Toxicology in Vitro 28 (2014) 916–925

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

A robotic BG1Luc reporter assay to detect estrogen receptor agonists

Matthew A. Stoner [a], Chun Z. Yang [a], George D. Bittner [a,b,*]

[a] CertiChem, Inc., Austin, TX, USA
[b] Neuroscience Department, University of Texas, Austin, TX, USA

ARTICLE INFO

Article history:
Received 23 January 2014
Accepted 21 March 2014
Available online 18 April 2014

Keywords:
BG1Luc
Bisphenol A
Estrogenic activity
Estrogen
Endocrine disruptor
Tritan™

ABSTRACT

Endocrine disrupting chemicals with estrogenic activity (EA) have been associated with various adverse health effects. US agencies (ICCVAM/NICEATM) tasked to assess *in vitro* transcription activation assays to detect estrogenic receptor (ER) agonists for EA have recently validated a BG1Luc assay in manual format, but prefer robotic formats. We have developed a robotic BG1Luc EA assay to detect EA that demonstrated 100% concordance with ICCVAM meta-analyses and ICCVAM BG1Luc results in manual format for 27 ICCVAM test substances, i.e. no false negatives or false positives. This robotic assay also consistently assessed other, more problematic ICCVAM test substances such as clomiphene citrate, L-thyroxin, and tamoxifen. Agonist responses using this robotic BG1Luc assay were consistently inhibited by the ER antagonist ICI 182,780, confirming that agonist responses were due to binding to ERs rather than to a non-specific agonist response. This robotic assay also detected EA in complex mixtures of substances such as extracts of personal care products, plastic resins or plastic consumer products. This robotic BG1Luc assay had at least as high accuracy and greater sensitivity and repeatability when compared to its manual version or to the other ICCVAM/OECD validated assays for EA (manual BG1Luc and CERI).

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1. Introduction

Endocrine disrupting chemicals (EDCs) mimic or otherwise alter the activities of hormones. Estrogenic activity (EA) is by far the most common type of hormonal activity for known or suspected EDCs (National Research Council, 1999; ICCVAM, 2003, 2006, 2010, 2011; vom Saal et al., 2005; Vandenberg et al., 2012). Many studies (National Research Council, 1999; ICCVAM, 2003; Calafat et al., 2005; Swan et al., 2005; vom Saal et al., 2005; Heindel and vom Saal, 2009; Talsness et al., 2009; Thompson et al., 2009; Gray, 2010; Adewale et al., 2011; de Cock et al., 2012; Hall and Korach, 2012; Vandenberg et al., 2012) have reported that xenobiotic chemicals with EA *in vitro* can produce adverse effects *in vivo* in laboratory animals and humans. These effects include decreased sperm counts, ovarian and uterine disorders, abnormalities in male reproductive organs, obesity, abnormal brain maturation, learning disabilities, attention disorders, increases in immune and autoimmune disease and increased incidence of some cancers. Fetal, infant, and juvenile mammals are especially sensitive to low dosages [nanomolar (nM) to <picomolar (pM) concentrations, or ppb to <ppt levels] of chemicals with EA (vom Saal et al., 2005; Gray, 2010; Vandenberg et al., 2012).

Many scientists and consumers are concerned about the potential public health effects of chemicals with EA that are released from commonly used products such as plastics and cosmetics (Gray, 2010). In the US, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program's Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) are tasked to co-ordinate the development, validation, and acceptance of *in vitro* toxicological tests. [These combined agencies are hereafter referred to as ICCVAM.] Acceptable *in vitro* toxicological tests to assess whether chemicals have EA include estrogen receptor (ER)-dependent transactivation assays such as BG1Luc and CERI, and cell proliferation assays such as those using MCF-7 cell lines

Abbreviations: CCi, CertiChem, Inc.; CS, calf serum; DMSO, dimethyl sulfoxide; E2, 17β-estradiol; EA, estrogenic activity; EC50, half-maximal response of a test substance in its dose-response curve; EDC, endocrine disrupting chemical; EFM, EA-free medium; ER, estrogen receptor; EtOH, ethanol; FBS, fetal bovine serum; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; ICI, ICI 182,780, an ER antagonist; NICEATM, National Toxicology Program's Interagency Center for the Evaluation of Alternative Toxicological Methods; NTP, National Toxicology Program; OECD, Organization for Economic Cooperation and Development; RLU, relative light unit [a measure of luciferase bioluminescence]; RPMI, Roswell Park Memorial Institute; SD, standard deviation; Sham Control, control solvent that went through all the steps that an extract did; VC, vehicle control.

* Corresponding author at: CertiChem, Inc., 11212 Metric Blvd., Suite 500, Austin, TX, USA. Tel.: +1 512 339 0550×201; fax: +1 512 339 0551.
E-mail address: gbittner@certichem.com (G.D. Bittner).

http://dx.doi.org/10.1016/j.tiv.2014.03.013
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(ICCVAM, 2003, 2006; Yang et al., 2011, 2013). Whenever possible, ICCVAM prefers robotic assays to manual assays (ICCVAM, 2003, 2006).

Only two EA assays are currently validated, or have been undergoing validation, by ICCVAM for regulatory use: the BG1Luc ER transactivation assay in manual format and the MCF-7:WS8 (MCF-7) cell proliferation assay in robotic format, respectively. A third assay (CERI) has been approved in manual format by the EU Organisation for Economic Co-operation and Development (OECD) and this validated assay is accepted by ICCVAM (2011). The validated assays for EA by ICCVAM are also accepted by the US Environmental Protection Agency (EPA).

In order to increase the high through-put and the repeatability, decrease the human errors and assay cost, we have developed a robotic version of the BG1Luc assay subsequently used to evaluate the EA of 44 test substances supplied by ICCVAM and of ICI 182,780 (ICI), a pure strong anti-estrogen. The 44 test substances were used in the ICCVAM validation study of the BG1Luc assay (2011). The half-maximum responses (EC50s) of individual test substances were calculated from concentration–response curves. From these EC50s, the test substances were classified as having strong EA ($EC50 \leqslant 1 \times 10^{-9}$ M, e.g., diethyl-stilbestrol), moderate EA (EC50 between 1.0×10^{-9} M and 1.0×10^{-7} M, e.g., coumestrol), weak EA ($EC50 \geqslant 10^{-7}$ M, e.g., genistein), or no detectable EA (e.g., atrazine). This robotic BG1Luc assay could detect EA in complex mixtures of chemicals. Furthermore, agonist responses detected for a test chemical, or a complex mixture, were suppressed by the ER-antagonist ICI 182,780 (ICI) to confirm that the agonist response is via ER pathway. That is, positive agonist responses classified as exhibiting EA were due to binding of chemicals to ERs, rather than non-specific ER activation, potentially resulting in a false positive classification for EA.

Twenty seven of the 44 ICCVAM test substances used by ICCVAM to assess the accuracy (concordance) of the manual BG1Luc assay with ICCVAM meta-analyses were used to assess the accuracy of this assay. This robotic BG1Luc assay had a 100% concordance with ICCVAM meta-analysis classifications (ICCVAM, 2003, 2006, 2011) for these 27 test substances. Robotic BG1Luc assays of individual test substances are very repeatable (reproducible). The EC50s of individual test substances tested in this robotic assay were usually lower (more sensitive) compared to EC50s previously reported by ICCVAM (2003, 2006, 2011) using other *in vitro* assays, including the validated manual BG1 and CERI assays for EA. We therefore conclude that this robotic BG1Luc assay is at least as accurate, and often more sensitive and reproducible, as the validated test methods accepted by the US ICCVAM/NICEATM, the US EPA and the EU OECD.

2. Materials and methods

2.1. Equipment

A Labconco Class II Biosafety Hood (Kansas City, MO, USA) equipped with a 254 nm fluorescent bulb to enclose EpMotion 5070 robotic workstations (Eppendorf, Hamburg, Germany) was used for all cell seeding, serial dilutions of test substances and for treatments with test substances (Yang et al., 2011, 2013). A Tristar Luminometer (Berthold Technology, Germany) was used to measure luminescence.

