Streptomycin (100 ug/ml).

Medium for the assay plate: Phenol Red Free D-MEM/F-12 supplemented with 5% DCC-FBS, Penicillin (100 units /mL), and Streptomycin (100 μg/ml).

15. Cell should be maintained in a $CO_2$ incubator (5% $CO_2$) at 37±1°C with Medium for cell propagation. Upon reaching 75-90% confluency, cells are subcultured to 10 mL at a density of 0.3-1.0 x 10 cell/mL in 10cm cell culture dish. To prepare the assay plate, cells should be suspended in the assay Medium and then plated into wells of a microplate containing 90 μL/well at a density of $1 \times 10^5$ cells/mL. Next, the cells should be pre-incubated in a 5% $CO_2$ incubator at 37°±1°C for 24 hours before chemical exposure.

16. To maintain the integrity of the response, the cells should be grown for more than one passage from the frozen stock in the conditioned media and should not be cultured for more than 40 passages. For the AR-EcoScreen™ cell line, this will be less than two months.

17. The DCC-FBS can be obtained from commercial sources. The selection of FBS is sometimes critical for the assay performance; therefore, the appropriate FBS should carefully be selected based on the cell response, as generally considered.

***Acceptability Criteria***

*Positive and Negative Reference Chemicals*

18. Prior to and during the study, the responsiveness of the test system should be verified using the appropriate concentrations of known reference chemicals as provided in Table 1-1 for the AR agonist assay and Table 1-2 for the AR antagonist assay. Acceptable range values derived from the validation study are given in Table 1-1 and Table 1-2 (2). These 3 concurrent reference chemicals for each AR agonist/antagonist assays should be included with each AR agonist/antagonist experiments and the results should fall within the given acceptable limits. If this is not the case, the cause for the failure to meet the acceptability criteria should be determined (*e.g.* cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. Once the acceptability criteria have been achieved, it is essential in order to ensure minimum variability of log PC50, log PC10, log IC30, log IC50 values, that use of materials for cell culturing is consistent. The three concurrent reference chemicals, which should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), can ensure the sensitivity of the assay because the log PC10s or log IC30 of the two positive reference chemicals should fall within the acceptable range, as should the log PC50s, or log IC50 where they can be calculated (see Table 1-1, 1-2).

**Table 1-1: Reference chemicals for AR agonist assay**

| Fold-induction | ≥ 6.4 | | |
|---|---|---|---|
| log PC10 value | Greater than 1 (fold-induction of VC) +2SD | | |
| Chemical Name [CAS No.] | logPC10 | logPC50 | Test range |
| 5α-Dehydrotestosterone (DHT) [521-18-6] | -12.08 ~-9.87 | -11.03 ~ -9.00 | $10^{-12}$ ~ $10^{-6}$M |
| Mestanolone [521-11-9] | -10.92 ~-10.41 | -10.15 ~ -9.26 | $10^{-12}$ ~ $10^{-6}$M |
| Di(2-ethylhexyl)phthalate (DEHP) [117-81-7] | - | - | $10^{-10}$ ~ $10^{-5}$M |

**Table 1-2: Reference chemicals for AR antagonist assay**

| Fold induction of positive control: [500 pM DHT]/[Vehicle Control] | ≥ 5.0 | | |
|---|---|---|---|
| $PC_{ATG}$ inhibition (%)* | ≤46 | | |
| Chemical Name [CAS No.] | log IC30 | log IC50 | Test range |
| Hydroxyflutamide (HF) [52806-53-8] | -8.37 ~ -6.41 | -7.80 ~ -6.17 | $10^{-10}$ ~ $10^{-5}$M |
| Bisphenol A (BisA) [80-05-7] | -7.52 ~ -4.48 | -7.05 ~ -4.29 | $10^{-10}$ ~ $10^{-5}$M |
| Di(2-ethylhexyl)phthalate(DEHP) [117-81-7] | - | - | $10^{-10}$ ~ $10^{-5}$M |

*$PC_{ATG}$ inhibition (%) is calculated by the following equation;

$$PC_{ATG}\text{ inhibition (\%)} = \text{Mean}\left(\frac{\text{RLU of } PC_{ATG} - \text{Mean RLU of VC}}{\text{Mean RLU of AG ref} - \text{Mean RLU of VC}} \times 100\right)$$

*Positive and Vehicle Controls*

19. **For the agonist assay**, positive control (PC) wells (n=6) treated with a natural ligand (10 nM of DHT) and vehicle control (VC) wells (n=6) treated with vehicle alone, should be prepared in each assay plate. **For the antagonist assay**, vehicle control (n=3), positive control for agonistic activity (PCago, 10 nM of DHT, n=3), positive control for antagonistic activity ($PC_{ATG}$, 0.1 μM of HF, n=3), positive control for cytotoxicity ($PC_{CT}$, 10 μg/mL of cycloheximide, n=3) and Agonist reference (AG ref 500 pM of DHT, n=12) should be set in each assay plate. The $PC_{ATG}$ inhibition (%) should be less than 46.

