should be set in each assay plate. As for the inhibitory ratio of PC_{ATG}, it should be greater than 0.46.

Fold-induction

20. The mean luciferase activity of the PC (10 nM DHT) should be at least 6.4-fold that of the mean VC on each plate for agonist assay, and at least 5.0-fold for antagonist assay. These criterion was 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 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 prioritisation purposes, the 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 prioritisation 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 by independent testing of the 10 proficiency chemicals listed in Table 2-1 and 2-2 for AR agonist and antagonist, 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

No.	Chemical name	CAS No.	Class
1	Flutamide	13311-84-7	N
2	4-*tert*-Octylphenol	140-66-9	N
3	Bisphenol A	80-05-7	N
4	Dexamethasone	50-02-2	P
5	Medroxyprogesterone acetate	71-58-9	P
6	Testosterone	58-22-0	P
7	4-Androstenedione	63-05-8	P
8	Spironolactone	52-01-7	P
9	Progesterone	57-83-0	P
10	17a-Methyltestosterone	58-18-4	P

Table 2-2 List of proficiency chemicals for antagonist assay

No.	Chemical name	CAS No.	Class
1	Methyltrienolone (R1881)	965-93-5	N
2	Fluoxymestrone	76-43-7	N
3	Medroxyprogesterone acetate	71-58-9	N
4	Cyproterone acetate	427-51-0	P
5	Flutamide	13311-84-7	P
6	Spironolactone	52-01-7	P
7	4- tert -Octylphenol	140-66-9	P
8	Procymidone	32809-16-8	P
9	Progesterone	57-83-0	P
10	Vinclozolin	50471-44-8	P

Vehicle

23. Dimethyl sulfoxide (DMSO), or appropriate solvent, at the same concentration used for the different positive and negative controls and the test chemicals should be used as the concurrent VC. Test substances should be dissolved in a solvent that solubilizes that 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 level should not exceed 0.1% (v/v). For any vehicle, it should be demonstrated that the maximum volume used is not cytotoxic and does not interfere with assay performance.

Preparation of Test Chemicals

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

Solubility and Cytotoxicity: Considerations for Range Finding.

25. A preliminary test should be carried out to determine the appropriate concentration range of chemical to be tested, and to ascertain whether the test chemical may have any solubility and cytotoxicity problems. Initially, chemicals are tested up to the maximum concentration of 1 μl/ml, 1 mg/ml, or 1 mM, whichever is the lowest. Based on the extent of cytotoxicity or lack of solubility observed in the preliminary test, the first definite run should test the chemical at log-serial dilutions starting at the maximum acceptable concentration (*e.g.* 1 mM, 100 μM, 10 μM, etc.) and the presence of cloudiness or precipitate or cytotoxicity noted. Concentrations in the second, and if necessary third run should be adjusted as appropriate to better characterise the concentration-response curve and to avoid

concentrations which are found to be insoluble or to induce excessive cytotoxicity.

26. For AR agonists, 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 testing methods that can provide information regarding 80% cell viability should be used, utilising an appropriate assay based upon laboratory experience. Cytotoxicity can be evaluated with renilla luciferase activity in AR-EcoScreen^{TM} cell line, which originally established to expresses renilla luciferase constitutively. Accordingly, AR-mediated transcriptional activity and cytotoxicity can be evaluated simultaneously in 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.

Chemical Exposure and Assay Plate Organisation

28. For AR agonist assay, the procedure for chemical dilutions (Steps-1 and 2) and exposure to 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 microtitre plate to achieve final serial concentrations as determined by the preliminary range finding test (typically in 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 the 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 for dilution.

Step-4: Chemical exposure of the cells: Add 10 μL of diluted chemical solution (prepared in Step-3) to an assay well containing 9×10^3 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			R1881			DEHP			Chemical-1		
	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)

29. For AR antagonist assay, the procedure for chemical dilutions (Steps-1 and 2) and exposure to 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 microtitre plate to achieve final serial concentrations as determined by the preliminary range finding test (typically in 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 concentration of 90 μL of media containing 56nM 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 for dilution.