2.2. Cell line maintenance, seeding and assay conditions

BG1Luc4E2 (BG1Luc) cells were obtained from Dr. Michael Denison (University of California-Davis). This human ovarian cell line expresses estrogen receptor (ER) alpha and beta receptors and is highly sensitive to 17β-estradiol (E2) at 1 pM (Rogers and Denison, 2000). These cells are modified to stably carry a firefly luciferase reporter vector that expresses luciferase enzyme under the control of multiple estrogen-response elements (ERE) sequences positioned in the gene enhancer/promoter sequence upstream of the luciferase coding sequence. The EA assay examines the ability of a substance to induce expression of luciferase enzyme.

As described in more detail below and by ICCVAM (2011) the BG1Luc assay consists of growing these cells in estrogen-free medium for three days, then exposing the cells to test substances or E2 for 24 h, then measuring any agonist-induced luciferase response against the E2 response (positive control) and the vehicle response (negative control). The enzymatic activity of luciferase is measured in relative light units (RLUs) with respect to the maximum E2 response to E2 (positive control) set at 100% activity obtained by a dose response-curve in each experimental run. Modified cell culture medium serves as the negative vehicle control (VC) and is set at 0% estrogenic activity.

Cell culture medium used to maintain the BG1Luc cells was RPMI (Roswell Park Memorial Institute)-1640 media supplemented with 10 μg/mL phenol red, 4 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 8% fetal bovine serum (FBS) and 1 mM sodium pyruvate. Cells were grown as monolayers in polystyrene tissue culture flasks (T-25 flask, CytoOne, USA Scientific, Ocala, FL; or T-75 flask, BD-Falcon, BD Biosciences, San Jose, CA) in a humidified incubator at 37 °C with 5%CO_2.

The EA-free medium (EFM) was prepared in two ways. ICCVAM EFM (used for ICCVAM validation study) was phenol red-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5% dextran-coated charcoal-stripped FBS, 1 mM sodium pyruvate, 4 mM L–glutamine and 100 units/mL penicillin and 100 μg/mL streptomycin solution (Invitrogen, Grand Island, NY). Alternatively, CCi EFM was phenol red-free RPMI-1640 medium supplemented with 1% charcoal-stripped FBS and 4% charcoal-stripped calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B solution, 4 mM L-glutamine, 1/100 (vol/vol) non-essential amino acids (100×: catalog number 11,140,050) purchased from Invitrogen, and 6 ng/mL insulin.

In preparation for experiments and in order to decrease the basal level expression of luciferase enzyme, BG1Luc cells were trypsinized, dispersed with a 22G needle on a 3 mL or 10 mL syringe, counted, re-plated in a T-75 flask and incubated for 1–4 days in EFM that was changed daily. (Three days was subsequently chosen as the standard incubation time.)

After the initial incubation time in EFM, BG1Luc cells were then seeded in 96-well, white-walled, clear bottom cell culture plates (Greiner Bio-One, Monroe, NC) at 10,000–40,000 cells per well in 0.1 mL EFM, followed by a 24 ± 6 h incubation after adding 0.1 mL of serially diluted test substances or extracts in triplicate of each testing concentration (see below). Water was distilled on-site in an all-glass system and collected directly into glass before use in extractions. Extractions were performed in borosilicate glass tubes.

2.3. Visual assessment of cell health/cytotoxicity observations

Some test substances were cytotoxic at high treatment concentrations. Since cytotoxicity can prevent measurement of EA and lead to false-negative interpretations, viability of BG1Luc cells was visually observed under an inverted light microscope immediately before terminating incubation. Cellular cytotoxicity was visually assessed using the following scoring parameters suggested by ICCVAM (NIEHS, 2011): 1 = normal cell morphology, 2 = low cytotoxicity (10–50% of cells with altered morphology), 3 = moderate cytotoxicity (50–90% of cells had altered morphology), and 4 = high

cytotoxicity (few or no cells visible). Additionally, any precipitate in the cell culture plate wells was also noted. Test substance concentrations that produced a cytotoxicity score of 2 or higher were excluded from data analyses.

2.4. Luminescence measurements

Incubation with test substances was terminated by aspirating cell culture medium and then adding a cell lysis solution and luciferase assay reagent (Promega, Madison, WI, USA), essentially following the manufacturer's protocol. The luminescence was measured by a Tristar Luminometer and exported into an Excel file. Luminescence data were analyzed on Excel spreadsheets (Microsoft Corp., Bellevue, WA) and graphed using GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA).

2.5. Preparation of test substances and extracts

2.5.1. Individual test chemicals

Test substances were provided originally by ICCVAM as part of a Phase I–IV inter-laboratory validation study commissioned by ICCVAM for the MCF-7 cell proliferation assay (Yang et al., 2013). Concentrated stocks of test substances were prepared in dimethyl sulfoxide (DMSO) at concentrations based on the concentrations used in the MCF-7 inter-laboratory validation study. The concentrated stocks in DMSO were initially diluted 50 times in EFM as the highest testing concentrations which were then serially-diluted in EFM with 2% DMSO in deep-well plates using epMoton 5070 (Eppendorf) liquid handling system. Test substance serial dilution factors were 2-, 2.5- or 5-fold based on the concentration–response curves obtained in MCF-7 validation study (Yang et al., 2013) and covered 11–12 concentrations (EA assays) or 5–6 concentrations (EA confirmation assays using ICI). Next, 0.1 mL of the diluted test substances or extracts was added to the 96 well plates that had BG1Luc cells in 0.1 mL of EFM so that the final DMSO concentration applied to cells was 1%. This vehicle control concentration of 1% DMSO in EFM was maintained for all dilutions of test substances.

2.5.2. Complex mixtures of chemicals (extracts of personal care products/cosmetics)

Sample products representing different classes of personal care products were purchased by the National Toxicology Program (Research Triangle Park, NC) from retail locations and shipped to CertiChem, Inc., to test their EA in MCF-7 cell proliferation assays. A subset of these products was also tested using BG1Luc cells. For such tests, three to five gram aliquots of cosmetic ingredients or products were combined with solvent (DMSO) in the ratio of 1 g product/1 ml solvent in a glass tube that was vortexed and placed on a shaker for 2–4 h at room temperature, then centrifuged at 1500 rpm for 10 min at room temperature. The liquid phase of each extract was transferred into a 1.5 mL tube, centrifuged at 13,000 rpm for 15 min at room temperature, and the transparent liquid phases were transferred to a 1.5 mL tube before diluting and testing for EA.

2.6. Complex mixtures of chemicals (extracts of plastic resins or products)

Some thermoplastic resins and products were purchased from 2010–2013 from the resin manufacturer or from retail outlets. Tritan™ resins were pressed into uniform plaques, then exposed to ultraviolet light at about 254 nm predominant wavelength by placing samples on aluminum foil in a Labconco Biosafety hood about 24″ from a germicidal fluorescent light for 24 h. As previously described for extracts of plastic products (Yang et al., 2011), samples of unstressed products or UVC stressed Tritan™ resin plaques were cut into 4 × 4 mm pieces, and then 2.0–5.0 g were added to sterile glass test tubes. The tubes were placed under a germicidal UV light for 30 min to sterilize the samples and tube openings before adding 100% EtOH as an extraction solvent to a final concentration of 1.0 g/mL. Most samples were extracted at 40 °C for 240 h in an incubator shaker. Some samples were extracted for 72 h at 37 °C in a static incubator. EtOH extracts were concentrated to dryness under vaccum. The residue was dissolved in DMSO and EFM to produce a highest product concentration of 0.375 g product/mL in 1% DMSO.

