumestrol	1.5E-08	4.4E-09	1.1E-09	4
Bisphenol B	8.8E-08	1.2E-08	4.9E-09	4
Flavone	ND	3.5E-08	1.6E-08	2
4-Cumylphenol	3.2E-07	5.1E-08	1.3E-08	4
Daidzein	2.9E-07	3.6E-08	8.3E-09	4
Mifpristone	ND	7.6E-08	1.1E-08	6
<i>o,p'</i> -DDT	6.6E-07	4.2E-08	1.0E-08	6
Kepone	ND	1.7E-07	3.6E-08	7
Apigenin	ND	1.7E-07	1.1E-07	3
Butylbenzyl phthalate	ND	4.3E-07	1.4E-07	3
Kaempferol	ND	7.5E-07	7.9E-08	6
Fenarimol	2.7E-05	5.8E-06	3.2E-06	2
<i>p,p'</i> -DDE	ND	6.6E-06	2.5E-06	5

ICCVAM data: Median Values from meta-study; n: number of experiments for that chemical; SEM: Standard Error of the Mean for multiple experiments; ND: Not determined, that is, chemicals were reported as EA positive by the ICCVAM meta-study, but no EC50s were determined by ICCVAM. EC50s are given in molar units.

Table 7: Comparison of ICCVAM Median EC50s with Mean EC50s Obtained at UM, CCI, and NU (Mean)

Chemical	ICCVAM Median EC50 (M)	Phase I/U. Missouri			Phase II/NWU			Phase II/CCI		
		Mean EC50 (M)	SEM	n	Mean EC50 (M)	SEM	n	Mean EC50 (M)	SEM	n
E2	1.0E-10	3.4E-12	4.4E-13	7	2.2E-13	7.4E-14	8	1.9E-13	3.7E-14	22
DES	1.9E-11	1.4E-11	1.8E-12	7	1.3E-11	7.3E-12	3	3.4E-11	7.2E-11	11
EE	1.1E-11	2.8E-12	5.2E-13	7	5.5E-12	3.4E-12	3	2.6E-11	1.5E-11	7
GEN	6.2E-08	1.1E-07	4.0E-08	7	NA			1.1E-08	3.7E-09	8
BPA	3.9E-07	2.6E-07	7.8E-08	7	1.4E-08	6.5E-09	4	2.6E-08	5.7E-09	6
MC	8.9E-06	1.3E-05	5.2E-06	7	3.0E-06	1.3E-06	3	3.7E-06	2.7E-06	4
ICI	NA	NG		7	NG		>10	NG		>10
PG	NA	NG		7				NG		4
NP	8.5E-08	5.8E-07	2.9E-07	5	1.2E-10	1.1E-10	2	4.3E-07	2.2E-07	4
OP	1.9E-07	5.3E-07	3.3E-07	5	4.7E-07	4.1E-08	4	2.7E-07	1.7E-07	5

ICCVAM data: Median Values from Meta-Study; NG: negative (no detectable EA); NA: not analyzed; n: number of experiments for that chemical; SEM: Standard Error of the Mean for multiple experiments; Phase I: data from Phase I studies on our NIH SBIR grant titled “An In Vitro Robotic Assay for Estrogenic Activity (1 R44 ES011469-01)”; Phase II: data from Phase II studies of our NIH SBIR grant titled An “In Vitro Robotic Assay for Estrogenic Activity (2 R44 ES011469-02)”. EC50s are given in molar units.

E2: 17β-Estradiol; DES: Diethylstilbestrol; EE: 17α-Ethyl estradiol; GEN: Genistein; BPA: Bisphenol A; MC: p,p'-methoxychlor; ICI: ICI 182, 780; PG: Propyl gallate; NP: p-n-Nonylphenol; OP: 4-tert-Octylphenol.

Note that NP and OP were purchased from ChemService for our Phase I studies at UM and from Sigma for our Phase II studies performed at CCI and NWU.

Note that the EC50 of NP at NWU is much lower than the EC50 obtained from ICCVAM, UM and CCI’s Austin laboratory, probably because the chemical was contaminated by a strong estrogen at NWU.

Table 8: Comparison of Relative EC50s* from ICCVAM (Median), UM (Mean), NU (Mean), and CCI (Mean)

Chemical	ICCVAM	UM	NWU	CCI
E2	1.0E+00	1.0E+00	1.0E+00	1.0E+00
DES	5.3E+00	2.4E-01	1.7E-02	5.5E-03
EE	9.1E+00	1.2E+00	4.0E-02	7.1E-03
GEN	1.6E-03	3.1E-05	NA	1.6E-05
BPA	2.6E-04	1.3E-05	1.6E-05	7.2E-06
MC	1.1E-05	2.5E-07	7.4E-08	5.1E-08
ICI	NG	NG	NG	NG
PG	NG	NG	NG	NG
NP	1.2E-03	5.8E-06	1.8E-03	4.3E-07
OP	5.3E-04	6.3E-06	4.7E-07	6.8E-07

Relative EC50* = Mean EC50 of E2 across multiple experiments/ Mean EC50 of test chemical across multiple experiments;

ICCVAM data: Median Values from meta-study; NG: negative (no detectable EA); NA: not analyzed; n: number of experiments;

E2: 17 β -Estradiol; DES: Diethylstilbestrol; EE: 17 α -Ethyl estradiol; GEN: Genistein; BPA: Bisphenol A; MC: p,p'-methoxychlor; ICI: ICI 182,780; PG: Propyl gallate; NP: p-n-Nonylphenol; OP: 4-tert-Octylphenol.

