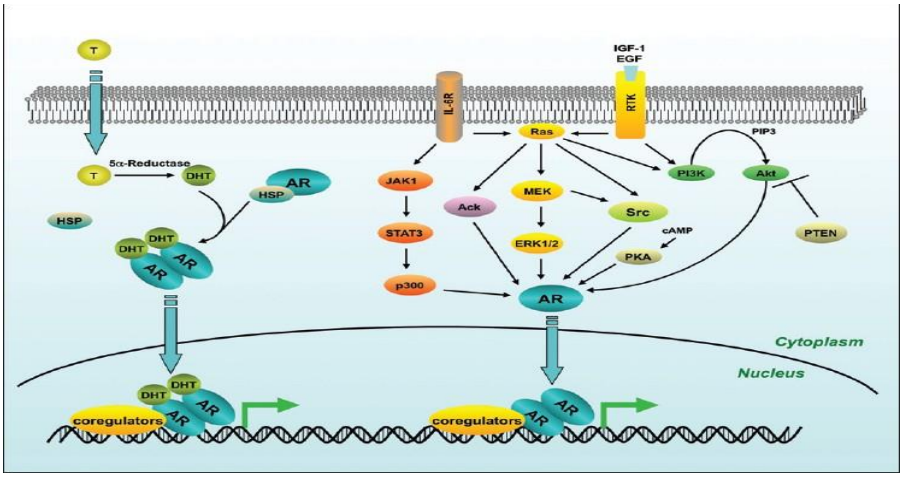
Overview of EDCs having androgenic or anti-androgenic activity (AnA)**

Endocrine disrupting chemicals (EDCs) mimic or otherwise alter the *in vitro* and/or *in vivo* activities of hormones such as estrogens and androgens. Many chemicals used in the manufacture of household products act as agonists or antagonists of androgenic or estrogenic hormones, while other chemicals interfere in multiple ways with the action of thyroid or other hormones in laboratory mammals as model systems. Similar EDC effects are almost certainly produced in humans, since basic endocrine mechanisms have been highly conserved across all classes of vertebrates. EDCs having AnA**can produce abnormal physical and/or behavioral effects at low (ppb to ppt) concentrations, particularly when exposure occurs during critical stages of development, from early fetal stages through puberty.

Mechanisms by which chemicals produce AnA and Anti-AnA

The mechanisms of action of androgenic and anti-androgenic EDCs are shared with natural androgens. These include affecting the actions of androgen receptors (ARs) and other members of the NR3C4 nuclear receptor superfamily as shown in **Figure 1**. Upon binding to dihydrotestosterone (DHT) the androgen receptor (AR) translocates to the nucleus, binds to its target genes and regulates their expression. ARs can also be transactivated in the absence, or in very low levels, of DHT by signals arising from several, non-mutually-exclusive mechanisms including extracellular peptides such as Insulin-like growth factor (IGF), Epidermal growth factor (EGF) and Interleukin-6 (IL-6). Thus, ARs bind a wide variety of natural and synthetic EDCs and, via parallel pathways, activate transcription of androgen-responsive genes, leading to synthesis of various proteins. Anti-AnA is produced by EDCs that bind to, but do not activate, ARs. Anti-AnA effects may be produced, in theory, by competitive inhibitors that bind to ARs but do not activate them. Furthermore, selective AR modulators (SARMs) bind to ARs, but subsequently activate cellular responses that differ from those activated by the endogenous androgens**.** It is also possible for a chemical to bind directly to an endogenous hormone, and thereby reduce its effect. Most chemicals that bind to ARs produce some effect on AR activation, either as a classical androgenic/anti-androgenic effect, or as a SARMs.

Figure 1



MDA-Kb2 Assay for AnA:** The stable MDA-Kb2 cell line is derived from MDA-MD-453 and was obtained from Dr. Wilson at the EPA. These MDA-Kb2 cells have androgen (AR) and glucocorticoid (GR receptors, but not progesterone (PR) or other nuclear receptors (e.g., ER) that might act through an MMTV promoter to mediate transcription of the luc gene to produce luciferase. Following guidelines established by ICCVAM AR TA assays, CCi has developed MDA-Kb2 AnA** assays in a novel robotized format (including a Confirmation Assay) to increase number of assays run simultaneously, reduce cost, and increase accuracy and repeatability. We recognize that the MDA-KB2 cell line has both androgenic (AR) and glucocorticoid receptors (GR) and that we cannot always distinguish between the two without Confirmation Assays.