2.7. Calculation of EA

The %RME2 was calculated by first adjusting raw luciferase RLU data by subtracting the VC RLU (negative control) from test substance RLU, and then normalized by dividing by the VC-adjusted highest E2 RLU response (positive control). Such a calculation accounts for any EA that may be released by the plastic labware or the effect of DMSO in the VC. The highest E2 RLU response was determined by a concentration–response curve run in triplicate for 8–12 concentrations for each assay. The highest normalized E2 response was set to 100%RME2 and the VC to 0%RME2. The concentration–response curve for EA of a substance or chemical mixture was plotted with log M or log g/mL test concentrations on the *X*-axis and %RME2 on the *Y*-axis. A dotted line was plotted at 15%RME2 and represents a value that is greater than VC + 3SD to greatly reduce the probability of producing false positive conclusions. A substance was classified as EA-positive when at least one test concentration had at least one data point greater than 15%RME2. In other words, at least one data point on the EA response-curve was above the VC + 3SD that is <15%RME2. (Note that this 15%RME2 value is a conservative measure of EA detectability.)

For an EA assay to be acceptable, the following three criteria had to be met: (1) The E2 RLU response had to be at least 3× greater than the assay VC RLU, (2) the "sham control" (solvent that went through all the steps for extracts) had to be lower than 15%RME2, and (3) the E2 positive control concentration–response curve had to have a positive slope, preferably with at least three data points on its ascending linear portion. For known individual pure test substances (pure chemicals), the EC50 (in M) was calculated from a best fit to dose–response data using GraphPad Prism software. Test substances were classified as EA negative if no points were ⩾15%RME2.

Some test substances were analyzed only one or two times, others multiple times. For those test substances that were analyzed four or less times, we provide the individual values for the EC50 for each of those runs in Table 1 of the results section. Each EC50, in turn, was calculated from a set of data points, each being the mean of 3–4 concentrations typically having little variance. When five or more independent assays were made of the same test substance, the mean and SD of their EC50s were calculated using Student's *T* test.

2.8. EA confirmation assay using ICI

Stimulation of BG1Luc Luciferase expression by some test chemicals or extracts of plastic resins or products was confirmed as estrogenic (EA positive, rather than non-specific effects) when the stimulation was suppressed by co-treatment with ICI (Yang et al., 2011, 2013). For EA confirmation assays, BG1Luc cells were seeded and grown as described for EA comprehensive assays, except that five to six concentrations of test substances were serially diluted in VC or in VC to which the ER antagonist ICI was added at 1×10^{-7} or 1.0×10^{-8} M to suppress any EA-induced luciferase expression (Yang et al., 2013). Data points that were obtained from wells where cytotoxicity was observed were excluded from analyses.

Table 1
Data for 44 ICCVAM test substances plus ICI from robotic BG1Luc assays, ICCVAM meta-analyses, BG1Luc manual assays, CERI manual assays, and YES assays.

Last updated: 2014.4.10 CY

TABLE 1

Substance	CCi					ICCVAM			
	BG1 Robotic				MCF-7 Robotic	Meta-Analysis	BG1 Manual	CERI Manual	YES Manual
	EC50 ± SD	class	n	ICI conf.	Mean EC50	Median EC50	Median EC50	Mean EC50	literature EC50
17ß-Estradiol	$5.5x10^{-12} \pm 2.6$	s	32	√	$2.9x10^{-12}$	$8.7x10^{-11}$	$3.4x10^{-12}$	$1.0x10^{-11}$	$4.9x10^{-10}$
17ß-Trenbolone	EA+ (MTP)	w	1		$1.0x10^{-6}$	neg		$2.7x10^{-7}$	$4.5x10^{-5}$
17α-Estradiol	(2.7, 3.6) $3.2x10^{-10}$	s	2		$1.6x10^{-10}$	$5.2x10^{-9}$	$3.0x10^{-10}$	$6.4x10^{-10}$	$1.1x10^{-8}$
17α-Ethinyl estradiol	(6.7, 3.1, 2.6, MBP)$4.1x10^{-12}$	s	4	√	$8.3x10^{-13}$	$5.2x10^{-11}$	$7.1x10^{-12}$	$1.0x10^{-11}$	$2.9x10^{-10}$
19-Nortestosterone	(1.4, 2.8) $2.1x10^{-8}$	m	2	√	$5.4x10^{-8}$	$2.0x10^{-7}$	$1.7x10^{-6}$	$2.7x10^{-7}$	
4-Cumylphenol	(3.1, 3.3) $3.1x10^{-7}$	w	2		$8.4x10^{-8}$	$3.2x10^{-7}$	$3.0x10^{-7}$	$1.6x10^{-6}$	$1.3x10^{-6}$
4-Hydroxytamoxifen	neg	ne	5	√	neg	I	neg		$1.9x10^{-9}$
4-*tert*- Octylphenol	$2.0x10^{-7}$	w	1		$1.9x10^{-8}$	$1.0x10^{-7}$	$2.1x10^{-8}$	$7.4x10^{-8}$	$1.4x10^{-7}$
5α-Dihydrotestosterone	$1.3x10^{-7} \pm 1.5$	w	5	√	$1.4x10^{-7}$	$1.3x10^{-7}$	$9.0x10^{-8}$		$3.7x10^{-6}$
Ammonium perchlorate	neg	neg	2		neg				EA+
Apigenin	(3.9, 4.2) $4.0x10^{-7}$	w	2		$4.4x10^{-7}$	$7.7x10^{-7}$	$1.4x10^{-6}$	$5.7x10^{-7}$	
Atrazine	neg	neg	4		neg	neg	neg	neg	neg
Benzophenone-2	$3.026x10^{-7}$	w	1		$1.3x10^{-7}$				
Bisphenol A	$1.9x10^{-7} \pm 1.6$	w	5	√	$6.5x10^{-8}$	$5.0x10^{-7}$	$4.0x10^{-7}$	$2.9x10^{-7}$	EA+
Bisphenol B	(1.3, 1.4) $1.3x10^{-7}$	w	2		$4.6x10^{-8}$	$9.2x10^{-8}$	$2.4x10^{-7}$	$2.1x10^{-7}$	
Butylbenzyl phthalate	$2.8x10^{-6} \pm 0.8$	w	6	√	$6.2x10^{-7}$	EA+	$2.7x10^{-6}$	$4.1x10^{-6}$	$4.7x10^{-5}$
Chrysin	(2.1, 1.3, 1.5) $1.6x10^{-6}$	w	3	√	$6.0x10^{-8}$	EA+	EA+		
Clomiphene citrate	neg	neg	2	√	I	EA+	I		neg
Corticosterone	neg	neg	1		neg	neg	neg	neg	$2.0x10^{-7}$
Coumestrol	$1.3x10^{-8} \pm 0.8$	m	6	√	$1.8x10^{-9}$	$1.6x10^{-8}$	$1.3x10^{-7}$	$2.0x10^{-8}$	$3.3x10^{-8}$
Daidzein	(3.0, 3.2) $3.1x10^{-7}$	w	2		$5.3x10^{-8}$	$4.9x10^{-7}$	$6.8x10^{-7}$	$1.5x10^{-7}$	-
Dexamethasone	neg	neg	2	√	neg	I			$6.3x10^{-5}$
Diethylstilbestrol	$1.2x10^{-11} \pm 1.0$	s	5		$2.9x10^{-12}$	$6.6x10^{-11}$	$2.1x10^{-11}$	$2.0x10^{-11}$	$6.4x10^{-10}$
Estrone	(2.2, 2.7, 2.4, MBP) $2.4x10^{-10}$	s	4	√	$2.7x10^{-11}$	$2.1x10^{-9}$	$2.2x10^{-10}$	$5.9x10^{-10}$	$4.5x10^{-9}$
Ethyl paraben	$3.4x10^{-5} \pm 0.5$	w	5	√	$3.9x10^{-6}$	EA+	EA+		$5.8x10^{-5}$
Fenarimol	(6.3, 4.5, 6.4) $5.7x10^{-6}$	w	3	√	$6.0x10^{-8}$	$7.0x10^{-6}$	$9.2x10^{-6}$		EA+
Flavone	neg	neg	1		neg	I			EA+
Genistein	$2.1x10^{-7} \pm 2.1$	w	14	√	$2.6x10^{-9}$	$6.8x10^{-8}$	$3.0x10^{-7}$	$2.5x10^{-8}$	$3.9x10^{-6}$
ICI	neg	neg	1						
Kaempferol	(1.6, 1.2) $1.4x10^{-7}$	w	2		$6.9x10^{-8}$	$1.6x10^{-7}$	$2.6x10^{-7}$	$1.2x10^{-6}$	neg
Kepone	(3.0, MTP) $3.0x10^{-6}$	w	2	√	$1.7x10^{-6}$	EA+	EA+	$7.7x10^{-6}$	neg
L-Thyroxine	neg	neg	2		I	EA+	neg		
meso- Hexestrol	(1.2, 0.8) $1.0x10^{-11}$	s	2		$1.4x10^{-12}$	$1.0x10^{-10}$	$1.6x10^{-11}$	$2.8x10^{-11}$	
Methyl testosterone	(1.9, 1.4) $1.7x10^{-6}$	w	2		$1.2x10^{-6}$	$1.6x10^{-8}$	$6.5x10^{-7}$	$4.1x10^{-6}$	$7.2x10^{-6}$
Mifepristone	(4.3, 5.8, 3.1, 0.5) $4.4x10^{-7}$	w	4	√	neg	neg	neg		EA+
Morin	(1.6, 1.5, 1.9, 1.3) $1.6x10^{-5}$	w	4	√	EA+		EA+	$4.2x10^{-6}$	
Norethynodrel	(12.6, 7.1, 7.2) $9.0x10^{-10}$	s	4	√	$2.8x10^{-10}$	$6.4x10^{-9}$	$1.3x10^{-7}$	$1.5x10^{-9}$	$1.4x10^{-4}$
p,p'- Methoxychlor	$2.2x10^{-6} \pm 0.6$	w	11		$3.4x10^{-6}$	$5.3x10^{-6}$	$8.4x10^{-7}$		-
p,p'- DDE	(2.1, MTP) $2.1x10^{-5}$	w	2	√	$2.3x10^{-5}$	EA+	I		$9.2x10^{-5}$
Progesterone	neg	neg	2	√	neg	I			EA+
Raloxifene HCl	neg	neg	1		neg	neg	neg		
Sodium azide	neg	neg	1		neg				neg
Spironolactone	neg	neg	2		neg	neg	neg	neg	
Tamoxifen	neg	neg	4	√	I	$5.3x10^{-7}$	$6.7x10^{-8}$		
TPA	neg	neg	1		neg				

