EA-Free™ Plastics: The only alternative for safer plastics

I: Biological Studies and Public Health Perspective

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Executive Summary: Part I

The Problem

Plastics are a fundamental building block for the technologies that have improved life for billions of people and are an indispensable component of modern societies. However, most plastic products sold today release chemicals that have estrogenic activity (EA). While estrogens occur naturally in men and woman, many scientific studies have shown that significant health problems can occur when chemicals are ingested that mimic or block the actions of these sex hormones in mammals; the fetus, newborn, and young child are especially vulnerable. These health-related problems include early puberty in females, reduced sperm counts in males, altered functions of reproductive organs, obesity, altered behaviors, learning disorders, and increased rates of breast, ovarian, testicular, and prostate cancers.

The Focus on BPA and Phthalates

Estrogenic chemicals leach from most plastic products sold today. Bisphenol A (BPA) and phthalates are only two of the hundreds of chemicals that have EA and are commonly used in the large majority of plastics. Because of early publicity surrounding these estrogenic chemicals, the current commercial approach is to solve this health-related problem by producing BPA-free and/or phthalate-free plastic products. Unfortunately this incremental solution to replace an individual chemical does not provide a comprehensive health solution. Furthermore, substitutes for BPA or phthalate-containing products can leach other chemicals having more total EA than the EA released by the original products; the solution can be worse than the problem.

Legislation to Date

The call to ban BPA and phthalates is growing rapidly. California has passed legislation banning phthalates in certain products; similar bills are pending in Connecticut, New York, Pennsylvania, Maryland, Maine and Minnesota. The US Congress has passed an amendment to the Consumer Product Safety Commission Reform Act that would implement a similar ban. The European Union and Canada have already passed this legislation. However, all current regulatory attempts try to solve this EA problem by banning just one or two chemicals having EA at a time. More radical approaches that are based primarily on consumer sentiment, like banning the use of all plastics for certain products (e.g., plastic bag bans), ignore the performance and ecological benefits of plastics, and are counter-productive.

The Health-Related Solution

The most appropriate regulatory solution is to require that all plastics be made without estrogenic chemicals, rather than ban specific EA-causing ingredients one at a time. This is not a pie-in-the-sky solution, as the technology already exists to produce EA-Free™ plastics that also have the same performance and ecological advantages of existing EA-releasing conventional plastics. In fact, some of the advanced-technology EA-Free™ plastics are already in the marketplace.

Part II. The Design and Manufacture of EA-Free™ Plastics and Plastic Products

In the second part of this White Paper, the cause of EA from plastics is related to molecular structure, technological solutions for plastics producers and plastic product manufacturers are described, and some case studies are outlined.

Technical Summary: Part I

Using a highly sensitive method of *in vitro* biological assay, PlastiPure has compiled extensive data showing that most existing commercially available plastics release chemicals that exhibit EA the most significant form of endocrine disruption. Steroid hormones, like estrogen, can have significant adverse biological effects at very low concentrations (micromolar (~ppm) to nanomolar (~ppb) or even picomolar (~ppt)), especially on fetal and newborn mammals, including humans. (NIEHS, 2006; EDSTAC, 1998; NRC, 1999; NTP, 2000; Welshons et al, 2003; Kabuto et al., 2004; vom Saal and Hughes, 2005; Swan et al. 2005; Rubin et al. 2006; vom Saal, 2006). This raises significant concern for human exposure because most plastic products leach chemicals having EA at concentrations greater than this nanomolar to picomolar range (Takao et al., 1999; Howdeshell et al, 1999; Yang and Bittner, 2007).

In **Part I** of this White Paper, we outline the methods for comprehensive EA testing, demonstrate that these methods are reliable, repeatable, sensitive, and correlate with older less precise methods.