*Fold-induction*

20. The mean luciferase activity of the PC (10 nM DHT) should be at least 6.4-fold higher than that of the mean VC on each plate for the agonist assay, and at least 5.0-fold for antagonism

assay. These criteria were established based on the reliability of the endpoint values from the validation study.

21. With respect to the quality control of the assay, the fold-induction corresponding to the log PC10 value of the concurrent PC (10 nM DHT) should be greater than 1+2SD of the fold-induction value (=1) of the concurrent VC. For prioritization purposes, the log PC10 value can be useful to simplify the data analysis required compared to a statistical analysis. Although a statistical analysis provides information on significance, such an analysis is not a quantitative parameter with respect to concentration-based potential, and so is less useful for prioritization purposes.

***Chemicals to Demonstrate Laboratory Proficiency***

22. Prior to testing unknown chemicals in the AR-STTA assay, the responsiveness of the test system should be confirmed by each laboratory, at least once for each newly prepared batch of cell stocks taken from the frozen stock. This is done by independent testing of the 10 proficiency chemicals listed in Table 2-1 and 2-2 for AR agonism and antagonism, respectively. This should be done at least in duplicate, on different days, and the results should be comparable to Table 2 and any deviations should be justified.

**Table 2-1: List of Proficiency Chemicals for agonist assay**

| Chemical Name | CASRN | Class[1] | log PC10 [1] (M) | log PC50 [1] (M) | Chemical Class [2] | Product Class [3] |
|---|---|---|---|---|---|---|
| 5α-Dehydrotestosterone | 521-18-6 | P | -12.08 ~ -9.87 | -11.03 ~ -9.00 | Steroid, nonphenolic | Pharmaceutical |
| Mestanolone | 521-11-9 | P | -10.92 ~ -10.41 | -10.15 ~ -9.26 | Steroid, nonphenolic | Pharmaceutical |
| Testosterone | 58-22-0 | P | -10.42 ~ -9.73 | -9.46 ~ -8.96 | Steroid, nonphenolic | Pharmaceutical |
| 17β-estradiol | 50-28-2 | P | -7.74 ~ -6.75 | -5.34 ~ -4.88 | Steroid, phenolic | Pharmaceutical |
| Medroxyprogesterone 17-acetate | 71-58-9 | P | -9.64 ~ -8.89 | -8.77 ~ -8.37 | Steroid, nonphenolic | Pharmaceutical |
| 17α-ethinyl estradiol | 57-63-6 | N | - | | Steroid, phenolic | Pharmaceutical |

| Butylbenzyl phthalate | 85-68-7 | N | - | Phthalate | Plasticizer |
|---|---|---|---|---|---|
| Di(2-ethylhexyl)phthalate | 117-81-7 | N | - | Phthalate | Chemical intermediate; Plasticizer |
| Hydroxyflutamide | 52806-53-8 | N | - | Anilide | Pharmaceutical metabolite |
| Bisphenol A | 80-05-7 | N | - | Bisphenol | Chemical intermediate |

Abbreviations; CASRN: Chemical Abstracts Service Registry Number, M: molar, P: Positive, N: Negative

[1] Validation report of Androgen Receptor (AR) Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities (2)

[2] Substances were assigned to one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at http://www.nlm.nih.gov/mesh).

[3] Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

**Table 2-2: List of proficiency chemicals for antagonist assay**

| Chemical Name | CASRN | Class[1] | log IC30 [1] (M) | log IC50 [1] (M) | Chemical Class [2] | Product Class [3] |
|---|---|---|---|---|---|---|
| Hydroxyflutamide | 52806-53-8 | P | -8.37 ~ -6.41 | -7.80 ~ -6.17 | Anilide | Pharmaceutical metabolite |
| Bisphenol A | 80-05-7 | P | -7.52 ~ -4.48 | -7.05 ~ -4.29 | Bisphenol | Chemical intermediate |
| Flutamide | 13311-84-7 | P | -6.20 ~ -5.69 | -5.66 ~ -5.43 | Anilide | Pharmaceutical |
| Prochloraz | 67747-09-5 | P | -5.77 ~ -5.47 | -5.44 ~ -5.12 | Imidazole | Pesticide |
| Vinclozolin | 50471-44-8 | P | -6.83 ~ -6.32 | -6.47 ~ -5.85 | Organochlorine | Pesticide |
| 5α-Dehydrotestosterone | 521-18-6 | N | - | | Steroid, nonphenolic | Pharmaceutical |
| Mestanolone | 521-11-9 | N | - | | Steroid, nonphenolic | Pharmaceutical |
| Di(2-ethylhexyl)phthalate | 117-81-7 | N | - | | Phthalate | Chemical intermediate; Plasticizer |

| | | | | | |
|---|---|---|---|---|---|
| Atrazine | 1912-24-9 | N | - | Triazine; Aromatic amine | Pesticide |
| 6-Propyl-2-thiouracil | 51-52-5 | N | - | Pyrimidines | Pharmaceutical |