The terms strong (EC50 $\leqslant 10^{-9}$ M), medium (EC50 1.0×10^{-9} –1.0×10^{-7} M) and weak (EC50 $\geqslant 10^{-7}$ M) were used as defined by ICCVAM (2011) to describe the agonist responses of test chemicals. The 1st column gives the name of the ICCVAM (2011) test substance or ICI. The 2nd–5th columns give robotic BG1Luc data. The 2nd column gives the mean EC50 and its standard deviation for that test chemical if it had 5 or more independent BG1 robotic assays, or otherwise up to 4 individual EC50s (in parentheses) and their mean. The symbol "neg" means no detectable EA. The 3rd column gives the classification of the test chemical as strong (s), moderate (m), weak (w) or no detectable (n). The 4th column gives the number of times at substance was independently assayed. The 5th column states whether an ICI confirmation assay was run (√) for the EA positive test chemical. The 6th column gives the mean EC50 of MCF-7 assay (Yang et al., 2013). The 7th–10th columns give the published ICCVAM data for meta-analyses (ICCVAM, 2006, 2011), or BG1Luc manual or CERI manual validated assays or the yeast (YES) assay (Sanseverino et al., 2009; Schultis and Metzger, 2004). Symbols are as given for 2nd–6th columns. A blank cell (no symbol or number) means that no data were reported for a BG1Luc or other assays or ICCVAM meta-analyses. "I" means an indeterminate or inadequate classification. Gray-highlighted test substances are those used by ICCVAM to validate the BG1Luc manual assay and also tested using this robotic BG1Luc assay. MTP or MBT mean missing top plateau or missing bottom plateau, respectively, for a concentration–response assay.

3. Results

3.1. Development of a robotized BG1Luc EA assay

To optimize the BG1Luc robotic assay, we initially performed various protocols using BG1Luc cells on ICCVAM EA controls (E2, genistein, and p,p′-methoxychlor) in which we varied the length of treatment (Fig. 1A), the composition of the EFM media (Fig. 1B), and the number of cells seeded per well (Fig. 1C and D) (see methods). In these early optimization experiments, test chemicals were usually run in triplicate, but sometimes in duplicate, for each assay. For this robotic assay, small SD variations are often

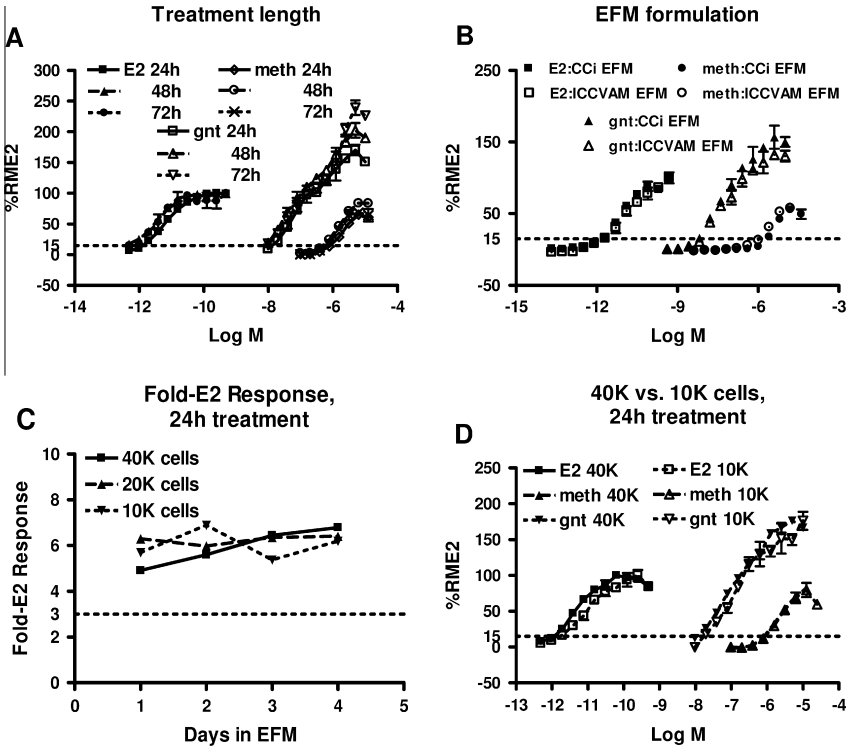

Fig. 1. Development of a robotic BG1Luc EA assay for test substances. Concentration–response curves (A, B, and D) plotted as log concentration in molarity (abscissa) *versus* normalized EA as %RME2 (see methods) or fold E2 response curves (ordinate) (C) for test substances given in the key as labeled in each panel A–D. The normalized vehicle control (VC) was set to 0% and the maximum normalized EA of E2 (positive control) was set to 100% in panels A, B, and D, as determined by E2 dose–response curve run in parallel with each test substance. Dotted line represents VC + 15%RME2 that is greater than 3 standard deviations (SD) of the VC for that experimental run. Each data point represents the normalized EA (%RME2) from three wells. Error bars display SDs for the mean of the three replicates. The SDs are often smaller than the space taken up by the symbol that designates the mean. Panels A, B, and D show multiple assays under different conditions as listed for the test substance, as stated in the key.

within the space taken up by the symbol for the mean in Fig. 1 and subsequent figures showing concentration–response data.