By far the large majority of plastics have tested positive for EA. Because of the lack of focus on endocrine disruption and use of much less sensitive EA testing modalities, reliable EA-Free™ plastics are only available from PlastiPure or its licensed partners. In fact, PlastiPure's data show that products advertised as BPA-free or phthalate-free can release chemicals that have more total EA than the total EA released by products containing BPA or phthalates. Products made from these highly estrogenic BPA-free materials are further contaminated by estrogenic colorants, additives, processing aids, and other materials. An example of this "marketing" solution to healthier plastics can be easily seen with the many reusable water and baby bottles currently being made from popular BPA-free materials, such as PES or PETG, which has replaced polycarbonate over the past two years but have consistently tested positive for significant EA. Millions of dollars have been spent to develop BPA-free plastics (and much more to market these materials) without addressing the underlying issue of EA.

However, it is possible to develop an extensive line of technologically advanced formulations and procedures for making safer plastics, food additives, and other products without sacrificing the desirable qualities of conventional plastics: flexibility, hardness, clarity, recyclability, small carbon footprint, etc. These materials can be produced from common chemicals, using existing tooling, and with only minor changes to operating procedures providing very price competitive solutions. The methods and materials for the design and manufacture of safer, EA-Free™ plastics are outlined in **Part II** of this White Paper.

Technical Presentation

Effects of endocrine disrupting chemicals (EDCs) on animals and humans

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-c, 2003, 2006; Palanza et al., 2002; Singleton and Khan, 2003; Copenhagen, 2007; Janer and Porte, 2007; Tan and Zoeller, 2007; Adewale et al., 2009). Wildlife and laboratory animals exposed to such endocrine disrupting chemicals (EDCs) exhibit adverse effects on 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; Murray et al., 2006; Patisaul et al., 2006). Such ED effects can be either gross or subtle when tested in animal model systems. A significant probability exists that similar ED effects are 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). EDCs can potentially 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 (Goldman, 2000; Baskin, 2001; Kawai et al., 2003; Markey et al., 2003; Goodman et al., 2006).

EA is the most common ED effect and 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; Fujimoto et al., 2006; Copenhagen, 2007; Newbold et al., 2004, 2009; Patisaul et al., 2006, 2008, 2009; Garner et al., 2008; Monje et al., 2009; Spivey 2009). *In vivo* data from mice and rats have shown that exposure to estrogenic EDCs at various developmental stages is associated with alterations in the reproductive organs of infants and adults (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, 2002; Kawai et al., 2003; Della Seta et al., 2006).

Federal Regulation of EDCs

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, 2003, 2006), commercial entities, non-profit organizations, and scientific panels or meetings (NRC, 1999; Jordan et al., 2000; NTP, 2001; Copenhagen, 2007). In response to such concerns about ED activity 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 that chemicals be tested for hormonal activity. To accomplish this goal, the EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to examine whether current toxicological testing procedures are adequate to determine EDC activity.

EDSTAC recommended that many thousands of chemicals be tested in an Endocrine Disruptor Screening Program 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 EDC testing. Tier 1 *in vitro* and *in vivo* tests are designed to

identify EDCs. Tier 1 robotic *in vitro* screening tests are especially desired as a way to more quickly identify EDCs – and at lower costs. The robotic assays for EA used by PlastiPure's testing partner, CertiChem, Inc., meet these criteria.

ICCVAM was established in 1997 (Public Law P.L.103-43) to develop and validate new *in vitro* test methods and authorized in 2000 (P.L. 106-545), as a 15 agency permanent committee, to co-ordinate the development, validation and acceptance of toxicological tests throughout the Federal Government. 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 of the validation status of assays for EDs. ICCVAM (2002a, b, 2006) recommended that ER-dependent Transcriptional Activation (TA) assays be developed because such "functional" assays are more sensitive than Relative Binding Affinity assays, can distinguish agonists from antagonists, and can be conducted with and without exogenous metabolic activation. The panel expressed a preference (ICCVAM, 2002b, 2003, 2006) for the use of human ER subtypes in any *in vitro* TA screening assay and developed minimum procedural standards for TA assays of EA.