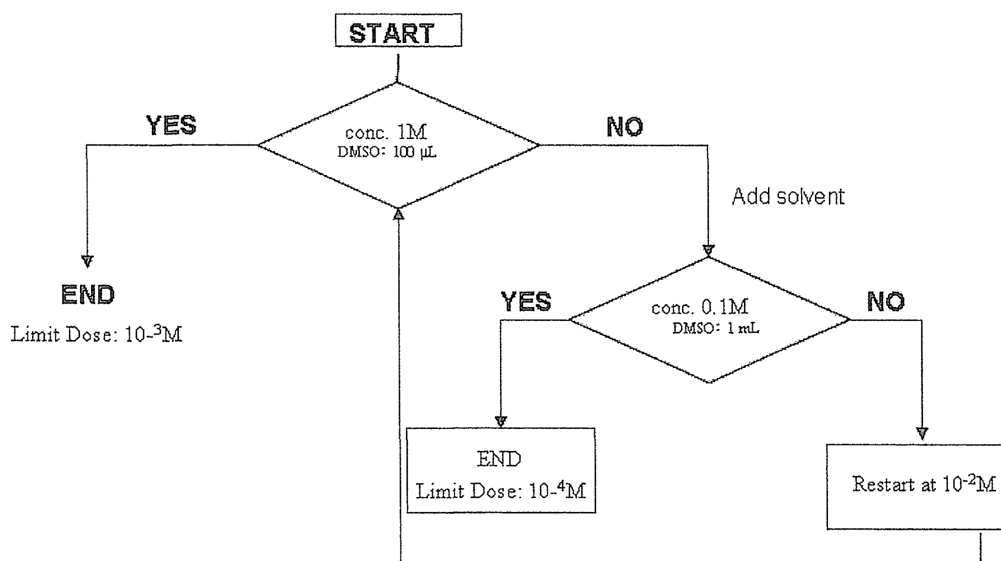
Abbreviations; CASRN: Chemical Abstracts Service Registry Number, M: molar, P: Positive, N: Negative
[1] Validation report of Androgen Receptor (AR) Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities (2)
[2] Substances were assigned to one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognized standardized classification scheme (available at http://www.nlm.nih.gov/mesh).
[3] Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

*Vehicle*

23. Dimethyl sulfoxide (DMSO), or another appropriate solvent, should be used as the concurrent VC at the same concentration for the different positive and negative controls and the test chemicals. Test substances should be dissolved in a solvent that solubilizes the test substance and is miscible with the cell medium. Water, ethanol (95% to 100% purity) and DMSO are suitable vehicles. If DMSO is used, the final level in the well should not exceed 0.1% (v/v). For any vehicle, it should be demonstrated that the maximum concentration used is not cytotoxic and does not interfere with the assay performance.

*Preparation of Test Chemicals*

24. Generally, the test chemicals should be dissolved in DMSO and serially diluted with the same solvent at a common ratio of 1:10 in order to prepare solutions for dilution with media as the diagram for solubility test indicated below.




25. A solubility test is a very important step to determine the maximum concentration for the assay and it may affect the sensitivity of the assay. The highest concentration should be selected based on the cell viability rather than the avoidance of some precipitation at higher concentration ranges.

26. For AR antagonists, the presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and should be considered when interpreting the data. Cytotoxicity can be evaluated with renilla luciferase activity in the AR-EcoScreen™ cell line, which was originally established to express renilla luciferase constitutively. Accordingly, AR-mediated transcriptional activity and cytotoxicity can be evaluated simultaneously in the same assay plate.

27. Should the results of the cytotoxicity test show that the concentration of the test substance has reduced the cell number by 20% or more, this concentration is regarded as cytotoxic, and the concentrations at or above the cytotoxic concentration should be excluded from the evaluation.

*28. Chemical Exposure and Assay Plate Organization*

29. For the AR agonist assay, the procedure for chemical dilutions (Steps 1 and 2) and for exposing cells (Step 3) can be conducted as follows:

Step 1: Each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtiter plate to achieve final serial concentrations as determined by the preliminary range finding test (typically a series of, for example 1 mM, 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM ($10^{-3}$-$10^{-11}$ M)) for triplicate testing.

Step 2: Chemical dilution: First dilute 10 μL of the test chemical in solvent into 90 μL of media.

Step 3: Then 10μL of the diluted chemical prepared in Step 2 should be diluted into 90 μL of the media.

Step 4: Chemical exposure of the cells: Add 10 μL of diluted chemical solution (prepared in Step 3) to an assay well containing $9 \times 10^3$ cells/90 μL/well.