Our data showed that treatment of BG1Luc cells with strong (E2), or weak (genistein, p,p′-methoxychlor) EA test chemicals for 24, 48 or 72 h had relatively little effect on their respective normalized (%RME2) dose–response curves (Fig. 1A). Similar results were obtained using different EFM formulations (Fig. 1B). Furthermore, cell seeding of 96 well plates with 10, 20, or 40 K cells/well had no consistent effect on the maximum fold-response to E2 for 24 h exposures (Fig. 1C) or on EC50 values obtained from dose–response curves for E2, p,p′-methoxychlor, or genistein (Fig. 1D). Given these data, in order to reduce the time and expense required to test each substance, we chose to use 24 h exposure times, to seed 10,000 cells/well, and to use CCi's EFM formulation. Most data presented in this paper are from this latter protocol. Since results from all protocols were qualitatively similar, results from different protocols that met all acceptance criteria are presented in this paper.

Columns 2–5 of Table 1 summarize the data for 44 ICCVAM test chemicals and ICI (column 1) examined using this BG1Luc assay. Assays for all test substances shown in Figs. 1–4 or Table 1 met all three acceptance criteria (see methods). As defined by ICCVAM (2011) for the validation of a manual version of the BG1Luc assay, we used the same terminology and EC50 definition of strong (EC50 $\leqslant 10^{-9}$ M), medium (EC50 1.0×10^{-9}–1.0×10^{-7} M) and weak (EC50 $\geqslant 10^{-7}$ M) to describe the agonist responses of test chemicals.

3.2. Test substances with strong, moderate, or weak EA

As identified in Table 1, seven ICCVAM test substances with an EC50 of $\leqslant 10^{-9}$ M were classified as having strong EA. Fig. 2 shows concentration–response curves for five such test chemicals: the natural hormone E2 (Fig. 2A), 17α-estradiol (Fig. 2A), the synthetic non-steroidal estrogen DES (Fig. 2B) that often binds to ERs with a comparable or greater affinity than E2 (Okulicz and Johnson, 1987), estrone (Fig. 2B), and 17α-ethinyl estradiol (Fig. 2C). Other test substances with strong EA (Table 1) were *meso*-hexestrol and norethynodrel (dose–response data not shown).

Two ICCVAM test substances with an EC50 between 1.0×10^{-9} M and 1.0×10^{-7} M in Table 1 were classified as having moderate EA: coumestrol (Fig. 2C) and 19-nortestosterone (Fig. 2D).

Twenty-one ICCVAM test substances with an EC50 $\geqslant 10^{-7}$ M were classified as having weak EA (Table 1), eleven of which are shown in Fig. 2: 4cumylphenol (Fig. 2D), genistein (Fig. 2E), apigenin (Fig. 2E), bisphenol A (Fig. 2F), diadzein (Fig. 2F), chrysin (Fig. 2G), ethyl paraben (Fig. 2G), fenarimol (Fig. 2H), morin (Fig. 2H), methyl testosterone (Fig. 2I), and p,p′-methoxychlor (Fig. 2I). Ten Other ICCVAM test substances having weak EA were 17β-trenbolone, 4-tert-octylphenol, 5α-dihydrotestosterone, benzophenone, bisphenol B, butylbenzylphthalate, kaempferol, kepone, mifepristone and p,p′-DDE (dose–response data not shown). No EC50 could be calculated for 17β-trenbolone (Table 1) because of toxic effects at higher concentrations that prevented a definitive determination of the concentration reached at its top plateau.

This robotic BG1Luc assay typically showed high data reproducibility as illustrated by EC50 values having small SDs or variation as given in column 2 of Table 1. For example, for 32 different assays, the mean EC50 and its SD for E2 (a test substance with strong EA) was $5.5 \pm 2.6 \times 10^{-12}$ and $1.3 \pm 0.8 \times 10^{-8}$ for 6 assays of coumestrol, a test substance with moderate EA. This high reproducibility held even for most test substances with weak EA. For example, the EC50 for butylbenzyl phthalate was $2.8 \pm 0.8 \times 10^{-6}$ ($n = 6$),

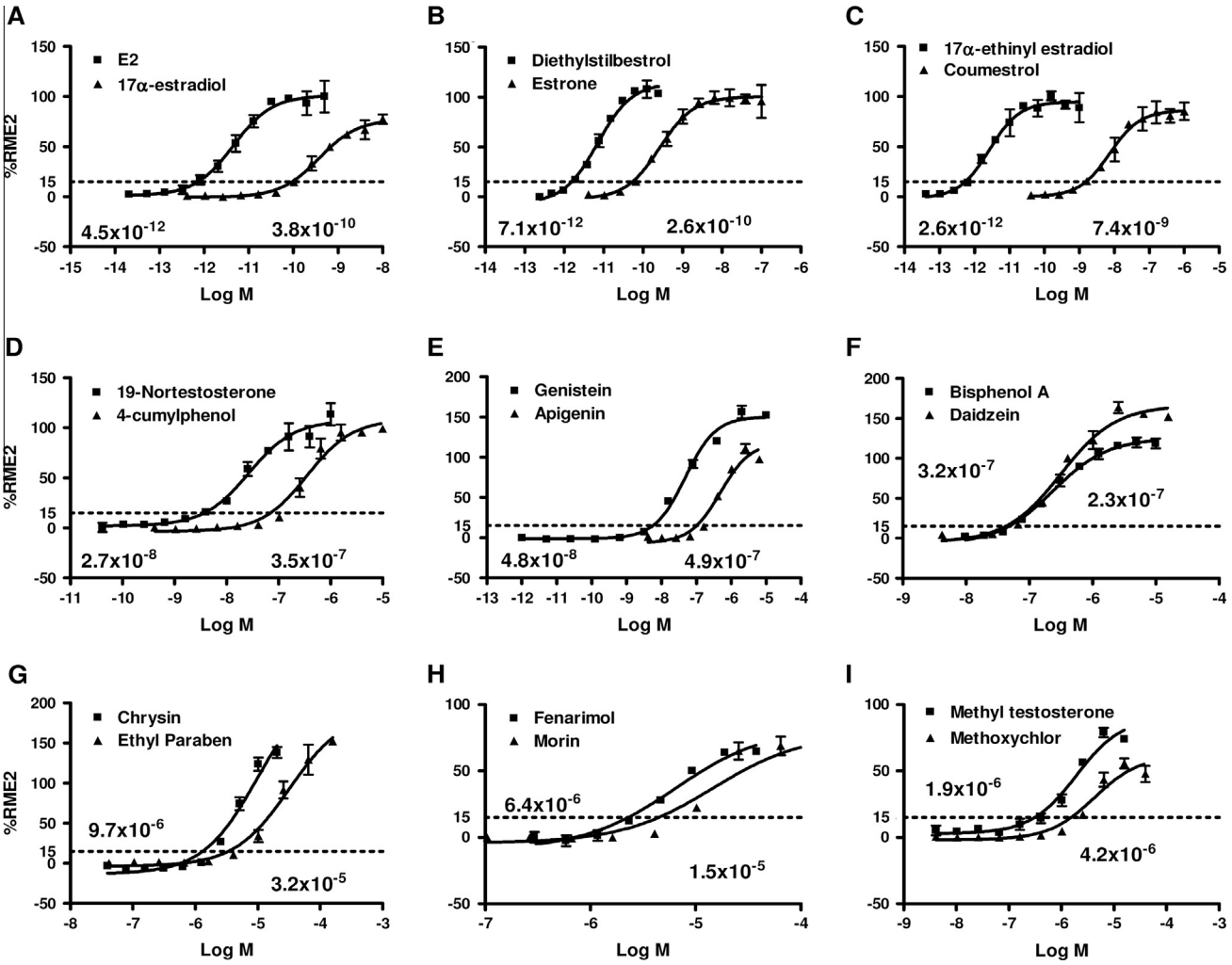

Fig. 2. Concentration–response curves of test chemicals with strong, moderate and weak EA. Data in this figure graphed as described for Fig. 1. Graphed data in panels show EA for E2 and 17α-estradiol (panel A) and diethylstilbestrol (panel B) having strong EA, estrone (panel B) and coumestrol (panel C) having moderate EA, and 19-nortestosterone and other test chemicals having weak EA as given in the key to each panel D-I.

ethyl paraben was $3.4 \pm 0.5 \times 10^{-5}$ ($n = 5$) and p,p′-methoxychlor was $2.2 \pm 0.6 \times 10^{-6}$ ($n = 11$).