1: Overview of the assays: The protocols for CCi's AnA** assays are modifications of our protocols developed in consultation with ICCVAM/NICEATM for MCF-7 and BG1-Luc validation. The AR transcriptional activation (TA) assays consist of several separate determinations. Test Substances are first tested for solubility (the highest soluble concentration in EtOH), then volatility. A Range Finder Assay is then conducted to determine the concentrations over which the test substance is active. A Comprehensive Assay is then conducted using concentrations determined by the Range Finder Assay and compared to DHT, a strong androgen used as the reference chemical for AnA similar to the use of 17β-estradiol for EA assays. A Confirmation Assay using the pure anti-androgen vinclozolin (Vinc) as the reference strong anti-androgen is then conducted on substances that appear to be "potentially positive" for AnA. [ICI-181,280 has a similar role

in anti-EA assays.] A Confirmation Assay is also conducted on substances that appear to be "potentially positive" for Anti-AnA.

2: Cell maintenance: MDA-Kb2 cells are maintained in cell maintenance medium that consists of Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 100U/mL penicillin (Invitrogen), 200μg/mL streptomycin (Invitrogen), and 0.25mg/mL amphotericin B (Invitrogen) in an incubator set at 37ºC without supplemental CO_2.

3: Preparation of Test Substances and solutions: Test solutions are prepared in a 96-well deep-well plate as follows: 8 chemicals in 100% EtOH are dispensed in the 1st column of a deep well plate that contains 1 part stock solution in 100% EtOH and 99 parts CMM (final EtOH concentration is 1%). The volume in the 1st column of the deep well plate (V in 1st column) is equal to the final volume (FV) multiplied by the dilution factor (DF) and divided by the dilution factor minus one (=FV*DF/(DF-1). The other columns of the DWP contain 1% EtOH diluted in CMM. The volume transferred from the higher concentration to the next concentration is equal to V in 1st column divided by the dilution factor. The EtOH concentration in all testing concentrations is therefore always 1% in any of the serial dilutions. Test solutions prepared for 384 well plates use similar concepts, but different volumes.

4: Volatility test: Prior to running Range Finder assays described below in this study, the volatility of Test Substances is determined to help design Range Finder, Comprehensive and Confirmation Assays described below to avoid cross-contamination. Stock test substance solution is diluted 100X in cell maintenance media in an Eppendorf tube (e.g., 5uL of stock chemicals in 495 uL of cell maintenance media). 100uL or 25uL of the solution is then placed in wells of 96- or 384-well plates surrounded by wells containing the vehicle control (VC: 0.5% EtOH). If the AnA in VC wells adjacent to the test substance is 20% greater than the average of VC wells far away from the test substance, the extract is classified as volatile and is tested separately from other non-volatile test substances.

5. Dilution factor and testing concentrations. Range Finder Assays typically have 8 testing concentrations with a dilution factor of 10, and Comprehensive Assays 10 testing concentrations with a dilution factor of 2.5. The dilution factor is typically 2.5 for a Confirmation Assay consisting of 5-6 testing concentrations.

6: AR Agonist Assay (AnA Assay): MDA-Kb2 cells are seeded in cell maintenance media onto 96-well plates or 384-well plates at 10,000 or 5,000 cells per well in 100uL or 25uL cell maintenance media, respectively, for 20±4 hours without CO_2. 100uL or 25uL testing solutions prepared in a deep well plate are added in duplicate in Range Finder Assays, triplicate in Comprehensive and Confirmation Assays for an additional 20±4 hours at 37ºC. The medium is removed from the plates and 24uL (for 96-well plates) or 8uL (for 384-well plates) of luciferase lysis buffer (Promega kit) is added for at least 2 minutes to lyse cells. 75uL (96-well plates) or 24uL (384-well plates) of luciferase assay buffer (Promega kit) is added before reading the relative luminescence units (RLU) in each well for 1 minute in a luminometer. The RLUs/well are exported in Excel format. The AnA of a Test Substance relative to a strong androgenic reference substance (DHT) is calculated according to **Equation 1** below:

Equation 1: $$\textbf{\%RMDHT}_{\textbf{TS}} \text{ of a Test Substance} = 100\% \times \frac{\mathrm{MaxRLU_{TS}} - \mathrm{RLU_{VC}}}{\mathrm{MaxRLU_{DHT}} - \mathrm{RLU_{VC}}}$$

That is, the AnA of a Test Substance (**$\%RMDHT_{TS}$**) is calculated relative to the maximum AnA of the positive control DHT, both RLUs for Test Substance and for DHT are corrected by the RLU of the Vehicle Control (VC). The $\mathrm{MaxRLU_{TS}}$ or the $\mathrm{MaxRLU_{DHT}}$ is the maximum AnA agonist response (greatest RLU) for any concentration of the Test Substance or DHT concentration. **$\%RMDHT_{TS}$** is a standard measure of AR response amplitude. To plot the data, the highest normalized DHT response is set to 100% and the VC response to 0%.

For an AnA assay to be acceptable, the following three criteria has to be met: 1) The DHT RLU response has to be at least 3x greater than the assay VC RLU. 2) The "sham control" (solvent that went through all the steps for extracts) has to be less than 15%RMDHT, and 3) The DHT positive control concentration-response curve has to have a positive slope, preferably with at least three data points on its ascending linear portion. For Test Substances (pure chemicals), the EC50 (in M) is calculated from a sigmoidal best fit to dose-response data using GraphPad Prism software.

If a Test Substance $\%RMDHT_{TS}$ has an agonist response that is 3 SD greater than the VC, then the Test Substance is characterized as AnA "potentially positive" and an AnA Confirmation Assay is performed by co-incubating the Test Substance with the AnA antagonist reference chemical (Vinc) at $1.0x10^{-6}$ M. A Test Substance is classified as having no detectable AnA if it does not induce a response > 3SD of the assay VC in any testing concentration, or if it induces a response that cannot be significantly ($p < 0.05$) inhibited at least 30% by a Vinc Confirmation Assay criteria similar to those described for our MCF-7 assay published in collaboration with scientists from ICCVAM (63). For example, dexamethasone is a GR agonist that stimulates luciferase expression in MDA-Kb2 cells (apparent AR agonist response) that is NOT inhibited by Vinc (see **Fig 6B**). Test substances are interpreted as positive for AnA if the calculated %RMDHT for at least two testing concentrations is significantly reduced by at least of 3SD of VC by the co-incubation with Vinc at $1.0x10^{-6}$ M.

7: AR Antagonist Assay (Anti-AnA Assay): MDA-Kb2 cells are seeded and treated as described in AR Agonist assays, except that all concentrations of a Test Substance are co-incubated with a "low dose" of $2x\ 10^{-10}$ M DHT (**LDHT**), a non-saturating DHT concentration that is the EC80 of DHT. That is, in AR antagonist assays, Test Substances are examined for their ability to reduce the agonist response by LDHT. The relative anti-AnA of a Test Substance is calculated using **Equation 2**

Equation 2: $\%RMVinc_{TS\ in\ LDHT}$ of a Test Substance = $\dfrac{(RLU_{LDHT} - RLU_{TS\ in\ LDHT})}{(RLU_{LDHT} - RLU_{Max\ Vinc\ in\ LDHT})}$ X 100%

$\%RMVinc_{TS\ in\ LDHT}$ of a Test Substance is the relative (%) reduction of the LDHT agonist response with respect to the maximum reduction of LDHT agonist response produced by the reference antagonist (Vinc). The normalized inhibition produced by a Test Substance is 100% if its inhibition of LDHT response equals that of Vinc. For an Anti-AnA Assay to be acceptable, all acceptance criteria for the AnA Assay need be met. In addition, the Vinc concentration response curve had to have a negative slope and Vinc had to reduce the LDHT response by at least 50% for at least two testing concentrations. For the Comprehensive Assay, Test Substances were classified as "potentially Anti-AnA positive" if their concentration-response curve consisted of a top plateau followed by a negative slope. Test Substances eventually classified as Anti-AnA positive preferably have at least two data points on the negative slope with non-overlapping error bars.