The recommended final volume of media required for each well is 100 μL.

Test samples and reference chemicals can be assigned as shown in Table 3-1.

**Table 3-1: Example of plate concentration assignment of the reference chemicals in the assay plate for agonist assay**

| Row | DHT | | | Mestanolone | | | DEHP | | | Test Chemical# | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 1 μM | → | → | 10 μM | → | → | 10 μM | → | → | 10 μM | → | → |
| B | 100 nM | → | → | 1 μM | → | → | 1 μM | → | → | 1 μM | → | → |
| C | 10 nM | → | → | 100 nM | → | → | 100 nM | → | → | 100 nM | → | → |
| D | 1 nM | → | → | 10 nM | → | → | 10 nM | → | → | 10 nM | → | → |
| E | 100 pM | → | → | 1 nM | → | → | 1 nM | → | → | 1 nM | → | → |
| F | 10 pM | → | → | 100 pM | → | → | 100 pM | → | → | 100 pM | → | → |
| G | 1 pM | → | → | 10 pM | → | → | 10 pM | → | → | 10 pM | → | → |
| H | VC | → | → | → | → | → | PC | → | → | → | → | → |

VC: Vehicle control (DMSO);
PC: Positive control (10 nM of DHT)
#: concentration of test chemical is an example

30. For the AR antagonist assay, the procedure for chemical dilutions (Steps 1 and 2) and for exposing cells (Step 3) can be conducted as follows:

Step 1: Each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtiter plate to achieve final serial concentrations as determined by the preliminary range finding test (typically a series of, for example 1 mM, 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, 1 nM and 100 pM ($10^{-3}$-$10^{-10}$ M)) for triplicate testing.

Step 2: Chemical dilution: First dilute 10 μL of the test chemical in the solvent to a

volume of 90 μL media containing 56 nM DHT/0.1% DMSO.

Step 3: Then 10μL of the diluted chemical prepared in Step 2 should be diluted into 90 μL of the media.

Step 4: Chemical exposure of the cells: Add 10 μL of diluted chemical solution (prepared in Step 2) to an assay well containing $9 \times 10^3$ cells/90 μL/well.
The recommended final volume of media required for each well is 100 μL.

31. Test samples and reference chemicals can be assigned as shown in Table 3-2.

**Table 3-2: Example of plate concentration assignment of the reference chemicals in the assay plate for antagonist assay**

| Row | HF | | | Bisphenol A | | | DEHP | | | Test chemical# | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 10 μM | → | → | 10 μM | → | → | 10 μM | → | → | 10 μM | → | → |
| B | 1 μM | → | → | 1 μM | → | → | 1 μM | → | → | 1 μM | → | → |
| C | 100 nM | → | → | 100 nM | → | → | 100 nM | → | → | 100 nM | → | → |
| D | 10 nM | → | → | 10 nM | → | → | 10 nM | → | → | 10 nM | → | → |
| E | 1 nM | → | → | 1 nM | → | → | 1 nM | → | → | 1 nM | → | → |
| F | 100 pM | → | → | 100 pM | → | → | 100 pM | → | → | 100 pM | → | → |
| G | AG ref | → | → | → | → | → | → | → | → | → | → | → |
| H | VC | → | → | $PC_{ago}$ | → | → | $PC_{ATG}$ | → | → | $PC_{CT}$ | → | → |

VC: Vehicle control (DMSO);
$PC_{ago}$: Positive control (10 nM of DHT);
$PC_{ATG}$-: Positive control (0.1 μM of HF) ;
$PC_{CT}$: Cytotoxicity control (10 μg/mL of cycloheximide);
AG ref (DMSO at 0.1% spiked with $5 \times 10^{-10}$ M DHT)
** Gray colored wells are spiked with $5 \times 10^{-10}$M DHT
#: concentration of test chemical is an example

32. The reference chemicals (DHT, Mestanolone and DEHP for the agonist assay; HF, BisA and DEHP for the antagonist assay) should be tested in every run (as indicated in Table 3-1 and 3-2). PC wells treated with 10 nM of DHT that can produce a maximum induction of DHT, and VC wells treated with DMSO (or appropriate solvent) alone should be included in each test assay plate for the agonist assay (Table 4-1). In case of the antagonist assay, $PC_{ATG}$: Antagonist positive control (0.1 μM of HF), $PC_{CT}$: Cytotoxicity control (10 μg/mL of cycloheximide) and AG ref (DMSO at 0.1% spiked with 500 pM DHT) should be prepared additionally (Table 4-2). If cells from different sources (*e.g.* different passage number, different lot, etc.,) are used in the same experiment, the reference chemicals should be tested for each cell source.