3.3. Test substances with no detectable EA

Fig. 3A–F graphs concentration–response data for 7 test substances that showed no detectable EA (<15%RME2) at any concentration: raloxifene (Fig. 3A), atrazine (Fig. 3A), corticosterone (Fig. 3B), ICI 182,780 (Fig. 3C), clomiphene citrate (Fig. 3D), L-thyroxine (Fig. 3E) and tamoxifen (Fig. 3F). As shown in Table 1, eight other of the 45 test substances examined were classified as EA-negative: 4OH-Tamoxifen, ammonium perchlorate, dexamethasone, flavone, progesterone, sodium azide, spironolactone, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). As described above for test substances with positive strong, moderate or weak EA, assays for test substances classified as negative were repeatable. For example, 4-hydroxy tamoxifen ($n = 5$), atrazine ($n = 4$), coumestrol ($n = 6$), tamoxifen ($n = 4$), and other test substances consistently tested EA-negative.

3.4. EA confirmation by the ER antagonist ICI on BG1Luc assays

ICI is often considered to have purely anti-estrogenic effects (Wakeling et al., 1991; Parker, 1993) and used to confirm that an EA agonist response is indeed due to agonist ER binding (ICCVAM, 2006, 2010; Yang et al., 2011, 2013). Fig. 4 shows the effects of 10^{-8} M ICI on BG1Luc dose–response curves for a strong (E2: Fig. 4A), a moderate (norethynodrel: Fig. 4B), and a weak (butylbenzl phthalate: Fig. 4C) ICCVAM test substance. ICI greatly reduced the agonist response for these test substances and others listed in column 5 of Table 1. For extracts of cosmetics or plastics (see Fig. 5 below), ICI also always suppressed BG1Luc agonist response of these complex mixtures of chemicals whose identity is not known to us (data not shown).

3.5. Ability to detect EA in complex mixtures of substances

We examined the ability of this robotic BG1Luc assay to detect chemicals with EA in complex mixtures of substances such as extracts of personal care products (hair or body lotions) or plastic resins or products (Fig. 5). Extracts of two body lotions creams and two hair recently reported to contain chemicals with EA using MCF-7 cell proliferation assays (data not shown) exhibited EA when tested using this robotic BG1Luc assay (Fig. 5A). Similarly, extracts of plastic resins or products (Fig. 5B) also exhibited EA in this robotic BG1Luc assay. These products have been reported to have EA in other studies using MCF-7 assays (Howdeshell et al., 2003; Yang et al., 2011) or *in vivo* assays for bisphenol A-based polycarbonate plastics (Howdeshell et al., 2003).

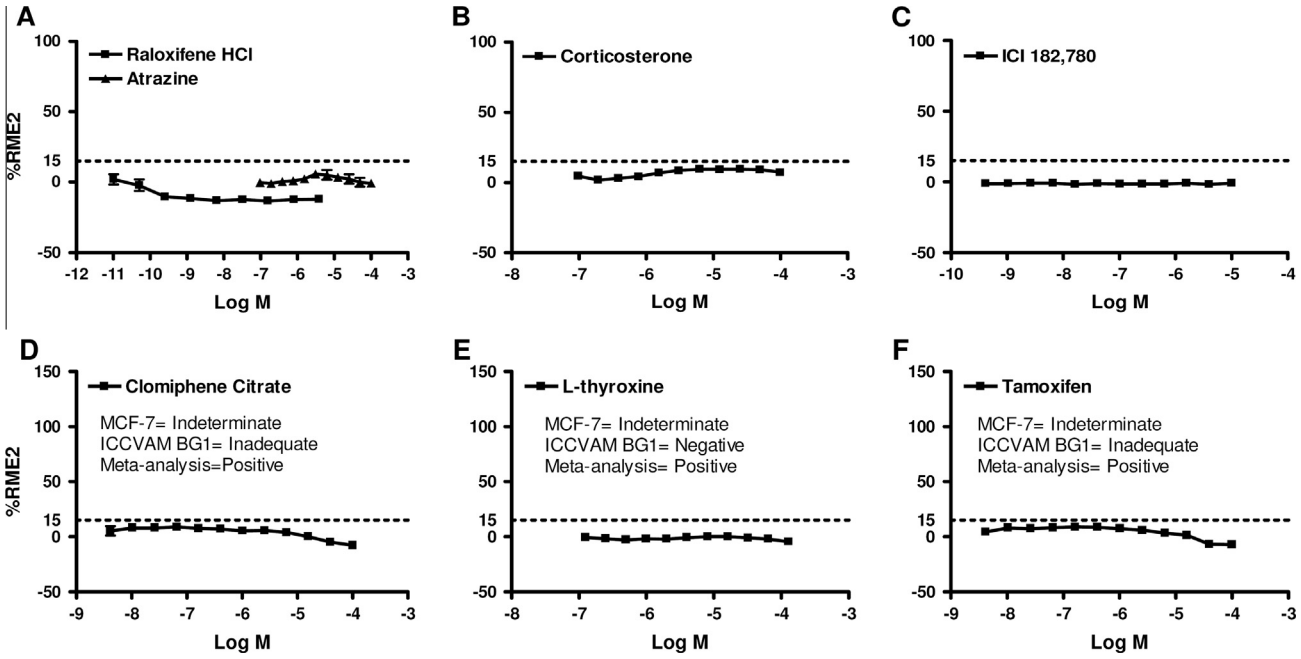

Fig. 3. Concentration–response curves of test chemicals having no detectable EA in robotic BG1Luc assays. Data graphed as described for Fig. 2. Keys in panels D–F give classifications of listed substances in other EA assays (see Table 1).

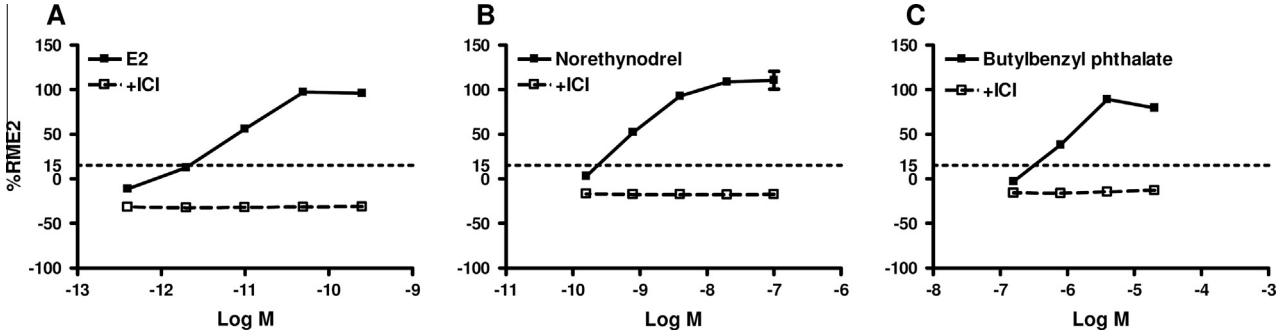

Fig. 4. EA and EA confirmation Assays for EA positive test chemicals in robotic BG1Luc assays. Data graphed as described for Fig. 3. Panels show suppression of EA by 1×10^{-8} M ICI (+ICI) for test chemicals listed in key having strong (A), moderate (B) or weak EA (C).