Table 9: Test Method Accuracy Assessment

Chemical	CASRN	Chemical Class	Results from CCI's Test Method (Mean) EC50 (M)	Results from CCI's Test Method (+/-)	Results from ICCVAM (Median) EC50 (M)	Results from ICCVAM (+/-)	Comments
17α-Ethinyl estradiol	57-63-6	Steroid, phenolic	4.0E-11	+	1.1E-11	+	Strong ER agonist
Diethylstilbestrol	56-53-1	Stilbene; Benzylidene; Diphenylalkene	1.5E-10	+	1.9E-11	+	Strong ER agonist
17α-Estradiol	57-91-0	Steroid, phenolic; Estrene	8.0E-11	+	4.6E-11	+	ER agonist
17β-Estradiol	50-28-2	Steroid, phenolic; Estrene	1.9E-13	+	1.0E-10	+	Strong ER and AR agonist; AR antagonist
meso -Hexestrol	84-16-2	Diphenylalkane; Bisphenol; Phenol	1.6E-11	+	2.0E-10	+	Strong ER agonist
Estriol	50-27-1	Resorecylic acid lactone; Phenol	2.8E-10	+	7.1E-10	+	ER agonist
Zearalenone	17924-92-4	Steroid, phenolic; Estrene	1.9E-10	+	2.0E-09	+	ER agonist
Estrone	53-16-7	Steroid, nonphenolic; Androstene	8.3E-12	+	3.2E-09	+	Strong ER agonist; AR agonist
Coumestrol	479-13-0	Coumestan; Benzopyranone; Coumarin; Ketone	4.4E-09	+	1.5E-08	+	ER agonist
Genistein	446-72-0	Flavanoid; Isoflavone; Phenol	1.1E-08	+	6.2E-08	+	Weak ER agonist and antagonist
<i>p</i>-n - Nonylphenol	104-40-5	Alkylphenol; Phenol	6.1E-07	+	8.5E-08	+	ER and AR antagonist ER agonist
Bisphenol B	77-40-7	Diphenylalkane; Bisphenol; Phenol	1.2E-08	+	8.8E-08	+	ER agonist
Daidzein	486-66-8	Flavanoid; Isoflavone; Phenol	3.6E-08	+	2.9E-07	+	Weak ER agonist
4-Cumylphenol	599-64-4	Phenol	5.1E-08	+	3.2E-07	+	Weak ER agonist
Bisphenol A	80-05-7	Diphenylalkane; Bisphenol; Phenol	2.6E-08	+	4.0E-07	+	ER agonist
<i>o,p'</i>-DDT	789-02-6	Organochlorine; Diphenylalkene	4.2E-08	+	6.6E-07	+	Weak ER and AR antagonist; Weak ER agonist
<i>p,p'</i>-Methoxychlor	72-43-5	Organochlorine; Chlorinated hydrocarbon	3.0E-06	+	8.9E-06	+	Weak ER agonist; AR antagonist
Fenarimol	60168-88-9	Heterocycle; Pyrimidine	5.8E-06	+	2.7E-05	+	Aromatase inhibitor
Apigenin	520-36-5	Flavanoid; Flavone; Phenol	1.7E-07	+	ND	+	ER agonist
Kepon	143-50-0	Organochlorine; Chlorinated bridged cycloalkane	1.7E-07	+	ND	+	Binds to ER and AR
Butylbenzyl phthalate	85-68-7	Phthalate	4.3E-07	+	ND	+	ER agonist

4-Hydroxy-tamoxifen	68047-06-3	Triphenylethylene; Benzylidene; Stilbene; Phenol	NG	-	ND	+	ER antagonist
Kaempferol	520-18-3	Flavonoid; Flavone; Phenol	7.5E-07	+	ND	+	ER agonist
4 - tert -Octylphenol	140-66-9	Alkylphenol; Phenol	2.7E-07	+	ND	+	ER agonist
p,p' -DDE	72-55-9	Organochlorine; Diphenylalkene	6.6E-06	+	ND	+	Weak AR agonist and antagonist
Flavone	525-82-6	Flavonoid; Flavone	3.5E-08	+	ND	+	Weak ER antagonist
Dexamethasone	50-02-2	Steroid, nonphenolic	NG	-	ND	+	AR agonist
2,4,5-Trichloro- phenoxyacetic acid	93-76-5	Organochlorine; Chlorinated aromatic hydrocarbon	NG	-	ND	+	Weak ER agonist
Dibenzo[a,h]anthracene	53-70-3	Polycyclic aromatic hydrocarbon; Anthracene	Not soluble in Ethanol	NA	ND	+	Polycyclic aromatic hydrocarbone; Anthracene
Clomiphene citrate	50-41-9	Chlorinated triphenylethylene; Benzylidene; Stilbene	NG	-	ND	+	Binds to the ER; Selective estrogen receptor modulator
Mifepristone	84371-65-3	Steroid, nonphenolic; Estrene	7.6E-08	+	ND	-	AR agonist and antagonist
ICI 182,780	129453-61-8	Steroid, phenolic	NG	-	ND	-	ER antagonist
Atrazine	1912-24-9	Aromatic amine; Triazine; Arylamine	NG	-	ND	-	Aromatic amine; triazine
Cycloheximide	66-81-9	Piperidine; Glutaramide	NG	-	ND	-	Protein synthesis inhibitor
Progesterone	57-83-0	Steroid, nonphenolic; Pregnenedione	NG	-	ND	-	Steroid; nonphenolic
Corticosterone	50-22-6	Steroid, nonphenolic	NG	-	ND	-	Binds weakly to AR
Vinclozolin	50471-44-8	Organochlorine; Cyclic imide; Carbamate	Not soluble in Ethanol	NA	ND	-	AR antagonist
Cyproterone acetate	427-51-0	Nitrile; Diphenyl ether; Organochlorine	NG	-	ND	-	AR agonist and antagonist
Flutamide	13311-84-7	Amide; Anilide; Nitrobenzene	NG	-	ND	-	AR antagonist
Linuron	330-55-2	Urea	NG	-	ND	-	Weak AR agonist and antagonist
Procymidone	32809-16-8	Organochlorine; Cyclic imide	NG	-	ND	-	AR antagonist
Haloperidol	52-86-8	Butyrophenone; Ketone; Piperazine	NG	-	ND	-	Dopamine D1/D2 receptor agonist;

NA: Not Analyzed; ND: Not Determined, that is, the chemical was reported as EA positive/negative by ICCVAM meta-study, but no EC50s were determined by ICCVAM. EC50s are given in molar units. Note that Mifepristone was reported as EA negative (NG) by ICCAM, but our assay shows Mifepristone is EA positive. 4-Hydroxy-tamoxifen, 2,4,5-Trichloro-phenoxyacetic acid, Clomiphene citrate, and Dexamethasone were reported as EA positive by ICCVAM, but they are EA negative by our assay.