If the $\%RMVinc_{TS\ in\ LDHT}$ for a Test Substance is determined to be "potentially Anti-AnA positive" in an Anti-AnA Comprehensive Assay, it is further tested in an Anti-AnA Confirmation Assay by co-incubating the Test Substance with a 100X higher (saturating) concentration of DHT ($2.0x10^{-8}$ M, aka High DHT or HDHT). This Confirmation Assay distinguishes Anti-AnA effects from other non-specific reductions of the LDHT agonist response, e.g., a cytotoxic effect or general transcription inhibition. Acceptance criteria for Anti-AnA confirmation assay are as described for the Anti-AnA Assay and the recovery of the Vinc-induced reduction of LDH agonist response when adding HDHT has to be greater than 3xSD of LDH for at least two testing concentrations.

Figure 2 shows that 96 well robotic data are very similar (repeatable, reproducible) for multiple agonist Comprehensive Assays of reference chemical (DHT) (**Fig. 2A**) or antagonist assays of reference chemical (Vinc) (**Fig. 2B**) and other Test Substances from comprehensive agonist and antagonist assays, respectively: **Table 1** shows that EC50s for DHT obtained from all three formats are similar and repeatable.

Consistency of pipetting accounts for some of the consistency of our data within and between formats. For example**, Figure 3 shows** that the relative luminescence units (RLUs) in different wells of a 96-well plate in manual format and a 384-well plate in robotic format have very little variation between wells**. Figure 4 A** shows that both manual and robotic formats give similar concentration-response curves for an agonist reference chemical DHT. **Figure 4B** shows a similar conclusion for an antagonist reference chemical (Vinc) in both manual and robotic formats.

Our data shows that Vinc is a purer AnA antagonist than OHF (**Fig 5D**). The dose-response curve for Vinc has a monotonic negative slope in our Anti-AnA assay (**Fig. 5D**), indicating that Vinc is a pure AR antagonist. However, OHF has a slope reversal (**Fig. 5B**), indicating that OHF is a partial AR antagonist and agonist. In addition, OHF is also a partial AR agonist (**Fig. 5A**). Some previous studies have used OHF for AR antagonist reference chemical in AR antagonist assays. However, CCi's data show that Vinc is a better choice than OHF as an Anti-AnA positive control and for the AnA confirmation Assay.

For all our agonist and antagonist protocols, we find that the inclusion of a Confirmation Assay is essential in reaching any determination that a Test Substance (or mixture of chemicals) has agonist and/or antagonist activity. For example, **Figure 6A** shows that the agonist induction of luciferase by three concentrations of DHT is reduced to near-background levels by the co-incubation of Vinc at 10^{-6}M. Hence DHT is confirmed as a strong AR agonist. In contrast, **Figure 6B** shows that dexamethasone (Dexa)-induced luciferase expression was **not** reduced by Vinc at 10^{-6}M, i.e., dexamethasone is not an AR agonist, even though it has previously been reported to be an AR agonist in the absence of a Confirmation assay (29,62). As additional examples of the theoretical and practical importance of our Confirmation Assays, **Figure 6C** shows that 5 of 6 dilutions of BPA reduces the agonist effect of $2x10^{-10}$M DHT that is in turn reversed by $2x10^{-8}$M DHT, i.e., BPA is confirmed as having anti-AnA. In contrast, **Figure 6D** shows that dilutions of Actinomycin D (ActD: a general transcription inhibitor(14), reduce the agonist effect of $2x10^{-10}$M DHT, that is **not** reversed by $2x10^{-8}$M DHT, i.e. Act D is **not** an AR antagonist.

