OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Androgen receptor (AR) mediated stably transfected transcriptional activation assay to detect AR agonist and antagonist using 22Rv1/MMTV_GR-KO cell line

INITIAL CONSIDERATIONS AND LIMITATIONS

1. The responses of nuclear receptors exert important roles in transcriptional activation (TA) (1, 2). Nuclear receptor families like the estrogen receptor (ER) or androgen receptor (AR), initiate the endocrine disruptor response. When ligands with hormonal functions bind to the nuclear receptors within cells, the ligand-receptor complex undergoes a conformational change that leads to a transcriptional response (3). Therefore, the TA mediated by endocrine disruptors is the plausible starting point for the hazard assessment. Considering the mechanisms by which hormones act, the reporter gene assay responses to the nuclear receptor complex can be used to detect the hormonal activity of exogenous chemicals (4).

2. The 22Rv1/MMTV_GR-KO androgen receptor (AR)-mediated stably transfected transcriptional activation (TA) assay is established to screen chemicals for potential endocrine disrupting activity via interaction with the AR using a human prostate cancer cell line, 22Rv1, that endogenously expressed the AR (5–7). This test method is specifically designed to detect human AR-mediated transcriptional activation and inhibition by measuring luciferase activity as the endpoint.

3. The 22Rv1 cell line has been developed to have minimal glucocorticoid receptor (GR)-mediated response. To solve the problem of GR expression in cells, we developed a GR-knockout 22Rv1/MMTV cell line using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system to avoid further controversy (7).

4. The 22Rv1/MMTV_GR-KO AR TA assay was validated with OECD VMG-NA to involve in the Performance Based Test Guideline (PBTG) for detecting the AR agonists and antagonists of level 2 in "OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals" (8, 9). The validation study of the

22Rv1/MMTV_GR-KO AR TA assay was conducted according to OECD Guidance Document (GD) 34, and it demonstrated the relevance and reliability of the assay for its intended purpose (10, 11).

5. The "General Introduction" and "AR TA test method components" should be read before using this test method for regulatory purpose.

PRINCIPLE OF THE TEST

6. The test system provided in this Test Guideline utilises the 22Rv1/MMTV_GR-KO cell line, which is derived from a 22Rv1 cell line. This cell line consists of stably transformed 22Rv1 cells with one pGL4 reporter plasmid containing the firefly luciferase gene under the control of an androgen-dependent promoter mouse mammary tumour virus (MMTV).

7. Data interpretation for an AR agonistic effect is based upon the maximum response level induced by a test chemical. If this response equals or exceeds 10% of the response induced by 10 nM 5α-dihydrotestosterone (DHT), the positive AR agonist control (PC_{AGO}) (i.e. the log PC_{10}), the test chemical is considered positive. Data interpretation for an AR antagonist effect of a test chemical is decided by two steps. i) A cut-off of a 30% inhibitory response of test chemical against 800 pM DHT (i.e. the log IC_{30}) and ii) R^2 value less than 0.9 in specificity control assay. If both criteria are addressed, then the chemical is considered a positive AR antagonist.

Name of method

8. Androgen receptor (AR)-mediated stably transfected transcriptional activation assay to detect androgen and anti-androgen activities using 22Rv1/MMTV_GR-KO cells.

Objectives and applications

9. The purpose of this test is to evaluate the potential for (anti)androgenic activity of chemicals via interaction with androgen receptor. This transcriptional activation assay can be used for *in vitro* screening of potential (anti)androgenic effects of industrial chemicals, drugs, cosmetic ingredients, pesticides, food additives and others (e.g. complex mixtures).

TEST METHODS

Stability of the cell line

10. To maintain the integrity of the response, the cells should not be cultured for more than 30 passages. Cell-doubling time is 48 hours.