Table 10: Accuracy of MCF-7 Assay for All Chemicals Tested

25 EA Positive ICCVAM Chemicals Found Positive by CCI

Chemical	ICCVAM	CCI	Chemical	ICCVAM	CCI
17 α -Ethinyl estradiol	+	+	4-Cumylphenol	+	+
Diethylstilbestrol	+	+	Bisphenol A	+	+
17 α -Estradiol	+	+	<i>o,p'</i> -DDT	+	+
17 β -Estradiol	+	+	<i>p,p'</i> -Methoxychlor	+	+
<i>meso</i> -Hexestrol	+	+	Fenarimol	+	+
<i>Estriol</i>	+	+	Apigenin	+	+
Zearalenone	+	+	Kepona	+	+
Estrone	+	+	Butylbenzyl phthalate	+	+
Coumestrol	+	+	Kaempferol	+	+
Genistein	+	+	4- <i>tert</i> -Octylphenol	+	+
<i>p-n</i> -Nonylphenol	+	+	<i>p,p'</i> -DDE	+	+
Bisphenol B	+	+	Flavone	+	+
Daidzein	+	+			

10 EA Negative ICCVAM Chemicals Found Negative by CCI

Chemical	ICCVAM	CCI	Chemical	ICCVAM	CCI
ICI 182,780	-	-	Cyproterone acetate	-	-
Atrazine	-	-	Flutamide	-	-
Cycloheximide	-	-	Linuron	-	-
Progesterone	-	-	Procymidone	-	-
Corticosterone	-	-	Haloperidol	-	-

4 EA Positive ICCVAM Chemicals Found Negative by CCI

Chemical	ICCVAM	CCI	Chemical	ICCVAM	CCI
Dexamethasone	+	-	Clomiphene citrate	+	-
2,4,5-Trichloro-phenoxyacetic acid	+	-	4-Hydroxytamoxifen	+	-

1 EA Negative ICCVAM Chemical Found Positive by CCI

Chemical	ICCVAM	CCI
Mifepristone	-	+

Note that Mifepristone was reported as EA negative (NG) by ICCAM, but our assay shows that Mifepristone is EA positive. 4-Hydroxy-tamoxifen, 2,4,5-Trichloro-phenoxyacetic acid, Clomiphene citrate, and Dexamethasone were reported as EA positive by ICCVAM, but they are EA negative by our assay.

Table 11: Comparison of the EC50 Rankings for Referenced Test Chemicals Published by ICCVAM, or Measured by UM, NWU, or CCI
11A: ICCVAM vs. CCI (18 Chemicals)

Chemicals	CASRN	Results Using CCI's Test Method EC50 (M)	Results Using CCI's Test Method (+/-)	Results from ICCVAM EC50 (M)	Results from ICCVAM (+/-)	ICCVAM Ranking	CCI Ranking
17α-Ethinyl estradiol	57-63-6	1.95E-11	+	1.10E-11	+	1	4
Diethylstilbestrol	56-53-1	2.80E-11	+	1.90E-11	+	2	5
17α-Estradiol	57-91-0	2.91E-11	+	4.60E-11	+	3	6
17β-Estradiol	50-28-2	1.44E-13	+	1.00E-10	+	4	1
meso -Hexestrol	84-16-2	3.77E-12	+	2.00E-10	+	5	2
Estriol	50-27-1	1.40E-10	+	7.10E-10	+	6	7
Zearalenone	17924-92-4	2.72E-10	+	2.00E-09	+	7	8
Estrone	53-16-7	8.27E-12	+	3.20E-09	+	8	3
Coumestrol	479-13-0	4.86E-10	+	1.50E-08	+	9	9
Genistein	446-72-0	1.89E-08	+	6.20E-08	+	10	10
p -n - Nonylphenol	104-40-5	5.01E-07	+	8.50E-08	+	11	16
Bisphenol B	77-40-7	1.21E-08	+	8.80E-08	+	12	11
Daidzein	486-66-8	5.11E-08	+	2.90E-07	+	13	14
4-Cumylphenol	599-64-4	4.70E-08	+	3.22E-07	+	14	13
Bisphenol A	80-05-7	2.52E-08	+	4.00E-07	+	15	12
o,p' -DDT	789-02-6	1.15E-07	+	6.60E-07	+	16	15
p,p' -Methoxychlor	72-43-5	3.68E-06	+	8.85E-06	+	17	17
Fenarimol	60168-88-9	2.47E-06	+	2.70E-05	+	18	18

This table shows the comparison of experimentally obtained CCI EC50 values (in molar concentrations) and potency rankings for 18 chemicals versus EC50 values (M) and rankings from ICCVAM meta-study. The most active test compound (lowest EC50) in each set is assigned the lowest (1) rank number and the least active chemical is assigned the highest (18) rank number.

Table 11: Comparison of the EC50 Rankings for Referenced Test Chemicals Published by ICCVAM, or Measured by UM, NWU, or CCI

11B: EC50 Rankings for ICCVAM vs. CCI, UM, and NWU for 8 Test Chemicals

Chemicals	Ranking			
	ICCVAM	CCI	UM	NWU
<i>17β-Estradiol</i>	3	1	2	1
<i>17α-Ethinyl estradiol</i>	1	2	1	2
Diethylstilbestrol	2	3	3	3
Genistein	4	4	4	5
Bisphenol A	6	5	5	6
<i>4 - tert -Octylphenol</i>	NA	6	6	4
<i>p -n -Nonylphenol</i>	5	7	7	7
<i>p,p'- Methoxychlor</i>	7	8	8	8

This table shows the comparison of potency rankings for 8 chemicals obtained at the CCI Austin facility (CCI), UM or NWU versus rankings from ICCVAM meta-study. The most active test compound (lowest EC50) in each set is assigned the lowest (1) rank number and the least active chemical is assigned the highest (8) rank number.

Table 12: CCI or ICCVAM EC50 Rankings of ICCVAM Reference Chemicals

Chemicals	CASRN	Results Using	Results Using	Results	Results	ICCVAM	CCI
		CCI's	CCI's	from	from		
		Test Method	Test Method	ICCVAM	ICCVAM	Ranking	Ranking
		EC50 (M)	(+/-)	EC50 (M)	(+/-)		
<i>17β-Estradiol</i>	50-28-2	1.44E-13	+	1.00E-10	+	4	1
<i>meso -Hexestrol</i>	84-16-2	3.77E-12	+	2.00E-10	+	5	2
<i>Estrone</i>	53-16-7	8.27E-12	+	3.20E-09	+	8	3
<i>17α-Ethinyl estradiol</i>	57-63-6	1.95E-11	+	1.10E-11	+	1	4
<i>Diethylstilbestrol</i>	56-53-1	2.80E-11	+	1.90E-11	+	2	5
<i>17α-Estradiol</i>	57-91-0	2.90E-11	+	4.60E-11	+	3	6
<i>Estriol</i>	50-27-1	1.40E-10	+	7.10E-10	+	6	7
<i>Zearalenone</i>	17924-92-4	2.72E-10	+	2.00E-09	+	7	8
<i>Coumestrol</i>	479-13-0	4.86E-10	+	1.50E-08	+	9	9
<i>Bisphenol B</i>	77-40-7	1.21E-08	+	8.80E-08	+	12	10
<i>Genistein</i>	446-72-0	1.89E-08	+	6.20E-08	+	10	11
<i>Bisphenol A</i>	80-05-7	2.52E-08	+	4.00E-07	+	15	12
<i>Flavone</i>	525-82-6	3.51E-08	+	ND	+	ND	13
<i>4-Cumylphenol</i>	599-64-4	4.70E-08	+	3.22E-07	+	14	14
<i>Daidzein</i>	486-66-8	5.11E-08	+	2.90E-07	+	13	15
<i>Mifepristone</i>	84371-65-3	6.84E-08	+	ND	-	ND	16
<i>o,p'-DDT</i>	789-02-6	1.15E-07	+	6.60E-07	+	16	17
<i>Kepone</i>	143-50-0	1.91E-07	+	ND	+	ND	18
<i>Apigenin</i>	520-36-5	2.14E-07	+	ND	+	ND	19
<i>4 - tert - Octylphenol</i>	140-66-9	3.31E-07	+	ND	+	ND	20
<i>p -n - Nonylphenol</i>	104-40-5	5.01E-07	+	8.50E-08	+	11	21
<i>Butylbenzyl phthalate</i>	85-68-7	5.59E-07	+	ND	+	ND	22
<i>Kaempferol</i>	520-18-3	7.70E-07	+	ND	+	ND	23
<i>p,p'-Methoxychlor</i>	72-43-5	3.68E-06	+	8.85E-06	+	17	24
<i>Fenarimol</i>	60168-88-9	2.47E-06	+	2.70E-05	+	18	25
<i>p,p'-DDE</i>	72-55-9	4.27E-06	+	ND	+	ND	26