**Table 4-1: Example of plate concentration assignment of test and plate control chemicals in the assay plate for agonist assay**

| Row | Test Chemical 1 | | | Test Chemical 2 | | | Test Chemical 3 | | | Test Chemical 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | conc 1 (10 µM) | → | → | 1 mM | → | → | 1 µM | → | → | 10 nM | → | → |
| B | conc 2 (1 µM) | → | → | 100 µM | → | → | 100 nM | → | → | 1 nM | → | → |
| C | conc 3 (100 nM) | → | → | 10 µM | → | → | 10 nM | → | → | 100 pM | → | → |
| D | conc 4 (10 nM) | → | → | 1 µM | → | → | 1 nM | → | → | 10 pM | → | → |
| E | conc 5 (1 nM) | → | → | 100 nM | → | → | 100 pM | → | → | 1 pM | → | → |
| F | conc 6 (100 pM) | → | → | 10 nM | → | → | 10 pM | → | → | 0.1 pM | → | → |
| G | conc 7 (10 pM) | → | → | 1 nM | → | → | 1 pM | → | → | 0.01 pM | → | → |
| H | VC | → | → | → | → | → | PC | → | → | → | → | → |

Plate controls = VC: Vehicle control (DMSO); PC: Positive control (10 nM of DHT)

**Table 4-2: Example of plate concentration assignment of test and plate control chemicals in the assay plate for antagonist assay**

| Row | Test Chemical 1 | | | Test Chemical 2 | | | Test Chemical 3 | | | Test Chemical 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | conc 1 (10 µM) | → | → | 1 mM | → | → | 1 µM | → | → | 10 nM | → | → |
| B | conc 2 (1 µM) | → | → | 100 µM | → | → | 100 nM | → | → | 1 nM | → | → |
| C | conc 3 (100 nM) | → | → | 10 µM | → | → | 10 nM | → | → | 100 pM | → | → |
| D | conc 4 (10 nM) | → | → | 1 µM | → | → | 1 nM | → | → | 10 pM | → | → |
| E | conc 5 (1 nM) | → | → | 100 nM | → | → | 100 pM | → | → | 1 pM | → | → |
| F | conc 6 (100 pM) | → | → | 10 nM | → | → | 10 pM | → | → | 100 pM | → | → |
| G | AG ref | → | → | → | → | → | → | → | → | → | → | → |
| H | VC | → | → | $PC_{ago}$ | → | → | $PC_{ATG}$ | → | → | $PC_{CT}$ | → | → |

VC: Vehicle control (DMSO);
$PC_{ago}$: Positive control (10 nM of DHT);
$PC_{ATG}$: Positive control (0.1 µM of HF) ;
$PC_{CT}$: Cytotoxicity control (10 µg/mL of cycloheximide);
AG ref ($5x10^{-10}$ M DHT)
** Gray colored wells spiked with $5x10^{-10}$M DHT

33. The lack of edge effects should be confirmed, as appropriate, and if edge effects are suspected, the plate layout should be altered to avoid such effects. For example, a plate layout excluding the edge wells can be employed.

34. After adding the chemicals, the assay plates should be incubated in a 5% $CO_2$ incubator at 37±1°C for 20-24 hours to induce the reporter gene products.

35. Special considerations will need to be applied to those compounds that are highly volatile. In such cases, nearby control wells may generate false positives, and this should be considered in light of expected and historical control values. In the few cases where volatility may be of concern, the use of "plate sealers" may help to effectively isolate individual wells during testing, and is therefore recommended in such cases.

36. Repeat definitive tests for the same chemical should be conducted on different days using freshly prepared assay reagents and dilutions of the test chemicals, to ensure independence.

***Luciferase assay***

37. A commercial luciferase assay reagent [e.g. Steady-Glo® Luciferase Assay System (Promega, E2510, or equivalents)] or a standard luciferase assay system (Promega, E1500, or equivalents) can be used for agonist detection. A Dual-luciferase assay system (e.g. Promega, E2920 and its equivalents) is preferable for antagonist detection where renilla luciferase is used to evaluate cytotoxicity, as long as the acceptability criteria are met. The assay reagents should be selected based on the sensitivity of the luminometer to be used. When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531, or equivalents) should be used before adding the substrate. When using Steady-Glo Luciferase Assay System (Promega, E2510) in the agonist assay, 40µL of prepared reagent should be directly added into the assay wells. When using Dual-Glo system (Promega, E2920) in the antagonist assay, 40µL of the first substrate should be added into the assay wells after removing 60 µL of supernatant to detect Firefly luciferase activity. Then 40µL of the second substrate should be added into the assay wells to detect Renilla luciferase activity.

## ANALYSIS OF DATA

38. **For the Agonist assay**, to obtain the relative transcriptional activity to PC (10 nM DHT), the luminescence signals from the same plate can be analyzed according to the following steps (other equivalent mathematical processes are also acceptable):
    - Step 1. Calculate the mean value for the VC.
    - Step 2. Subtract the mean value of the VC from each well value to normalize the data.
    - Step 3. Calculate the mean for the normalized PC.
    - Step 4. Divide the normalized value of each well in the plate by the mean value of the normalized PC (PC=100%).