Step-4: Chemical exposure of the cells: Add 10 μL of dilutchemical solution (prepared in Step-2) to an assay well containing 9×10^3 cells/90 μL/well.

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

30. 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			DEHP		
	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	SPK	→	→	→	→	→	→	→	→	→	→	→
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}: Positive control (10 μg/mL of cycloheximide);
SPK (DMSO at 0.1% spiked with $5x10^{-10}$ M DHT)
** Gray colored wells are spiked with $5x10^{-10}$M DHT

31. The reference chemicals (DHT, R1881 and DHEP for agonist assay; HF, BisA and DEHP for antagonist assay) should be tested in every run (as exampled in Table 3-1 and 3-2). PC wells treated with 10 nM of DHT that can produce maximum induction of DHT and VC wells treated with DMSO (or appropriate solvent) alone should be included in each test assay plate for agonist assay (Table 4-1). In case of antagonist assay, PC_{ATG}: Antagonist positive control (0.1 μM of HF), PC_{CT}: Cytotoxicity positive control (10 μg/mL of cycloheximide) and SPK-in control (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	SPK	→	→	→	→	→	→	→	→	→	→	→
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}: Positive control (10 µg/mL of cycloheximide);
SPK (DMSO at 0.1% spiked with $5x10^{-10}$ M DHT)
** Gray colored wells spiked with $5x10^{-10}$M DHT

32. 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.

33. 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.

34. 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.

35. Repeat definitive tests for the same chemical should be conducted on different days, to ensure independence.

Luciferase assay

36. A commercial luciferase assay reagent, Steady-Glo Luciferase Assay System (Promega, E2510 and its equivalents) or a standard luciferase assay system (Promega, E1500 and its equivalents) can be used for the agonism detection and Dual-Glo (Promega, E2920 and its equivalents) can be used for the antagonism detection, as long as the acceptability criteria is 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. In case of using Steady-Glo Luciferase Assay System (Promega, E2510) in the agonist assay, 40μL of prepared reagent should be directly added into the assay wells. In case of 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

37. **For Agonist assay**, to obtain the relative transcriptional activity to PC (10 nM of DHT), the luminescence signals from the same plate can be analysed according to the following steps (other equivalent mathematical processes are also acceptable):

    Step 1. Calculate mean value for the VC.

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

    Step 3. Calculate the mean for the normalised PC.

    Step 4. Divide the normalised value of each well in the plate by the mean value of the normalised 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).

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

Step 1. Calculate mean value for the VC.

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

Step 3. Calculate the mean for the normalised SPK.

Step 4. Divide the normalised value of each well in the plate by the mean value of the normalised mean SPK (SPK=100%).

The final value of each well is the relative transcriptional activity for that well compared to the maximum SPK 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).

EC50, PC50, PC10, IC50, lin.IC50 and lin.IC30 induction considerations

39. The full concentration-response curve is required for the calculation of the EC50 (IC50), but this may not always be achievable or practical due to limitations of the test concentration range (for example due to cytotoxicity or solubility problems). However, as the EC50 (IC50) and maximum induction level (corresponding to the top value of the Hill-equation) are informative parameters, these parameters should be reported where possible. For the calculation of EC50 (IC50)and maximum induction level, appropriate statistical software should be used (*e.g.* Graphpad Prism statistical software).

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

41. If the Hill's logistic equation is applicable to the concentration response data, the EC50 (IC50) should be calculated by the following equation (15):

Y=Bottom + (Top-Bottom) / (1+10 exp ((log EC50 (IC50) -X) x Hill slope))

Where:

X is the logarithm of concentration; and,

Y is the response and Y starts at the Bottom and goes to the Top in a sigmoid curve.

Bottom is fixed at zero in the Hill's logistic equation.