ND: Not Determined; that is, chemicals were reported as EA positive by ICCVAM meta-study, but no EC50s were determined by ICCVAM, so no rankings were made for those chemicals.

The most active test compound (lowest EC50) in each set is assigned the lowest (1) rank number and the least active chemical is assigned the highest (18 or 26) rank number.

Table 13

EA of foodstuffs stored in glass vs. plastic containers and sampled at 0 & 2 weeks

Casein: no detectable EA at 0 weeks or at 2 weeks in glass

- 1) Center: No EA difference between any type plastic (n =4)* or glass (n=1) containers
- 2) Edge: EA in all 4 plastics > EA in glass
- 3) Edge **vs.** Center: EA at edge of all 4 plastics > EA at center; edge = center for glass

Gluten: significant EA at 0 weeks that does not increase in glass

- 1) Center: No EA difference between any container
- 2) Edge: EA in 3 plastics > EA in glass and zip bag
- 3) Edge **vs.** Center: EA at edge of 3 plastics > EA at center; edge = center for glass and zip bag

Oats: no detectable EA at 0 or 2 weeks in any container

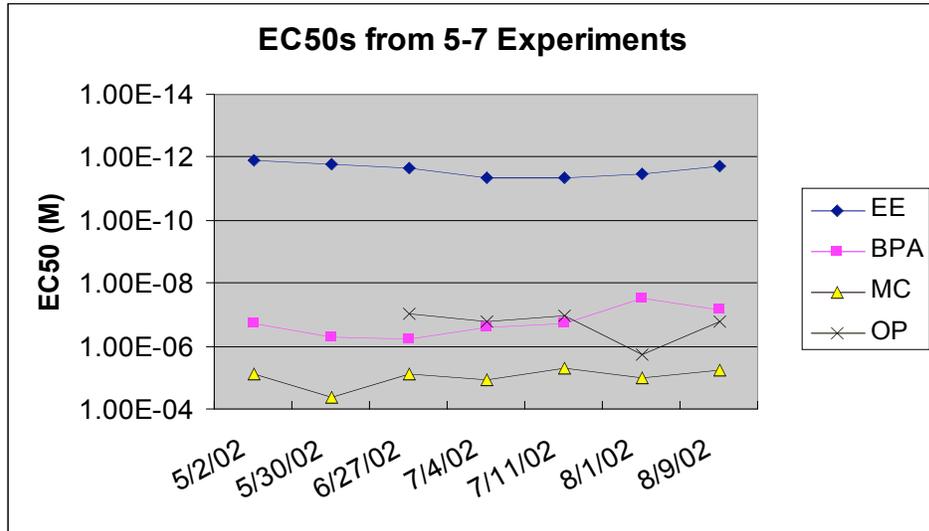
- 1) Center: No EA difference between any container
- 2) Edge: No EA difference between any container
- 3) Edge **vs.** Center: No EA difference between edge and center of any container

Yeast: significant EA at 0 weeks that does not increase in glass

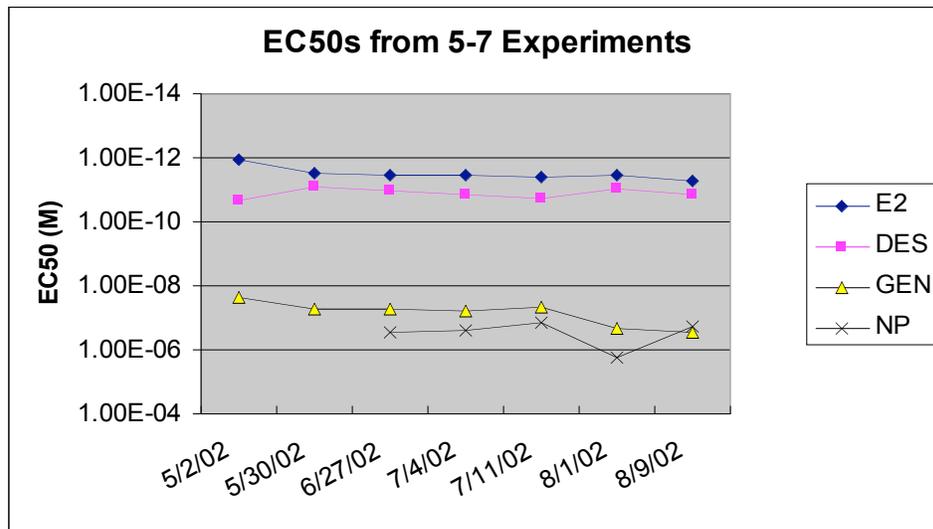
- 1) Center: EA in 4 mil and 8 mil bag > EA in glass, zip bag, and whirl bag
- 2) Edge: EA in all 4 plastics > EA in glass
- 3) Edge **vs.** Center: EA at edge of all 4 plastics > EA at center; edge = center for glass

n: number of types of containers; (n=4)*: four types of plastic bags including 4 mil bag, 8 mil bag, zip bag, and whirl bag.