The final value of each well is the relative transcriptional activity for that well compared to the PC response.

Step 5. Calculate the mean value of the relative transcriptional activity for each concentration group of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).

39. **For the Antagonist assay**, to obtain the relative transcriptional activity, the luminescence signals from the same plate can be analyzed according to the following steps (other equivalent mathematical processes are also acceptable):

Step 1. Calculate the mean value for the VC.

Step 2. Subtract the mean value of the VC from each well value to normalize the data.

Step 3. Calculate the mean for the normalized AG ref.

Step 4. Divide the normalized value of each well in the plate by the mean value of the normalized mean AG ref (AG ref=100%).

The final value of each well is the relative transcriptional activity for that well compared to the maximum AG ref response.

Step 5. Calculate the mean value of the relative transcriptional activity for each concentration group of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).

**log PC50, log PC10, log IC50 and log IC30 induction considerations**

40. To evaluate cytotoxicity in the antagonist assay, cell viability should be expressed as the percentage of renilla luciferease activity of the chemically-treated wells to the mean renilla luciferase activity of the VC wells.

41. In the case of the agonist assay, the following should be provided for each test chemical:

(i) The RPCmax which is the maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 10 nM DHT on the same plate

(ii) For positive chemicals, the concentrations that induce the log PC10 and, if appropriate, the log PC50.

42. Descriptions of log PC values are provided in Figure 1 below.

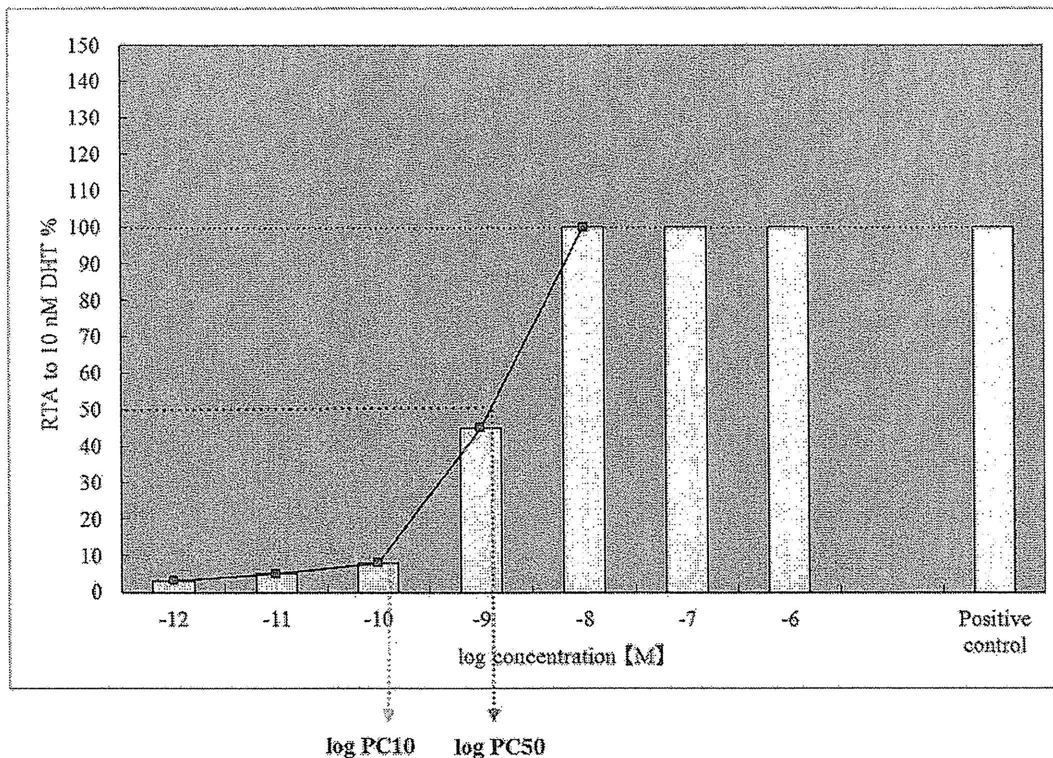



**Figure 1: Schematic descriptions of log PCx values**
The PC (Positive control; 10 nM of DHT) is included on each assay plate.