Non-Federal Regulation of EDCs

Non-Federal legislation directed at EDCs has gained momentum recently. Last year's SB 484, also known as the California Safe Cosmetics Act, forces cosmetics makers to reveal harmful ingredients having endocrine disruptor activity. In New York, regulations in effect since September of 2006 now require all public and private schools to use chemicals that are free of reproductive hormonal activity. In late 2006, the European Union passed laws to protect people from thousands of toxic chemicals. REACH, or Registration Evaluation and Authorization of Chemicals will force industries to register chemicals, submit health and safety data, and replace the most hazardous ones with safer alternatives by 2009. A new European Chemicals Agency based in Helsinki, Finland will become the central regulatory authority. These regulations include the first steps to eliminate use of plastics containing polyvinylchloride (PVC), phthalates and many other chemicals; similar regulations have also been passed in Japan. The Economist (9/22/07) believes that these REACH regulations will quickly be accepted as world regulatory standards for chemicals having hormonal activities.

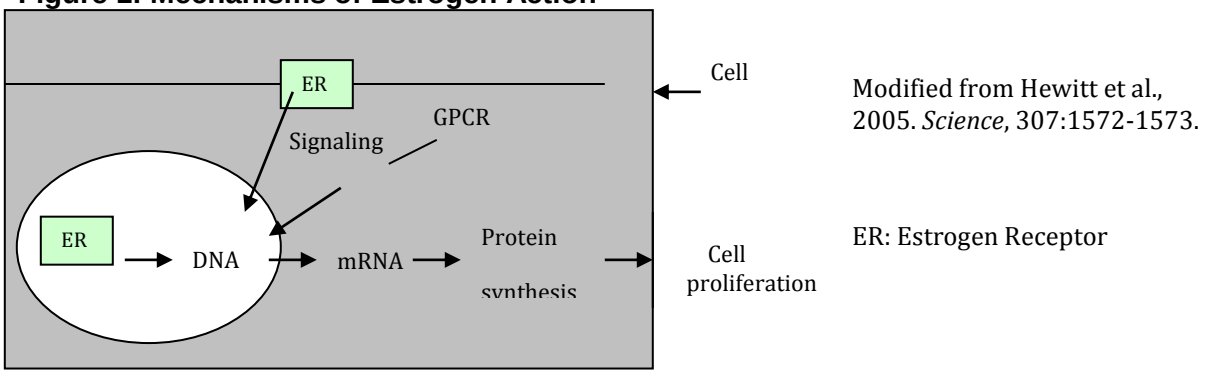
Cellular/Molecular mechanisms by which chemicals produce EA and Anti-EA

The cellular/molecular mechanisms of action of estrogenic and anti-estrogenic EDCs are shared with natural estrogens. Synthetic exogenous EDCs present in the environment, such as monomers or additives released from plastic products, mimic endogenous estrogenic hormones by affecting 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 EDCs and activate transcription of estrogen-responsive genes, leading to cell proliferation (**Fig. 1**; Matthews et al., 2002; Revankar et al., 2005). Anti-EA effects may be produced by competitive inhibitors that bind to ERs but do not activate them (e.g., ICI 182,780 and ICI 164,384; Wakeling, 1993) or agonists that bind strongly to ERs, but do not activate as strong an ER response (Jordan and Murphy, 1990; Muller et al., 2002).

Furthermore, selective ER modulators (SERMs) bind to ERs, but subsequently activate cellular responses that differ from those activated by the endogenous estrogen, 17β-estradiol (E2) (Black et al., 1983; Yang et al., 1996; Shang and Brown, 2002; Lonard and Smith, 2002). It is also possible for a chemical to bind directly to an endogenous hormone, and thereby reduce its effect.