Figure 1: EC50s of 8 Chemicals Repetively Tested at UM
1A: The First Four of 8 Test Chemicals



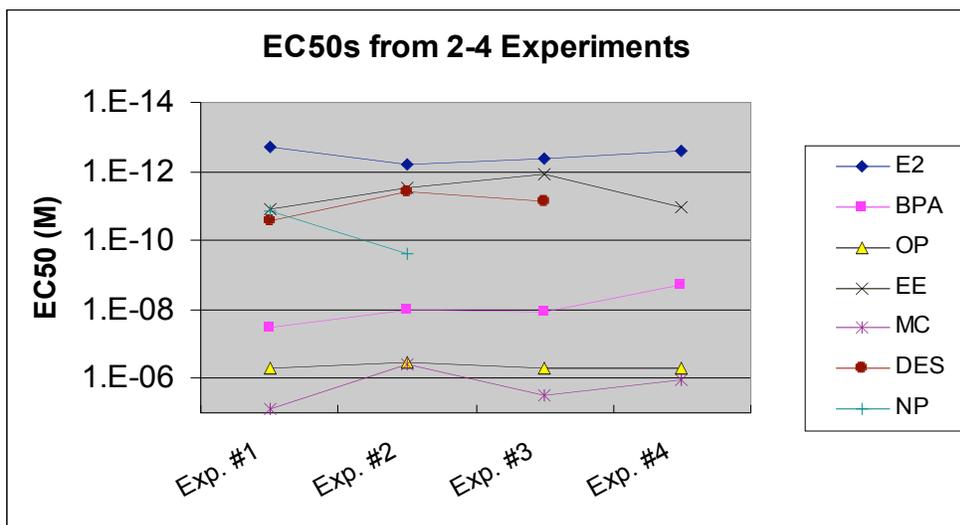
1B: The Second Four of 8 Test Chemicals



Ten Chemicals were repeatedly tested in 5-7 experiments at UM by robotic MCF-7 assay in the laboratory of Dr. Wade Welshons from 5/2002- 8/2002 using an earlier version of our robotic EA assay (see Table 1 in Appendix A). The figure gives the EC50 for each of 8 chemicals exhibiting estrogenic activity (EA). Two (ICI 182,780 and PG) of the 10 test chemicals were EA negative (no detectable EA), and are not presented in this figure. BPA: Bisphenol A; DES: Diethylstilbestrol; E2: 17 β -Estradiol; EE: 17 α -Ethyl estradiol; GEN: Genistein; ICI: ICI 182,780; MC: p,p'-methoxychlor; NP: p-n-Nonylphenol; OP: 4-tert-Octylphenol; PG: Propyl gallate.

The EC50 data point for each experiment was calculated from a dose-response curve plotted using the mean of three replicates at each concentration. Note consistency (reliability, repeatability) of the mean EC50 for each chemical for each experiment.

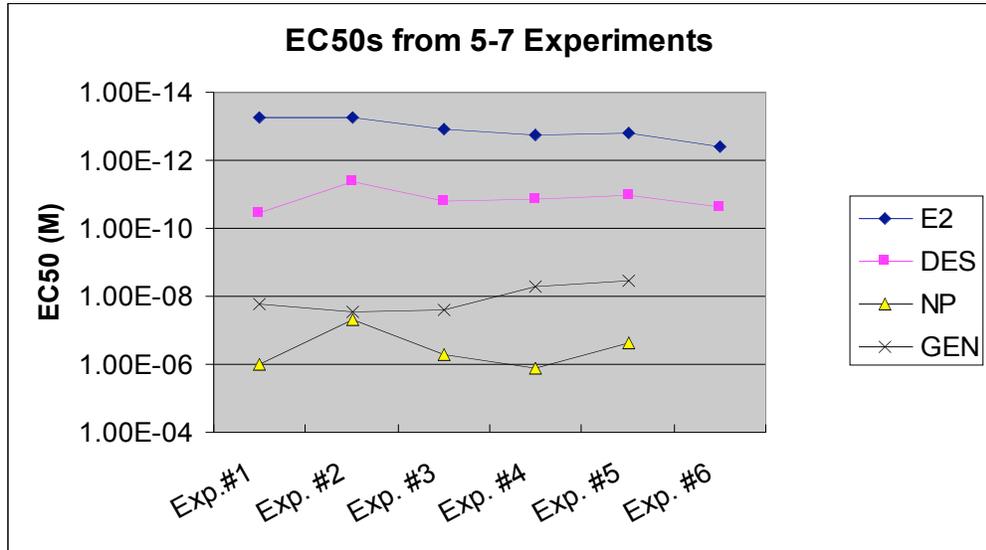
Figure 2: EC50s of 7 Chemicals Repeatedly Tested at NWU



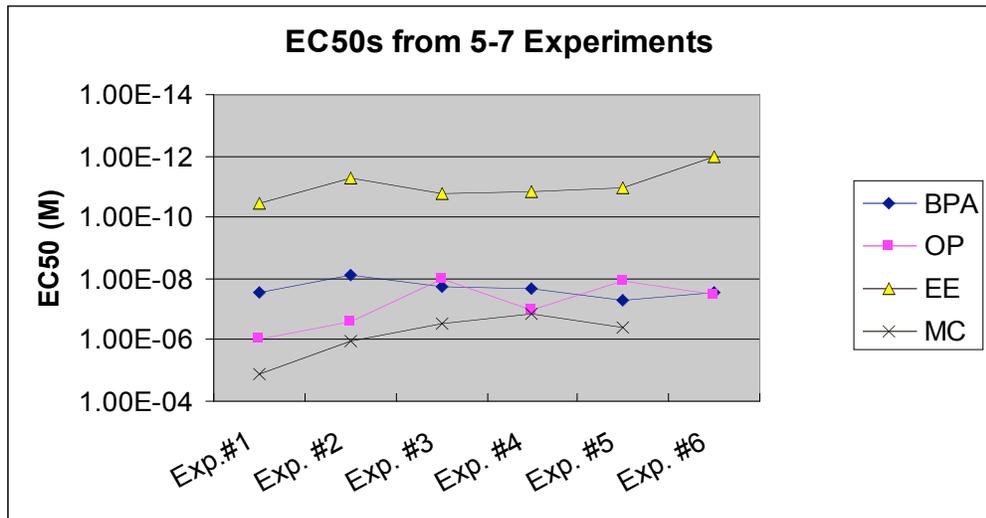
Nine test chemicals were repeatedly tested in four experiments at NWU by manual MCF-7 cell proliferation assay in 2004 and 2005 in the laboratory of Dr. V. Craig Jordan. EC50s of seven chemicals that exhibited EA are plotted in the figure. ICI 182,780 and PG were EA negative and are not presented in this figure. Only two experiments are presented in the figure for NP, because the other two experiments did not meet our data acceptance criteria. Note that the EC50 of NP at NWU is much lower (probably due to contamination by a strong estrogen) than the EC50 obtained from ICCVAM, UM or CCI's Austin laboratory. BPA: Bisphenol A; DES: Diethylstilbestrol; E2: 17 β -Estradiol; EE: 17 α -Ethyl estradiol; GEN: Genistein; ICI: ICI 182,780; MC: p,p'-methoxychlor; NP: p-n-Nonylphenol; OP: 4-tert-Octylphenol; PG: Propyl gallate.