43. The log PCx value can be calculated by interpolating between 2 points on the X-Y coordinate, one immediately above and one immediately below a log PCx value. Where the data points lying immediately above and below the log PCx value have the coordinates (a,b) and (c,d) respectively, then the log PCx value may be calculated using the following equation and Figure 2:

$$\log[PCx] = c+[(x-d)/(b-d)](a-c)$$

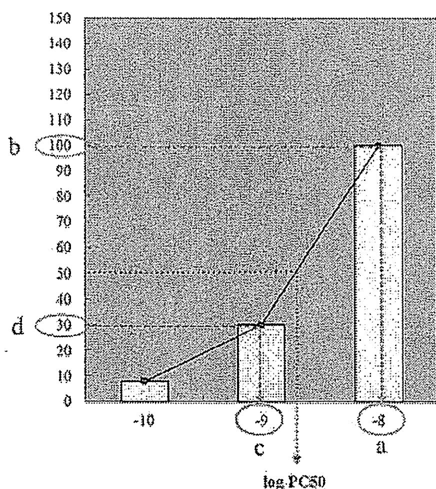



**Figure 2: Example for calculation of log PC50.**

44. Descriptions of log ICx values are provided in Figure 3 below.

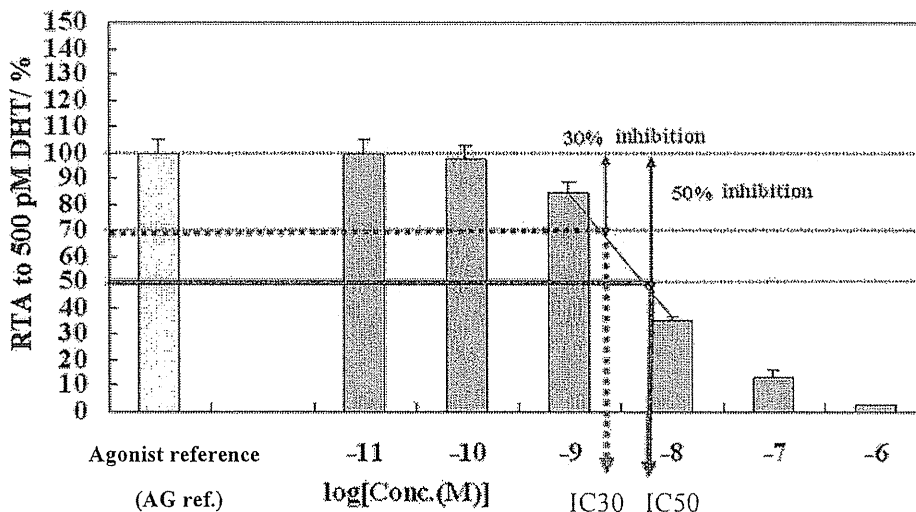



**Figure 3: Schematic descriptions of log IC values.**
The AG ref (DMSO at 0.1% spiked with 500 pM DHT) is included on each assay plate.

45. In the case of the antagonist assay, log IC50 and log IC30 values can be defined as the test chemical concentrations estimated to elicit either a 50% or a 30% inhibition of transcriptional activity induced by 500 pM DHT that can be calculated in the same way as the log PC values. Each log ICx value can be calculated by a simple linear regression using two variable data points for the transcriptional activity. Where the data points lying immediately above and below the log ICx value have the coordinates (c,d) and (a,b) respectively, then the log ICx

value may be calculated using the following equation and Figure 4:

$$\log [ICx] = a-[(b-(100-x)) /(b-d) ] (a-c)$$

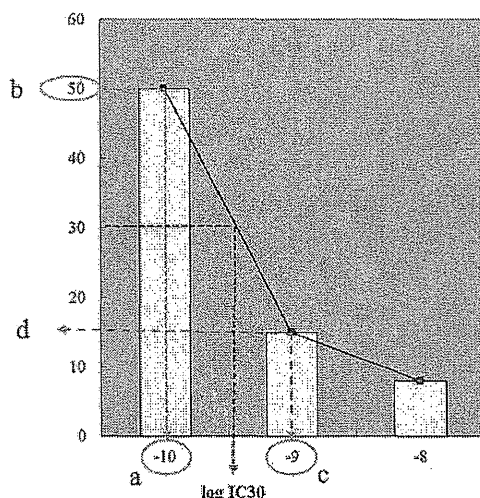



**Figure 4: Example for calculation of log IC30.**

46. To distinguish pure antagonism from a cytotoxicity-related decrease of luciferase activity, AR-EcoScreen is designed to express two kinds of luciferase: firefly luciferase inducibly expressed by the AR response element and renilla luciferase stably and non-inducibly expressed.

47. By using Dual-luciferase reporter assay technology, both cell viability and the antagonism can be evaluated in the same cells in a single plate run. PCct (10μg/mL of cycloheximide) is used to adjusti renilla activity by substracting the PCct values "renilla activities" from those of all sample wells. To evaluate the true cytotoxicity of chemicals with the AR Ecoscreen assay, such revised cell viability should be used. log ICx value can be accepted if cell viability is recorded as higher than 80% at the specific test concentration.