While binding affinities differ between estrogenic ligands (Kuiper, 1997), ER ligands typically bind to both receptors (ICCVAM, 2002a, 2003, 2006; Routledge et al., 2000). 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). EDCs with EA or anti-EA can bind to nuclear or extra-nuclear receptors (**Fig. 1**; Hewitt et al., 2005; Evinger and Levin, 2005; Raz et al., 2008; Vasudevan and Pfaff, 2008).

Figure 1: Mechanisms of Estrogen Action



CertiChem's *In Vitro* EA and anti-EA Assays

The MCF-7 cell proliferation assay has been used in manual format as the gold standard for many years to measure EA: human breast-derived MCF-7 cells divide when stimulated by chemicals having EA. The ability of PlastiPure to develop EA-Free™ plastics has depended, in part, on the development by CertiChem of highly sensitive, reliable, and accurate EA and an *anti-*EA MCF-7 cell assays in robotic format that meet all duplicate wells, positive and negative controls, etc (See ICCVAM, 2002a, b, 2003, 2006, 2007 for details and references to CertiChem's EA assay).

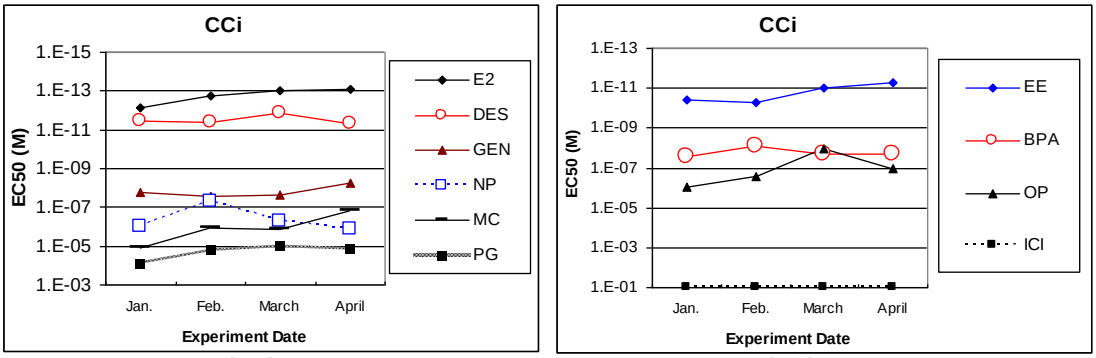
For example, CertiChem has extensively shown that its robotic MCF-7 assay for EA is repeatable (reproducible/reliable) both within its own laboratory (**Fig. 2A-B**) and among laboratories at CertiChem, U Missouri, and Northwestern Medical School (**Fig. 2C-D**). CertiChem has also shown that its cell proliferation assay is very versatile, i.e., is capable of assaying EA in many foodstuffs, feeds, plastics, etc (**Fig. 3**). In addition, CertiChem has completed extensive analyses of chemicals suggested by ICCVAM to show that this robotic assay is:

(1) extremely sensitive: concentration producing 50% of maximum response (EC50) for E2 =~10^{-13} M (see **Fig. 2, Table 1**);

And

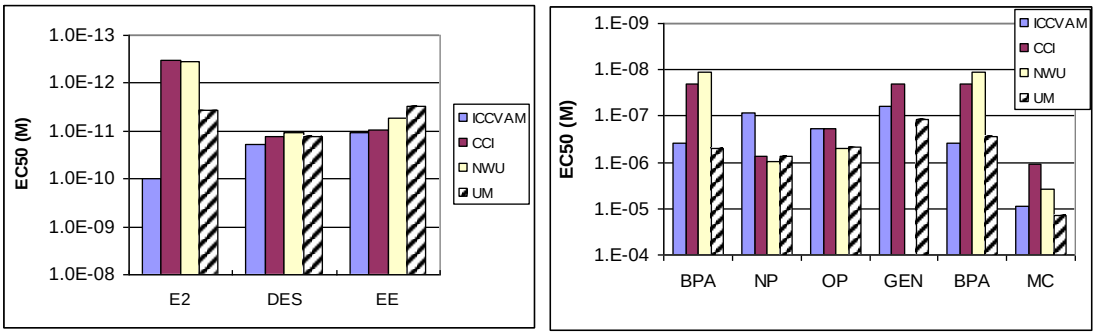
(2) very accurate (almost no false negatives or positives in over 75 chemicals analyzed to date; see **Table 2, Fig. 3**).