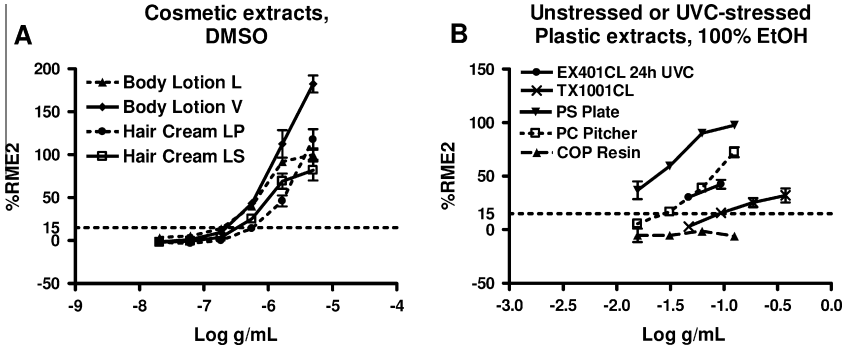

Fig. 5. (A and B) Dilution–response curves from robotic BG1Luc assays for EA for extracts of personal care and plastic products. Data graphed as described for in Figs. 1–3. Panel A shows data from assays for each extract of four personal care products listed in the key. Panel B shows data from assays for each extract of three plastic resins and two products listed in the key. Plastic extracts are from hard and clear plastic resins and products as follows: EX401CL and TX1001CL are different grades of Tritan™ resins; polystyrene (PS) cell culture plate; polycarbonate (PC) beverage pitcher; and cyclo-olefin polymer (COP) resin.

4. Discussion

4.1. Development of a robotic BG1Luc assay for EA

Data shown in Figs. 1–5 demonstrated that this robotic BG1Luc assay appropriately detected EA in strong, moderate and weak ICCVAM test chemicals and in chemical mixtures previously reported to exhibit EA. The assay did not find EA in ICCVAM test chemical that were not known to have EA. This BG1Luc assay also showed high repeatability when run according to the developed protocols. Compared to the manual version of the BG1Luc assay (ICCVAM, 2011), this robotic version is more repeatable and sensitive, and at least as accurate, as described below.

4.2. Qualitative accuracy of this assay versus ICCVAM meta-analyses

A total of 44 ICCVAM test substances were examined using this robotic BG1Luc assay. Data on the EA of these test substances have previously been reported by ICCVAM meta-analyses, an ICCVAM-validated manual BG1Luc assay and by ICCVAM analyses of the CERI assay validated by the OECD (ICCVAM, 2003, 2006, 2011; NIEHS, 2011). Columns 7–10 of Table 1 show previously reported data for test substances categorized as EA positive (EA+) or with their EC50s, Inadequate (I), or negative for each of these data sets as reported. Not all test chemicals have been analyzed for EA in each data set, represented by a cell with no symbol or numerical value in Table 1.

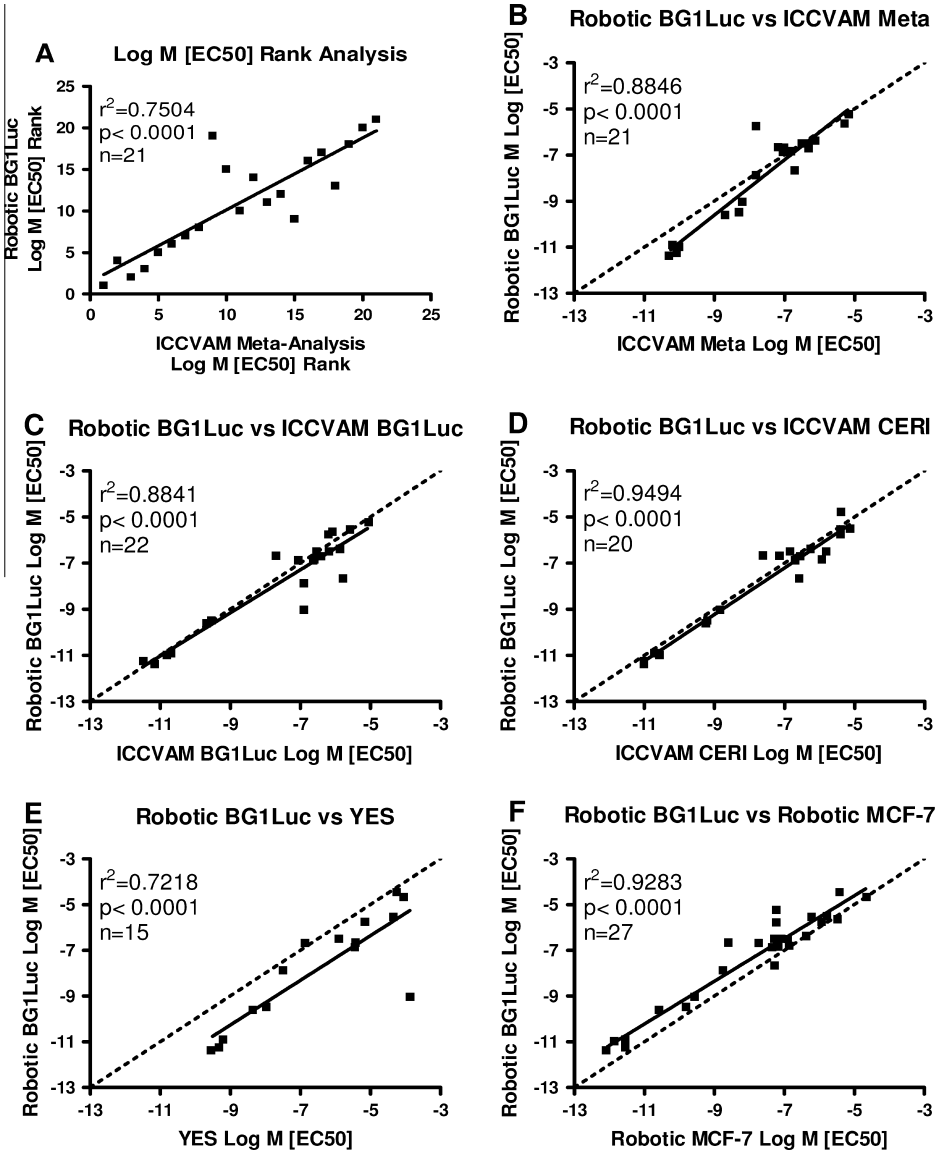

Fig. 6. Scatter plot comparisons of mean EC50s from robotized BG1Luc *versus* meta-analysis data or data for other assays taken from (ICCVAM (2003, 2006, 2011); also see Yang et al., 2013). In all panels, BG1Luc EC50s from Table 1 plotted on ordinate, EC50s in Table 1 from other assays as given in each panel plotted on abscissa. In all panels, the solid line is the best-fit regression line, r^2 is the regression correlation coefficient, p is the significance level of the regression correlation for that panel. In panels B–F, a data point on the dotted line means equal sensitivity for the two EC50s. A data point below the dotted line means that the BG1Luc assay is the more sensitive of the two EC50s. Scatter plots graph: Panel A: rank-order of 21 mean robotic BG1Luc EC50s *versus* median EC50s rank-order of the same test substances from ICCVAM (2011) meta-analyses. Panel B: 21 mean robotic BG1Luc EC50s *versus* median EC50s of the same test substances from ICCVAM (2011) meta-analyses. Panel C: 22 mean robotic BG1Luc EC50s *versus* median EC50s of the same test substances from ICCVAM (2011) manual BG1Luc assay. Panel D: 20 mean robotic BG1Luc EC50s *versus* mean EC50s of the same test substances from ICCVAM (2011) manual CERI assay. Panel E: 15 mean robotic BG1Luc EC50s *versus* mean EC50s of the same test substances from yeast (YES) assay (Sanseverino et al., 2009; Schultis and Metzger, 2004). Panel F: 27 mean robotic BG1Luc EC50s *versus* mean EC50s of the same test substances from Yang et al. (2013) robotic MCF-7 assay.