42. For each test chemical, the following should be provided:

(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, as well as the PCMax (concentration associated with the RPCMax); and

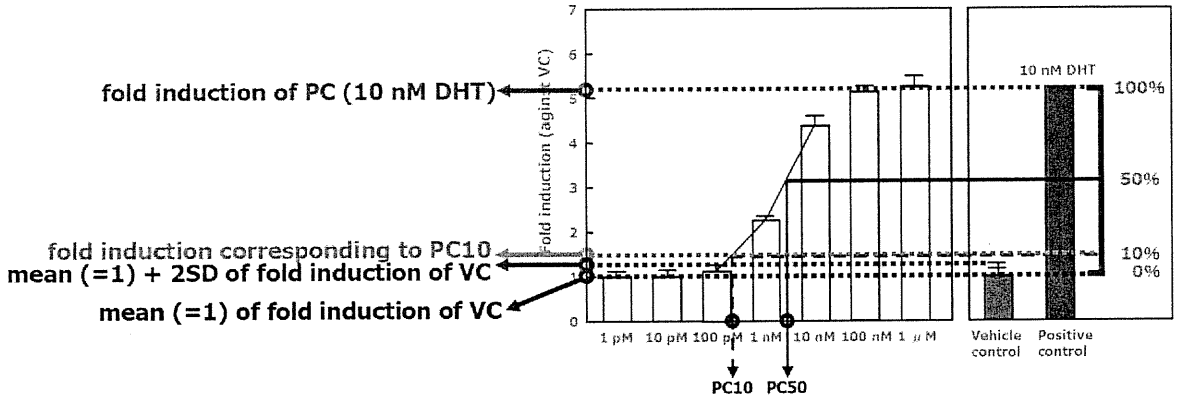
(ii) For positive chemicals, the concentrations that induce the PC10 and, if appropriate, the PC50.

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

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

44. Descriptions of PC values are provided in Figure 1 below.

Figure 1: Example of how to derive PC-values.
The PC (Positive control; 10 nM of DHT) is included on each assay plate

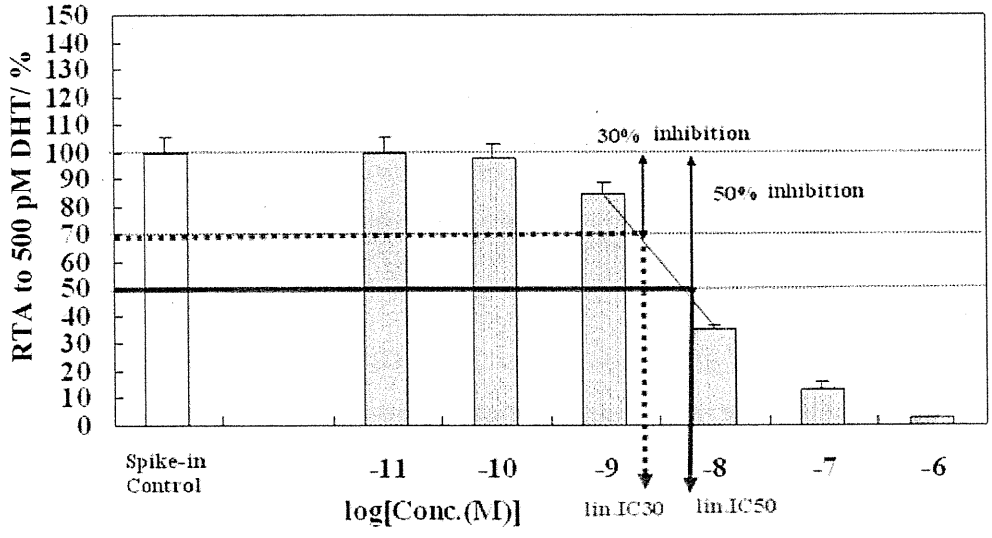


45. In case of antagonist assay, (i) The RICMax which is the maximum inhibition level of luciferase induced by a test chemical, expressed as a percentage of the response induced by 500 pM DTH on the same plate, as well as the PCMax (concentration associated with the RPCMax); and if the RICmax is exist, the lin.IC50 and lin.IC30 values should be calculated. These lin.IC50 and lin.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 same as PC values. Each lin.ICx value can be calculated by a simple linear regression using two variable data points in the transcription activity same as PCx values.