Figure 2: Reliability/Reproducibility of CertiChem's EC50 assays



(2A) **(2B)**

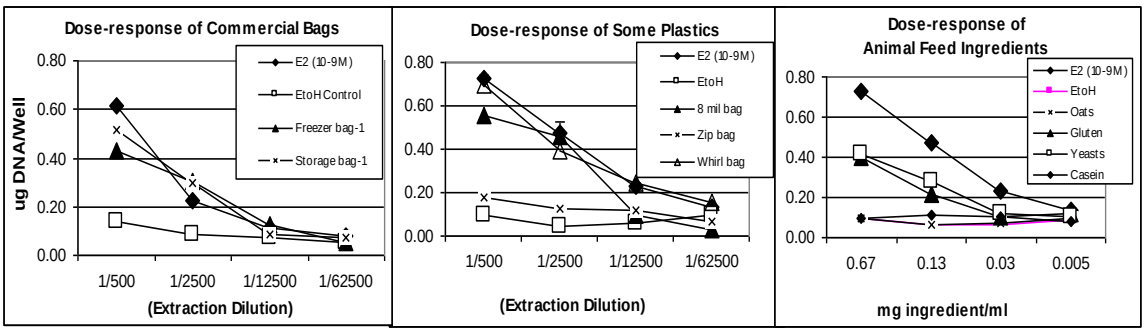
Figures 2A, B: Repetitive testing of 10 test chemicals by CertiChem laboratory once each month for four months using the current version of robotic EA assay.



(2C) **(2D)**

Figures 2C, D: Comparison of EC50's of 8 test chemicals as published by ICCVAM (2003 meta-analysis, median value of manual assays) vs. MCF-7 assay at CertiChem (average of robotic assays), Northwestern University Medical School (NWU) in the laboratory of Dr. V. Craig Jordan (average of manual assays), and UM in the laboratory of Dr. Wade Welshons (average of robotic assays). 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 the consistency (reliability/repeatability) of EC50 values among the three labs using this assay and its accuracy with respect to data from the ICCVAM (2003) meta-analysis.**

Figure 3: Versatility of CCi's Robotic EA assay



(3A) **(3B)** **(3C)**

Figures 3A-C: For plastics **(Figs. 3A-B)**, 1g finely cut plastic pieces are covered by 1ml 100% EtOH at 37°C for at least 24 hrs. For foodstuffs **(Fig. 3C)**, 1g of finely ground ingredient is dissolved in 1ml 100% EtOH for 30 minutes at 20°C; supernatant obtained by centrifuging at 3000 rpm for 10 min. The

supernatants from feed and plastics are then serially diluted in culture medium by robotics at 1:500 to 1/62,500 and applied to MCF-7 cells in 96 well plates as described for EC50 analyses.