Figure 3: EC50s of 8 Chemicals Repetitively Tested by CCI

3A: The First Four of 8 Test Chemicals

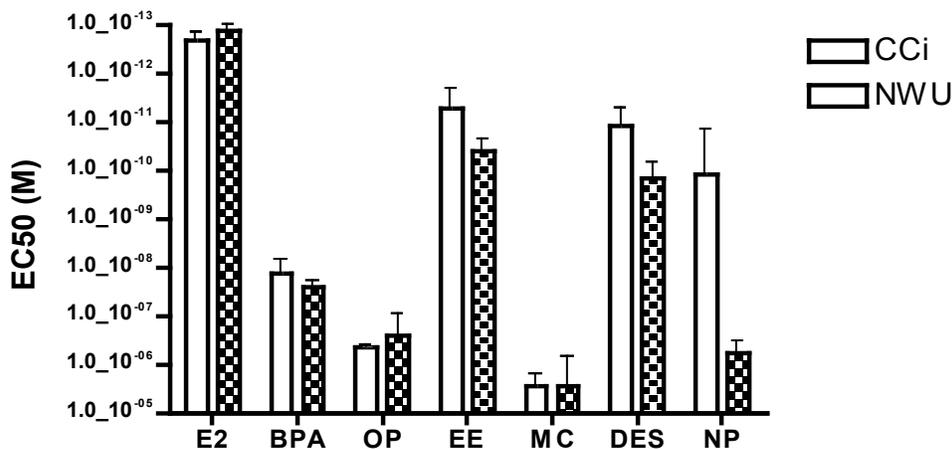


3B: The Second Four of 8 Test Chemicals



Ten chemicals were repeatedly tested in CCI's facility at Austin by robotic MCF-7 cell proliferation assay in 2004 and 2005. EC50s of the eight chemicals that exhibited EA are plotted in the figure. Two (ICI 182,780 and P) of the ten test chemicals were EA negative and are not presented in this figure. BPA: Bisphenol A; DES: Diethylstilbestrol; E2: 17 β -Estradiol; EE: 17 α -Ethyl estradiol; GEN: Genistein; ICI: ICI 182,780; MC: p,p'-methoxychlor; NP: p-n-Nonylphenol; OP: 4-tert-Octylphenol; PG: Propyl gallate.

Figure 4: Comparison of EC50s Obtained by CCI (robotic format) and by NWU (manual format)

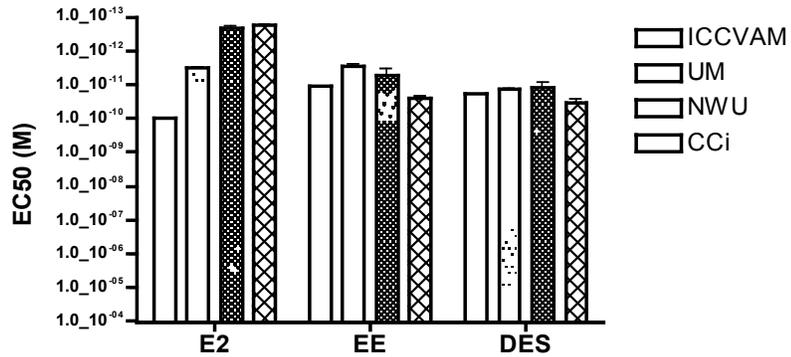


EC50s (in molar concentrations) obtained at CCI’s facility in Austin in a robotic format and those obtained at NWU in a manual format are plotted in the figure. The error bars indicate the SEM of the EC50s for each test chemical. The number of experiments for each test chemical is presented in the table below.

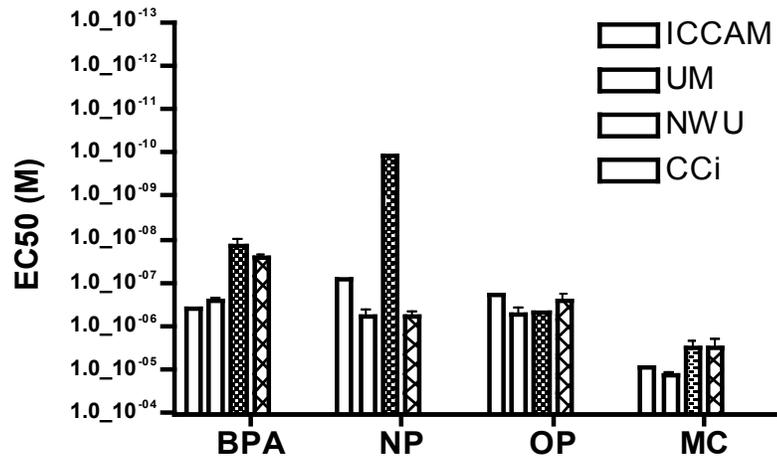
Chemical	Number of Experiments	
	NWU	CCI
E2	8	22
BPA	4	6
OP	4	5
EE	3	7
MC	3	5
DES	3	11
NP	2	5

E2: 17β-Estradiol; BPA: Bisphenol A; OP: 4-tert-Octylphenol; EE: 17α-Ethyl estradiol; MC: p,p’-methoxychlor; DES: Diethylstilbestrol; NP: p-n-Nonylphenol.

Figure 5: Comparison of EC50s from ICCVAM, CCI, NWU and UM
5A: Comparison of the First Three Test Chemicals



5B: Comparison of the Second Four Test Chemicals



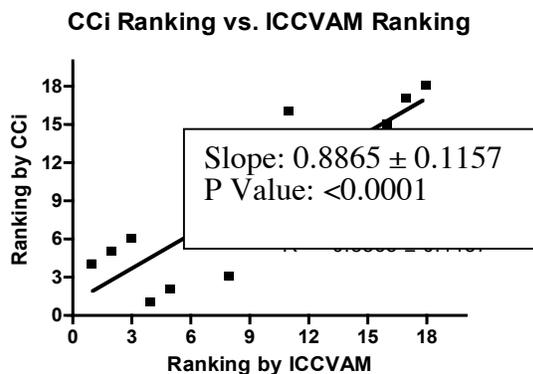
EC50s published by ICCVAM or obtained at UM, NWU or CCI's facility in Austin are plotted in the figure. The error bars indicate the SEM of the EC50 for each test chemical. The number of experiments for each test chemical is presented in the table below.