46. Descriptions of lin.ICx values are provided in Figure 2 below.

Figure 2.: Example of how to derive lin.IC-values.
The SPK-in control (DMSO at 0.1% spiked with 500 pM DHT) is included on each assay plate.



47. 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 that of the mean VC on each plate
  - The fold induction corresponding to the 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 3 reference chemicals should be within the acceptable range (Table 1-1).

  <for AR antagonist assay>

  - Fold induction of spike-in ([Spike-in of 500 pM DHT]/[Vehicle Control]) should be at least 5.0.
  - The PC_{ATG} (0.1 μM of HF) inhibitory ratio should be greater than 0.46.
  - The results of 3 reference chemicals should be within the acceptable range (Table 1-2).
- Be reproducible.

Data Interpretation Criteria

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

Positive	If the 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 the RPCMax fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

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

Positive	If the RICMax is obtained that is equal to or exceeds 30% inhibition against of the PC_{ATG} response (500 pM DHT) in at least two of two or two of three runs.
Negative	If the RPCMax fails to achieve at least 30% inhibition against of the PC_{ATG} response in two of two or two of three runs.

48. Data interpretation criteria are shown in Table 5-1 and 5-2. Positive results will be characterised by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (PC50) or 10% (PC10) **for antagonist assay**, and 50% (lin.IC50) or 30% (lin.IC30) **for antagonist assay** are reached accomplishes both of these goals. However, a test chemical is determined to be positive, if the maximum response induction or inhibition by the test chemical (RPCMax or RICMax) is equal to or exceeds 10% or 30% of the the positive control responses in at least two of two or two of three runs, while a test chemical is considered negative if the RPCMax or RICMax fails to achieve at least 10% or 30% of the response of the positive control in two of two or two of three runs.

49. The calculations of PC10, PC50 and PCMax for antagonist assay, and lin.IC50, lin.IC30 and RICMax can be calculated by using a spreadsheet available with the Test Guideline on the OECD public website2.

50. It should be sufficient to obtain PCx or lin.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 may 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 PC10 should be less than 20%.

51. 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 insurance that accurate data were produced, see paragraphs 47 and 48.

52. Where more information is required in addition to the screening and prioritisation purposes of this TG for positive test compounds, particularly for PC10-PC49 chemicals, it can be confirmed that the observed luciferase-activity is solely an AR-specific response, using an AR antagonist.

TEST REPORT

53. 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 logEC50, logPC50, logPC10 and Hill slope values for concurrent reference chemicals.

Results:

- Raw and normalised data of luminescent signals;
- Concentration-response relationship, where possible;
- PC10, PC50 and PCMax for antagonist assay, and lin.IC50, lin.IC30 and RICMax 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

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ANNEX 1

Definitions and abbreviations

Agonist: A substance that binds to a specific receptor and triggers a response in the cell. It mimics the action of an endogenous ligand binds to the same receptor.

Androgenic activity, the capability of a chemical to mimic 5α-Dehydrotestosterone in its ability to bind to and activate androgen receptors. AR mediated specific androgenic activity can be detected in this Test Guideline.

Antagonist: A type of receptor ligand or chemical that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses.

Anti-androgenic activity, the capability of a chemical to suppress the action of 5α-Dehydrotestosterone mediated through androgen receptors. AR mediated specific anti-androgenic activity can be detected in this Test Guideline.

AR: Androgen receptor

ARTA: Androgen Receptor Transcriptional Activation Assay.

BisA: Bisphenol A

CV: Coefficient of variation

Cytotoxicity: the harmful effects to cell structure or function ultimately causing cell death and can be a result of a reduction in the number of cells present in the well at the end of the exposure period or a reduction of the capacity for a measure of cellular function when compared to the concurrent vehicle control.

DCC-FBS: Dextran-coated charcoal treated fetal bovine serum.

DEHP: Di(2-ethylhexyl)phthalate

DHT: 5α-Dehydrotestosterone

DMSO: Dimethyl sulfoxide

EC50 value, the concentration of agonist that provokes a response halfway between the baseline (Bottom) and maximum response (Top).