Figure 2: Repeatability of AR assays for reference chemicals

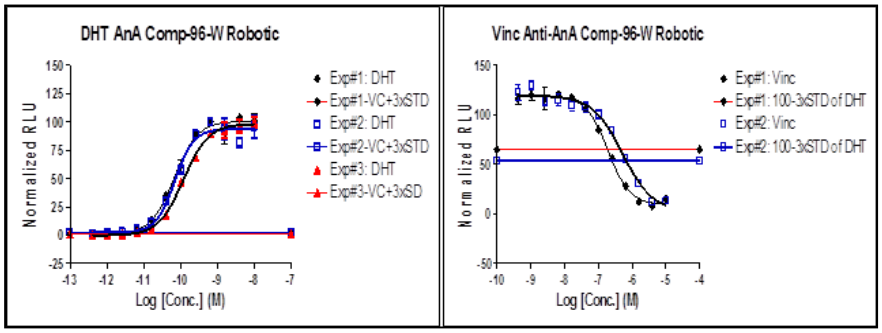


Figure 3: Pipetting Consistency

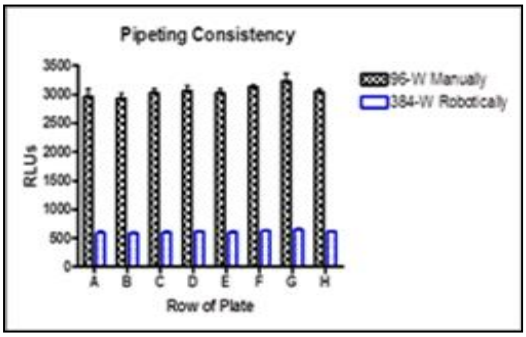


Figure 4: Agonist and antagonist AnA assays in manual (M) and robotic (R) formats

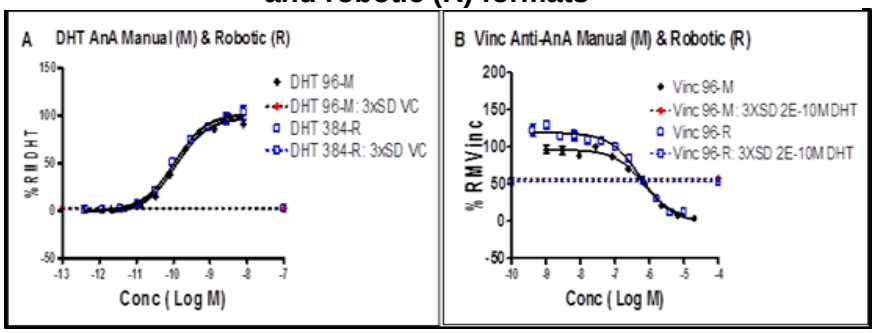


Table 1: Summary of EC50s of DHT in Three Assay Formats

Assay Format	Mean (M)	SD	# of Exp
EC50 0f DHT in 96-well plates (Robotic)	1.8E-10	7.0E-11	7
EC50 0f DHT in 384-well plates (Robotic)	1.3E-10	4.9E-11	8
EC50 0f DHT in 96-well plates (Manual)	1.2E-10	9.3E-12	2