Table 1: CertiChem or ICCVAM EC50 Values for ICCVAM Reference Chemicals

TEST CHEMICALS	CAS No.	CCi Mean EC50 (M)	CCi EA (+ or -)	ICCVAM Median EC50 (M)	ICCVAM EA (+ or -)	ICCVAM EC50 ranking	CCi EC50 ranking
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
Estrone	53-16-7	8.27E-12	+	3.20E-09	+	8	3
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	2.90E-11	+	4.60E-11	+	3	6
Estriol	50-27-1	1.40E-10	+	7.10E-10	+	6	7
Zearalenone	17924-92-4	2.72E-10	+	2.00E-09	+	7	8
Coumestrol	479-13-0	4.86E-10	+	1.50E-08	+	9	9
Bisphenol B	77-40-7	1.21E-08	+	8.80E-08	+	12	10
Genistein	446-72-0	1.89E-08	+	6.20E-08	+	10	11
Bisphenol A	80-05-7	2.52E-08	+	4.00E-07	+	15	12
Flavone	525-82-6	3.51E-08	+	No EC50	+	No EC50	
4-Cumylphenol	599-64-4	4.70E-08	+	3.22E-07	+	14	13
Daidzein	486-66-8	5.11E-08	+	2.90E-07	+	13	14
Mifepristone	84371-65-3	6.84E-08	+	No EC50	-	No EC50	
o,p'–DDT	789-02-6	1.15E-07	+	6.60E-07	+	16	15
Kepone	143-50-0	1.91E-07	+	No EC50	+	No EC50	
Apigenin	520-36-5	2.14E-07	+	No EC50	+	No EC50	
4-tert-Octylphenol	140-66-9	3.31E-07	+	No EC50	+	No EC50	
p-n-Nonylphenol	104-40-5	5.01E-07	+	8.50E-08	+	11	16
Butylbenzyl phthalate	85-68-7	5.59E-07	+	No EC50	+	No EC50	
Kaempferol	520-18-3	7.70E-07	+	No EC50	+	No EC50	
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
p,p'–DDE	72-55-9	4.27E-06	+	No EC50	+	No EC50	

No EC50: Reported as EA positive by ICCVAM meta-study, but no EC50s given and therefore no ranking comparison. 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 2: Accuracy of CertiChem's MCF-7 EA Assay for All Chemicals Tested

24 EA Positive ICCVAM (2003, 2006) test chemicals assayed positive by CCi **0/24 false negatives**	17α-estradiol, 17β-estradiol, 4-Cumyphenol, 4-tert-Octylphenol, Apigenin, BPA, BPB, Butylbenzyl phthalate, Coumestrol, Daidzein, Diethylstilbestrol, o,p'-DDT, p,p'-DDE, Estriol, Estrone, Fenarimol, Flavone, Genistein, Kaempferol, Kepone, meso-Hexestrol, p,p'-Methoxychlor, p-n-Nonylphenol, Zearalenone.
15 EA negative ICCVAM (2003, 2006) test chemicals assayed negative by CCi **0/15 false positives**	Atrazine, Clomiphene citrate*, Corticosterone, Cycloheximide, Cyproterone acetate, Dexamethasone*, Flutamide, Haloperidol, Hydroxytamoxifen**, ICI 182, 780, Linuron, Procymidone, Progesterone, Trichloro-phenoxyacetic acid. *,**: Chemicals once considered EA positive by ICCVAM (2003), but now (ICCVAM, 2006) considered to be negative* or uncertain**.
34 other EA negative chemicals assayed by CCi. No EA has been reported for these chemicals by ICCVAM, nor do CCi's QSAR analyses predict any EA for these chemicals **0/34 false positives**	Acetaminophen, Acetonitrile, Acetylsalicylic acid, Amitriptyline HCl, Carbamazepine, Catechin, Cycloheximide, DL Propranolol, Eserine, Ethanol, Ethylene glycol, Glycerol, Glycyrrhizic acid, Haloperidol, Hexachlorophene, Lactic acid, Lithium carbonate, Methyl viologen, n-Phenylthiourea, Potassium chloride, Procainamide HCl, Sodium Chloride, Sodium fluoride, Sodium hypochlorite, Sodium oxalate, Sodium selenate, Strychnine, Tert-butylhydroquinone (TBHQ), Trichloroacetic acid, Trihydrobutyrophenone (TBHP), Triphenyltin hydroxide, Valprioc acid, Verapamil HCl, Vitamin E.

A least squares regression analysis (**Fig. 4a**) shows that the EC50 rankings produced by CertiChem's robotic EA assay and ICCVAM meta-analysis rankings for EC50 measures of EA of various chemicals do not differ significantly (null hypothesis, $p < 0.001$). CertiChem's robotic assay also includes several positive (E2, genistein) and negative (saline) controls, as well as an anti-EA control to insure that any cell growth is EA-dependent. As a check, this assay easily detects BPA (**Fig. 4b**).