ER; Estrogen receptor

ERE: Estrogen Response Element

Estrogenic activity, the capability of a chemical to mimic 17β-estradiol in its ability to bind to and activate estrogen receptors.

FBS: Fetal bovine serum

hERα: Human estrogen receptor alpha

HF: Hydroxyflutamide

lin.IC50: the concentration of a test chemical at which the measured activity in an antagonist assay inhibits at level of 50% of the maximum activity induced by 500 nM DHT in each plate

lin.IC30: the concentration of a test chemical at which the measured activity in an antagonist assay inhibits at level of 30% of the maximum activity induced by 500 nM DHT in each plate

PC: Positive contro l (DHT at 10 nM)

PC10: the concentration of a test chemical at which the response in an agonist assay is 10% of the response induced by positive control l (DHT at 10 nM) in each plate

PC50: the concentration of a test chemical at which the response in an agonist assay is 50% of the response induced by positive control l (DHT at 10 nM)in each plate

PCMax: the concentration of a test chemical inducing the RPCMax

R1881: Methyltrienolone

RICMax: maximum level of luciferase activity inhibited by a test chemical, expressed as a percentage of the response induced by 500 pM DHT on the same plate

RPCMax: maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate

RT PCR: Real Time polymerase chain reaction

SD: Standard deviation

STTA: Stably Transfected Transcriptional Activation Assay.

TA: Transcriptional activation

Validation, a process based on scientifically sound principles by which the reliability and relevance of a particular test, approach, method, or process are established for a specific purpose.

Reliability is defined as the extent of reproducibility of results from a test within and among laboratories over time, when performed using the same standardised protocol. The relevance of a test method describes the relationship between the test and the effect in the target species and whether the test method is meaningful and useful for a defined purpose, with the limitations identified. In brief, it is the extent to which the test method correctly measures or predicts the (biological) effect of interest, as appropriate (16).

VC (Vehicle control): The vehicle that is used to dissolve test and control chemicals is tested solely as vehicle without dissolved chemical.

ANNEX 2

Preparation of Serum treated with Dextran Coated Charcoal (DCC)

1. The treatment of serum with dextran-coated charcoal (DCC) is a general method for removal of estrogenic compounds from serum that is added to cell medium, in order to exclude the biased response associated with residual estrogens in serum. 500 mL of fetal bovine serum (FBS) can be treated by this procedure.

Components

2. The following materials and equipment will be required:

Materials

Activated charcoal
Dextran
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)
Sucrose
1 M HEPES buffer solution (pH 7.4)
Ultrapure water produced from a filter system

Equipment

Autoclaved glass container (size should be adjusted as appropriate)
General Laboratory Centrifuge (that can set temperature at 4°C)

Procedure

3. The following procedure is adjusted for the use of 50 mL centrifuge tubes:

[Day-1] Prepare dextran- coated charcoal suspension with 1 L of ultrapure water containing 1.5 mM of $MgCl_2$, 0.25 M sucrose, 2.5 g of charcoal, 0.25 g dextran and 5 mM of HEPES and stir it at 4°C, overnight.

[Day-2] Dispense the suspension in 50 mL centrifuge tubes and centrifuge at 10000 rpm at 4°C for 10 minutes. Remove the supernatant and store half of the charcoal sediment at 4°C for the use on Day-3. Suspend the other half of the charcoal with FBS that has been gently thawed to avoid precipitation, and heat-inactivated at 56°C for 30 minutes, then

transfer into an autoclaved glass container such as an Erlenmeyer flask. Stir this suspension gently at 4°C, overnight.

[Day-3] Dispense the suspension with FBS into centrifuge tubes for centrifugation at 10000 rpm at 4°C for 10 minutes. Collect FBS and transfer into the new charcoal sediment prepared and stored on Day-2. Suspend the charcoal sediment and stir this suspension gently in an autoclaved glass container at 4°C, overnight.

[Day-4] Dispense the suspension for centrifugation at 10000 rpm at 4°C for 10 minutes and sterilise the supernatant by filtration through 0.2 μm sterile filter. This DCC treated FBS should be stored at -20°C and can be used for up a year.