Figure 5: Vinc is a purer reference antagonist

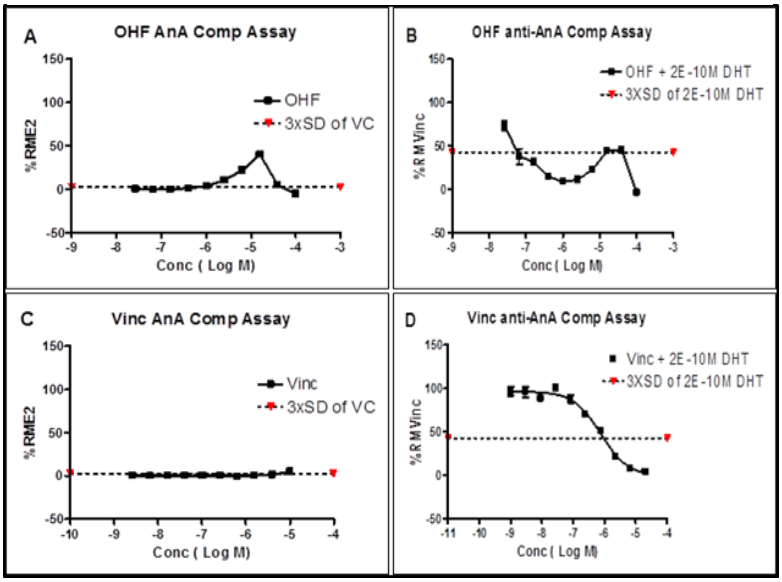
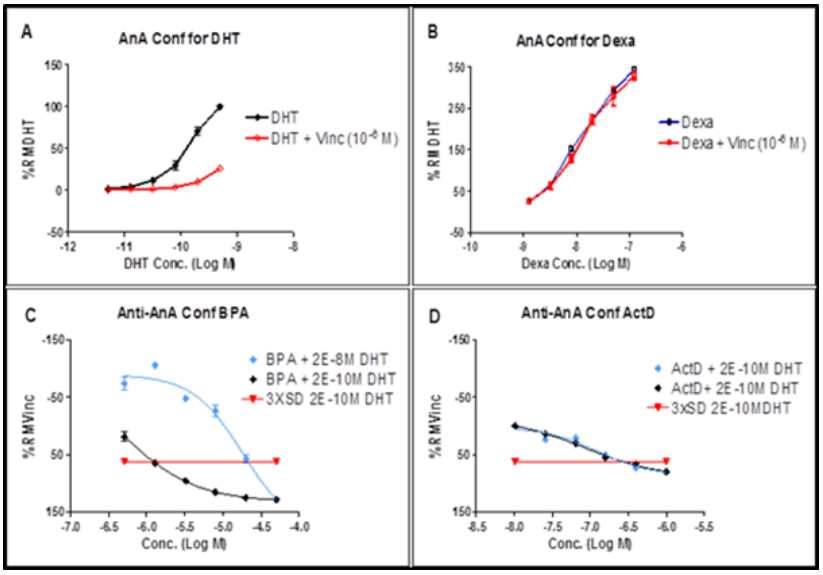


Figure 6: AnA and Anti-AnA Confirmation Assays



Comparison of our robotic AnA assays (*in italics*) with applicable ICCVAM (*28-30*) Standards for Transcriptional Activation (TA) assays AnA** (in normal typeface):**

1) Assays using cells (e.g., MDA-MB-453) with an endogenous AR that has been transduced with an adenovirus carrying a Luc reporter gene were the most effective and reliable. *We use this MDA cell line*.

2). The reference androgen was originally R-1881 but was later changed to DHT; the TA response with DHT should be demonstrated by a full concentration response curve. *We use DHT at eight concentrations ranging from 10^{-9}M to 10^{-16}M to calculate EC50 and EC80 (see Methods).*

3). 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. *We typically dissolve test substances in absolute ethanol and then dilute with AnA-free cell culture medium as our negative vehicle control.*

4). The solvent/vehicle volume must equal that in the reaction mixtures containing the test substances, and should remain constant throughout the concentration range tested. *We do this.*

5). A known AR antagonist (e.g., OHF) should be included as a positive antagonist control in each antagonism study. The concentration of the reference antagonist that is used should be one that reduces the ability of the reference androgen to induce TA in the test system by 70 to 90%. *We use VINC as the positive control because of its minimal agonist effects compared to OHF previously used by other laboratories.*

6). It might be useful to include in each study a positive control androgen with a maximal TA response two to three orders of magnitude lower than the reference androgen DHT. The necessity for inclusion of an additional positive control androgen in each study should be evaluated during the validation process. *We will use Medroxyprogesterone 17-acetate (MPA) as the weak positive control*

7). The limit concentration should be 1 mM, but the solubility characteristics of each test substance must be taken into consideration. *We do this.*

8). 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. *We typically use **eight** concentrations for each test chemical in RF, **Ten** in Comp Assays.*

9). Triplicate measurements should be performed at each test substance concentration level. *We do triplicate or quadruplicate measurements.*

10). 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. *We do this.*

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

12). 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. *We do this.*

13). 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. *We test 5 times in the Phase I and 3 times in II studies.*

14). The assays should be performed following Good Laboratory Practice guidelines. *We do this.*

Additional or modified minimum procedural standards

15). 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. *We do this.*

16). A measure of cellular cytotoxicity should be incorporated into the assay to help define the upper limit for test material concentrations. *We do this.*

17)*. Most importantly, we now include a confirmation assay as now recognized by ICCVAM/NICEATM as necessary to help determine whether apparent agonist/antagonist activity is indeed due to AR-binding effects.*