48. The results should be based on two (or three) independent runs. If two runs give comparable and therefore reproducible results, it is not necessary to conduct a third run. To be acceptable, the results should:
   - Meet the performance standard requirements
     for AR agonist assay:
     - The mean luciferase activity of the PC (10 nM DHT) should be at least 6.4-fold higher than the mean VC on each plate

- The fold induction corresponding to the log PC10 value of the concurrent PC (10 nM DHT) should be greater than 1+2SD of the fold induction value (=1) of the VC.
- The results of the 3 reference chemicals should be within the acceptable range (Table 1-1).

- Meet the performance standard requirements

  for AR antagonist assay:
  - Fold induction of AG ref ([AR ref. of 500 pM DHT]/[Vehicle Control]) should be at least 5.0.
  - The $PC_{ATG}$ inhibition (%) should be less than 46.
  - The results of the 3 reference chemicals should be within the acceptable range (Table 1-2).
- Be reproducible.

*Data Interpretation Criteria*

49. **For agonist assay,** data interpretation criteria are shown in Table 5-1. Positive results will be characterized by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (log PC50) or 10% (log PC10) accomplishes the goal. However, a test chemical is determined to be positive, if the maximum response induction or inhibition by the test chemical (RPCmax) is equal to or exceeds 10% of the positive control responses in at least two of two or two of three runs, whereas a test chemical is considered negative if the RPCmax fails to achieve at least 10% in agonist assay in two of two or two of three runs.

**Table 5-1: Positive and negative decision criteria for agonist assay**

| | |
|---|---|
| **Positive** | If a RPCmax is obtained that is equal to or exceeds 10% of the response of the positive control in at least two of two or two of three runs. |
| **Negative** | If a RPCmax fails to achieve at least 10% of the response of the positive control in two of two or two of three runs. |

50. **For antagonist assay,** data interpretation criteria are shown in Table 5-2. Positive results will be characterized by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (log IC50) or 30% (log IC30) accomplishes the goal. However, a test chemical is determined to be positive, if the log IC30 could be calculated in at least two of two or two of three runs, whereas a test chemical is considered as negative if the log IC30 could not be calculated in two of two or two of three runs.

**Table 5-2: Positive and negative decision criteria for antagonist assay**

| | |
|---|---|
| **Positive** | If the log IC30 is calculated in at least two of two or two of three runs. |
| **Negative** | If the log IC30 fails to calculate in two of two or two of three runs. |

51. The calculations of log PC10, log PC50 and RPCmax for agonist assay, and log IC50 and log IC30 for antagonist assay can be calculated by using a spreadsheet available with the Test Guideline on the OECD public website (to be addressed after submission).

52. It should be sufficient to obtain log PCx or log ICx values at least twice. However, should the resulting base-line for data in the same concentration range show variability with an unacceptably high coefficient of variation (CV; %) the data should not be considered reliable and the source of the high variability should be identified. The CV of the raw data triplicates (*i.e.* luminescence intensity data) of the data points that are used for the calculation of log PCx or log ICx should be less than 10%.

53. Meeting the acceptability criteria indicates the assay system is operating properly, but it does not ensure that any particular run will produce accurate data. Duplicating the results of the first run is the best assurance that accurate data were produced, see paragraphs 48-52.

54. In the case of the agonist assay, if more information is required in addition to the screening and prioritization purposes of this TG for positive test compounds, it can be confirmed that the observed luciferase-activity is solely an AR-specific response, using an AR antagonist.

## TEST REPORT

55. The test report should include the following information:

***Test substance:***

- identification data and CAS Number, if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance.

*Solvent/Vehicle:*

- characterisation (nature, supplier and lot);
- justification for choice of solvent/vehicle;
- solubility and stability of the test substance in solvent/vehicle, if known.

***Cells:***

- type and source of cells;
- number of cell passages;
- methods for maintenance of cell cultures.

***Test conditions:***

cytotoxicity data (and justifications for the method of choice) and solubility limitations should be reported, as well as:

- composition of media, $CO_2$ concentration;
- concentration of test chemical;
- volume of vehicle and test substance added;
- incubation temperature and humidity;
- duration of treatment;
- cell density during treatment;
- positive and negative reference chemicals;
- duration of treatment period;
- Luciferase assay reagents (Product name, supplier and lot);
- acceptability and data interpretation criteria.

***Reliability check:***

- Fold inductions for each assay plate.
- Actual log PC50, log PC10 (or log IC50 and log IC30) values for concurrent reference chemicals.

***Results:***

- Raw and normalised data of luminescent signals;
- the maximum fold induction level;
- cytotoxicity data;
- Concentration-response relationship, where possible;
- log PC10, log PC50 and PCmax for agonist assay, and log IC50 and log IC30 values for antagonist assay, as appropriate;
- EC50 values, if appropriate;
- Statistical analyses, if any, together with a measure of error (*e.g.* SD, CV or 95% confidence interval) and a description of how these values were obtained.

***Discussion of the results***

***Conclusion***

**LITERATURE**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