ICCVAM used 35 of 78 test substances to assess the qualitative accuracy of the BG-1Luc ER transcriptional activation assay in manual format *vs.* its most-recent meta-analysis data (ICCVAM, 2006, 2011). We have tested 27 of these 35 test substances (gray-highlighted in Table 1) having EA positive or negative assessments for congruence between this robotic BG1Luc assay and their ICCVAM meta-analysis. This robotic BG1Luc EA assay had 100% concordance (accuracy) with 0% false positives and 0% false negatives for the 27 ICCVAM reference test substances for which ICCVAM meta-analyses and this robotic assay report positive or negative classifications.

4.3. Problematic test substances

We have classified three chemicals as negative for EA, two of which (clomiphene citrate, tamoxifen) were similarly classified as "inadequate" for EA using the ICCVAM manual BG-1Luc assay. ICCVAM validation studies by XDS (Xenobiotic Detection Systems, Inc., Durham, North Carolina, USA), JaCVAM (Japanese Center for the Validation of Alternative Methods, Hiyoshi Coperation, Japan), and ECVAM (European Centre for the Validation of Alternative Methods, Ispra, Italy) have also reported that EA assays of tamoxifen were inadequate for determining its EA (ICCVAM, 2011). As previously reported (Yang et al., 2013), tamoxifen has been classified as "+, inconclusive (I), and +" and clomiphene citrate classified as "no relevant data were identified (n.d.)" until 2011 (ICCVAM, 2003, 2006, 2011). L-thyroxine was twice classified as negative for EA and twice as "n.d." by ICCVAM (2003, 2011).

We suspect that these negative classifications in the robotic BG1Luc are not inaccurately reporting what should be positive EA classifications. For example, the "no detectable EA" criteria for the ICCVAM BG1Luc manual assay was set at 20%RME2 (compared to our 15%RME2) in part because of the much greater variability (less reproducibility) for assays of these and some other chemicals in the ICCVAM validation process. Furthermore, some test substances classified as indeterminate or inadequate in BG1Luc, MCF-7, CERI and other assays may have tissue-specific EA or may be specific for certain concentrations of a chemical with EA (Barkhem et al., 1998; ASRM, 2006; ICCVAM, 2011). As one example, tamoxifen has been reported to be an ER agonist in the endometrium and an antagonist in breast tissue (Barkhem et al., 1998). As other examples, clomiphene citrate exhibits EA at lower, but not higher, levels of endogenous estrogens (Clark and Markaverich, 1981; ASRM, 2006). L-thyroxine has EA in rat pituitary adenomas and human cervical carcinoma (HeLa) cells, indeterminate (Yang et al., 2013) or negative (Takeyoshi, 2004) in human breast adenocarcinoma (MCF-7) cells.

In brief, we recognize that a test chemical response using a given cell line in an *in vitro* assay may have a different response if another cell line is used, and that *in vivo* responses to test chemicals have yet-more-variables and complications.

4.4. No false EA positives due to non-specific effects

Non-specific activation of BG1Luc cells was not observed in any assay for 17 test substances with EA that were confirmed with ER antagonist ICI (Table 1), i.e., ICI greatly reduced the their agonist response (Table 1 and Fig. 4). A similar result was obtained for other chemicals with EA that are not ICCVAM test chemicals and for over a hundred chemical mixtures in various commercial products assayed to date. Hence, a BG1Luc ICI Confirmation Assay is not absolutely necessary to determine if a chemical has EA using this BG1 robotic assay. A BG1Luc ICI Confirmation assay does provide additional evidence that a BG1Luc assay classification that a substance "has EA" is indeed correct.

4.5. Comparisons with other assays

As discussed above, this robotic BG1Luc assay had high (100%) concordance for the presence or absence of detectable EA with ICCVAM meta-analyses for 27 test chemicals. When chemicals tested in common by both assays are compared (Table 1), this robotic BG1Luc assay has 100% concordance with the ICCVAM manual BG1 assay for 27 test chemicals, 100% concordance with CERI for 20 test chemicals, and 100% concordance with a robotic MCF-7 assay for 27 test chemicals. In contrast, Table 1 shows that the yeast estrogen screening (YES) assay has only 47% (7/15) concordance with any of these other assays for 15 test chemicals, having three false positives (ammonium perchlorate, corticosterone, and progesterone) and five false negatives (apigenin, daidzein, kaempferol, and kepone, and p-p′-methoxychlor) (Schultis and Metzger, 2004; Sanseverino et al., 2009) when compared with ICCVAM (2011) meta-analysis data, MCF-7 and BG1Luc assays (see Table 1).

When sensitivities of these different assays are compared to detect the EA of the same test chemical as defined by its EC50, this robotic BG1Luc assay is more sensitive for 15/20 and one tie out of 21 chemicals reported by ICCVAM meta-analyses (Fig. 6B), i.e., is more sensitive ($p < 0.001$, Chi Squared test) for 15 chemicals whose EC50s can be directly compared. Compared to ICCVAM BG1 manual data for 22 chemicals Fig. 6C), the robotic BG1Luc assay is more sensitive for 14/22 ($p < 0.0.001$). Compared to CERI manual assays (Fig. 6D), the robotic BG1 is more sensitive for 18/20 test chemicals ($p < 0.0001$). Compared to the YES assay, the robotic BG1 assay is more sensitive ($p < 0.0001$) for 15/15 chemicals whose EC50s can be directly compared. In contrast, with respect to the robotic MCF-7 assay as reported for ICCVAM validation results (Yang et al., 2013), the BG1Luc is more sensitive for only 4/27 chemicals whose EC50 can be directly compared (Table 1), i.e. the MCF-7 assay is more sensitive with a high significance ($p < 0.0001$) compared to this robotic BG1Luc, ICCVAM manual BG1Luc, CERI, YES and ICCVAM EC50 meta-analysis EC50s.

In a scatter-gram plot (Fig. 6A), the rank order of EC50s obtained with this robotic BG1Luc assay correlated well (fairly high r^2 value of 0.75, $p < 0.0001$) with the rank order of EC50s reported for ICCVAM meta-analyses (ICCVAM, 2011). Although usually more sensitive, the EC50s obtained by this robotic BG1Luc assay were very well correlated with EC50s reported in ICCVAM meta-analyses (Fig. 6B: $r^2 = 0.89$), ICCVAM manual BG1Luc assays (Fig. 6C: $r^2 = 0.88$) and CERI manual assays (Fig. 6D: $r^2 = 0.95$). CERI mean EC50s have a higher regression score vs ICCVAM meta-analysis median EC50 data (Fig. 6D) compared to robotic BG-1Luc assays (Fig. 6B) in part because CERI data from an OECD validation study were used to calculate the ICCVAM (2011) meta-analysis data (Yang et al., 2013). In contrast, YES assay EC50s do not correlate as well with ICCVAM median meta-analysis data (not shown) or robotic BG1Luc EC50 data (Fig. 6E: $r^2 = 0.72$). Robotic BG1Luc EC50 values correlated well with robotic MCF-7 EC50 values (Fig. 6F: $r^2 = 0.93$), although MCF-7 EC50s were usually lower (more sensitive assay) compared to BG1Luc EC50s, as described above.

5. Conclusion

Considering all these data, we conclude that BG1Luc EA assays to detect EA provide repeatable, reproducible, sensitive (as defined by EC50s) and accurate results in high concordance ICCVAM meta-analyses. That is, this robotized BG1Luc EA assay has accuracy, sensitivity and specificity values at least equivalent to, and often exceeding, validated test methods accepted by the US ICCVAM and EPA and the EU OECD.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This work was supported by the following NIH Grants: R44 ES011469, 01-03 (CZY) and 1R43/44 ES014806, 01-03 (CZY).

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