Chemical	Number of Experiments		
	NWU	CCI	UM
E2	8	22	7
BPA	4	6	7
OP	4	5	5
EE	3	7	7
MC	3	5	7
DES	3	11	7
NP	2	5	5

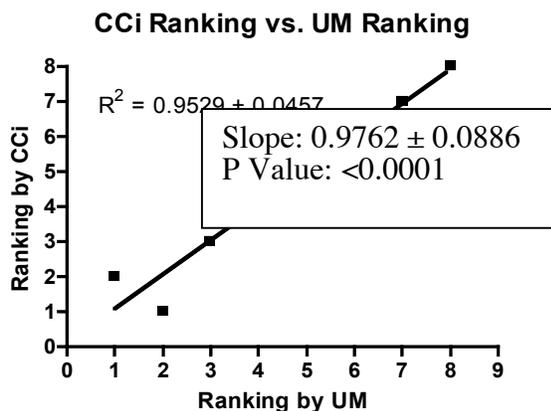
E2: 17β-Estradiol; BPA: Bisphenol A; OP: 4-tert-Octylphenol; EE: 17α-Ethyl estradiol; MC: p,p'-methoxychlor; DES: Diethylstilbestrol; NP: p-n-Nonylphenol.

Figure 6: Correlation of CCI EC50 rankings vs ICCVAM, UM, or NWU EC50 rankings

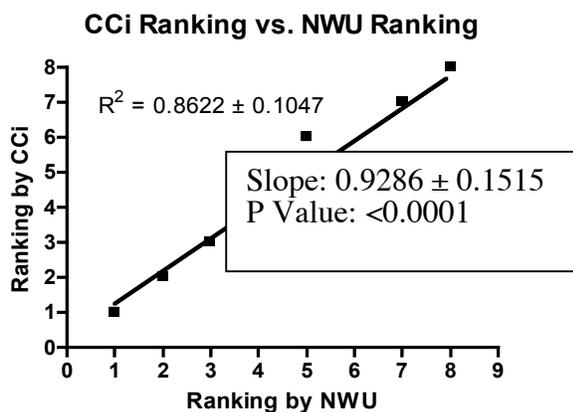
6A



6B

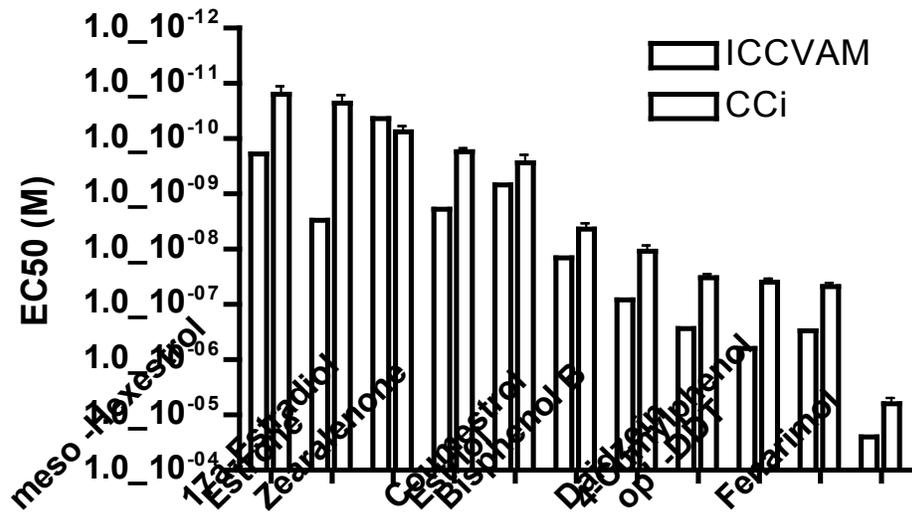


6C



$P < 0.001$ for Null Hypothesis in all three cases indicates that CCI's rankings do **not** differ significantly from those obtained by ICCVAM, UM, or NWU. The most active test compound (lowest EC50) in each set is assigned the lowest (1) rank number and the least active chemical is assigned the highest (8 or 18) rank number.

Figure 7: Comparison of EC50s from CCI and ICCVAM



The EC50s published by ICCVAM and obtained by CCI (see Table 6 in Appendix A) are plotted in the figure. The error bars indicate the Standard Error of the Mean for the EC50s obtained in CCI's Austin facility by a robotic format. Note that the EC50's obtained by CCI are lower than the EC50's published by ICCVAM, indicating that CCI's assay is more sensitive than some other assays.

Figure 8-9: Versatility of CCI's Robotic Assay

Fig. 8: EA of two types of plastic bags

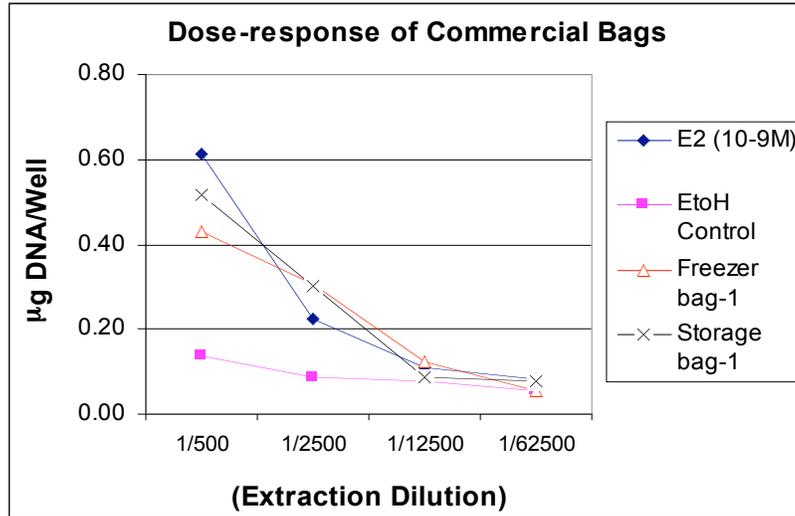
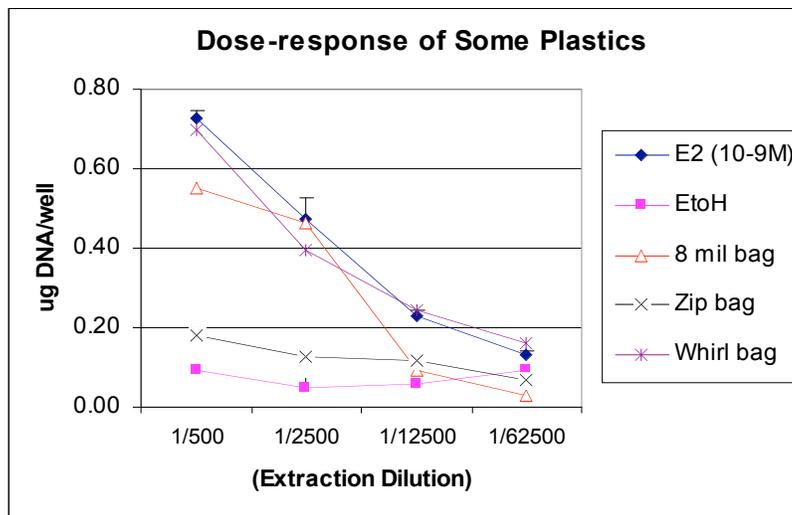