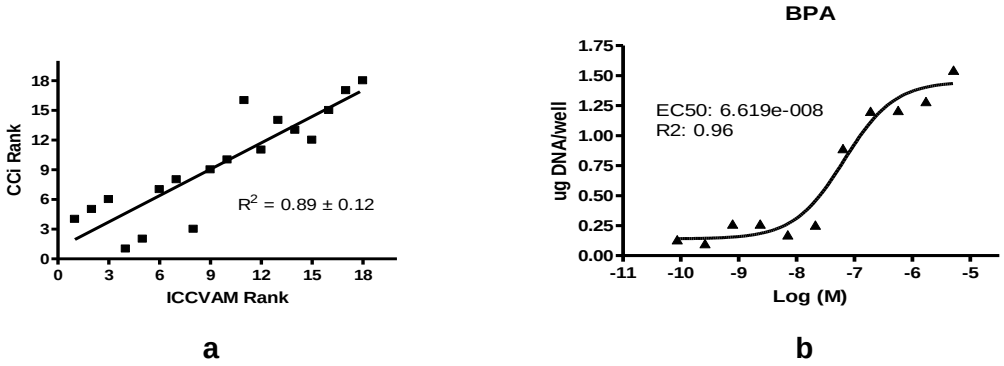


Figure 4: Accuracy of CCi's Robotic Assay a) CCi (mean) *versus* ICCVAM (median) EC50 rankings for 18 chemicals ranked by ICCVAM (2003) meta-study; b) Response of MCF-7 assay to BPA

Similar to its EA assay, CertiChem's anti-EA robotic *in vitro* assay is in compliance with ICCVAM recommended protocols. CCi's assay is highly sensitive, i.e. capable of detecting chemicals with high anti-EA (e.g., ICI, HDT) at picomolar concentrations and chemicals with low anti-EA (apigenin, flavone) at less than micromolar concentrations. This assay can measure the anti-EA of single chemicals, as well as complex mixtures of known and/or unknown chemicals in small amounts (data not shown).

CertiChem's EA assay is the most sensitive EA assay commercially available, having an EC50 of about 10^{-13} M for 17β–estradiol. For example, in contrast, the EC50 of 17β–estradiol is about 10^{-11} (100 times less sensitive) for the EA assay offered by some commercial laboratories; other ICCVAM reference test chemicals tested by CertiChem versus other commercial laboratory's assays usually show that CertiChem's assay is on average 20 to >100 fold more sensitive. In addition, CertiChem's most sensitive extraction protocols are also 5-20 times more robust than those published by other laboratories. Hence, CertiChem's sensitive EA assay can be100-10,000 times more sensitive in detecting EA in plastic extractives than other commercial laboratories.

Summary

- At least several thousand chemicals are now known to have EA, several hundred of which are commonly used in making plastics.
- Estrogens are the predominately female sex hormones, but are of critical importance in regulating multiple systems in both men and women. Chemicals with EA mimic or block the actions of these hormones.
- Many scientific studies have now shown that chemicals with EA produce a wide range of health problems in mammals, including early puberty in females, reduced sperm counts in males, altered functions of reproductive organs, obesity, altered behaviors, learning disorders, and increased rates of some breast, ovarian, testicular, and prostate cancers.
- PlastiPure, in conjunction with its testing partner, CertiChem, Inc, uses a cell proliferation *in vitro* assay protocol for determining EA in plastics, which is demonstrated to be highly sensitive, accurate, reliable, and repeatable. This method looks at EA directly through cell growth, rather than just individually assaying for one or two of the thousands of possible chemicals that can cause EA. These tests are generally much more sensitive than other *in vitro* (e.g. gene expression) or *in vivo* testing (e.g. Sprague-Dawley) modalities.

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