Fig. 9: EA of three types of plastic bags



One gram each of some commercial bags were cut into small pieces and then extracted with 0.5ml EtOH in glass tubes at 37°C for at least 24 hours. The extractions were diluted 100x with EA-free medium (final concentration of EtOH is 1% that is used as our negative control). The initial 100x dilution of the extractions was then diluted 5x with EA free medium containing 1% EtOH (the extraction becomes 1:500); this 5x dilution step was then repeated three more times by diluting with EA free medium containing 1% EtOH (1:2500, 1:12500, and 1:62500). EA-induced cell proliferation is then plotted as dose-response curves as µg DNA/well produced by the test substance at 1:500, 1:2500, 1:12500, and 1:62500.

Figure 10-11: Versatility of CCI's Robotic Assay

Fig. 10: EA of cell culture plates and flasks

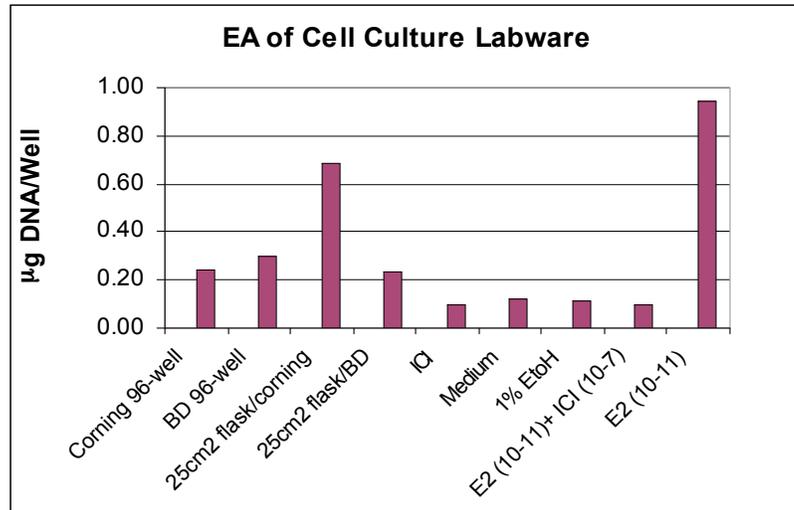
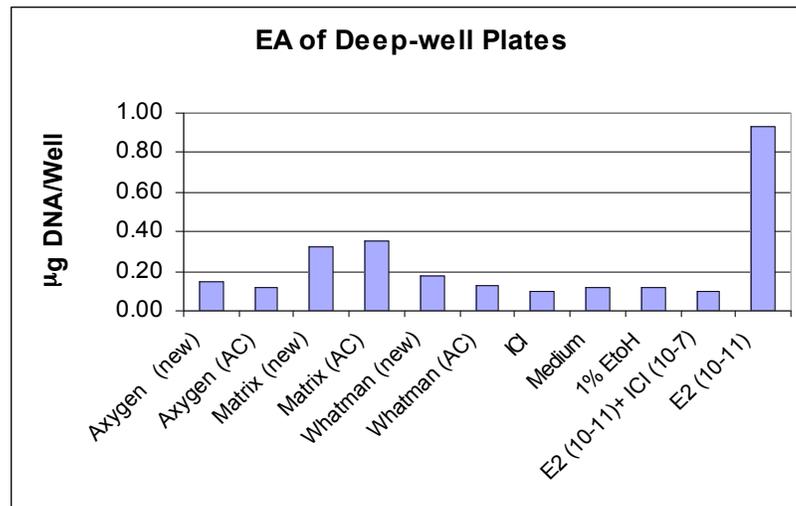
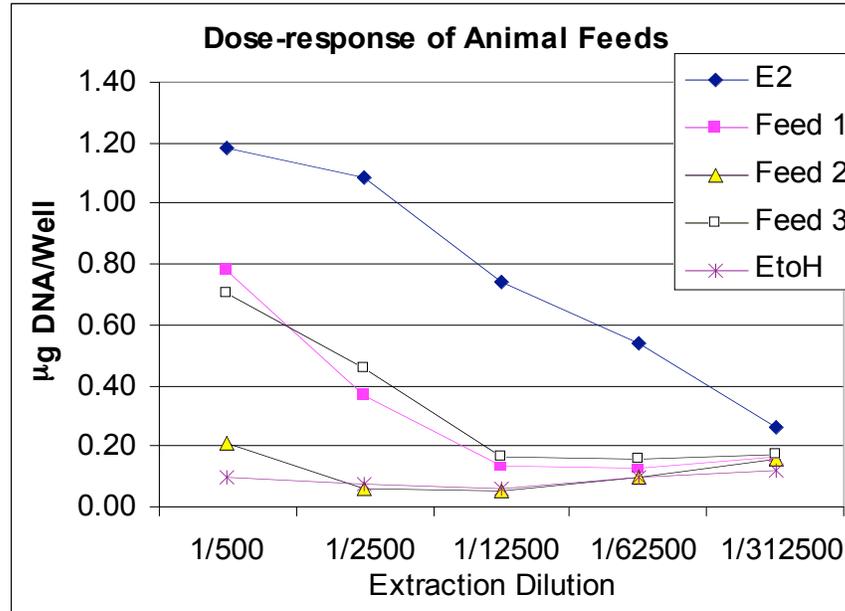


Fig. 11: EA of different types of deep-well plates



EA of some plastic labware extracted as described above. EA-induced cell proliferation plotted as $\mu\text{g DNA/well}$ produced by the test substance at 1:500 dilution. Each graph also gives the response to (1) medium, 1% ETOH, and 10^{-7}M ICI 182,780 as negative controls and, (2) 10^{-11}M E2 as positive control and reference standard, and (3) 10^{-11}M E2 + 10^{-7}M ICI 182,780 as a positive control.

Figure 12: Versatility of CCI's Robotic Assay



0.5 grams of some animal feeds were extracted with 3ml of EtOH by rotating 30 minutes at room temperature. The animal feed extractions were then centrifuged for 30 minutes at 15,000xg. The supernatants were then diluted as described for **Figs. 8-9**. EA-induced cell proliferation is then plotted as $\mu\text{g DNA/well}$ produced by the test substance at 1:500, 1:2500, 1:12500, and 1:62500.