Cell line maintenance and plating conditions

11. The maintenance protocol for the 22Rv1/MMTV_GR-KO cell line is based on the ATCC 22Rv1 maintenance protocol. 22Rv1/MMTV_GR-KO cells are maintained in a culture medium that includes 200 µg/mL hygromycin as a luciferase gene selection marker to be used the first time after thawing cells. 2× Trypsin-EDTA is preferred over 1× Trypsin-EDTA for passage of 22Rv1/MMTV_GR-KO cell line, because the higher concentration improves cell dissociation from the cell culture plate. All of the cell culture media should be warmed in a 37 °C water bath before use. For the assay, cells should be suspended at 3.0×10^5 per 1 mL with test medium (5% Dextran-Coated Charcoal treated FBS, 1% GlutaMAX and 1% anti–anti in phenol red-free RPMI 1640). 100 µL aliquots of suspended cells (at a density of 3×10^4 cells /well) should be transferred into a 96-well white plate. Cells are pre-incubated for 48 hours at 37 °C in a 5% CO_2 incubator prior to exposure.

Positive and vehicle controls

12. For the AR agonist assay, positive control (PC_{AGO1}) wells (n = 6) treated with a 10 nM dihydrotestosterone (DHT) and vehicle control (VC) wells (n = 6) containing only 0.1%

DMSO, should be prepared on each plate. The 10 nM DHT concentration is selected in order to achieve 100% response in the AR agonist assay.

13. For the AR antagonist assay, VC wells (n = 3), positive agonist control (PC_{AGO2}; 800 pM DHT) wells (n = 3), positive AR antagonist control (PC_{ANTA}; 800 pM DHT and 1 μM of bicalutamide) wells (n = 3), and positive cytotoxicity control (PC_{CT}; 1 mM SDS) wells (n = 3) should be included for each plate.

Positive and negative reference substances

14. For conducting the AR agonist assay, three well-characterised reference substances (positive substances: DHT and mestanolone, negative substance: DEHP) should be verified. In addition, reference substances for the AR antagonist assay include two positive (bicalutamide and bisphenol A) and one negative (DEHP) substance. These reference substances should always be included in AR agonist/antagonist assays.

Quality criteria for AR agonist/antagonist assay

15. The mean luciferase activity of the PC_{AGO} (AR agonist assay: 10 nM DHT (PC_{AGO1}); AR antagonist assay: 800 pM DHT (PC_{AGO2})) should be at least 13-fold greater than the mean VC on each plate for the AR agonist assay, and at least 10-fold greater than the mean VC for the AR antagonist assay. With respect to the quality control of the assay, the fold-induction corresponding to the $logPC_{10}$ (10%) of PC_{AGO1} (10 nM DHT) should be greater than 1+2SD of the fold-induction value (= 1) of the concurrent VC. Relative transcriptional activity (RTA) of PC_{ANTA} (800 pM DHT and 1 μM bicalutamide) should be less than 53.6% of the PC_{AGO2} in the AR antagonist assay.

Table 1. Acceptability criteria for AR agonist assay

Chemicals	Log PC_{10}	Log PC_{50}	Test Range
5α-Dihydrotestosterone	−12.2 to −9.7	−10.6 to −9.0	10^{-6} to 10^{-12} M
Mestanolone	−12.3 to −9.8	−10.2 to −8.6	10^{-6} to 10^{-12} M
Diethylhexyl phthalate	–	–	10^{-5} to 10^{-11} M
Fold induction of PC_{AGO1}	≥ 13		
Fold-induction of PC_{10}	Greater than 1+2SD (fold-induction of VC)		

Fold-induction of PC_{AGO1} is calculated using the following equation:

$$\text{Fold-induction of } PC_{AGO1} = \frac{\text{Mean RLU of } PC_{AGO1} \text{ (10 nM DHT)}}{\text{Mean RLU of Vehicle control}}$$

- RLU: relative light units

Table 2. Acceptability criteria for AR antagonist assay

Chemicals	Log IC_{30}	Log IC_{50}	Test Range
Bicalutamide	−7.5 to −6.2	−7.0 to −5.8	10^{-4} to 10^{-10} M
Bisphenol A	−6.6 to −5.4	−6.2 to −5.0	10^{-5} to 10^{-11} M
Diethylhexyl phthalate	-	-	10^{-5} to 10^{-11} M
Fold induction of PC_{AGO2}	≥ 10		
RTA of PC_{ANTA} (%)	≤53.6		

Fold-induction of PC_{AGO2} is calculated using the following equation:

$$\text{Fold-induction of } PC_{AGO2} = \frac{\text{Mean RLU of } PC_{AGO2} \text{ (800 pM DHT)}}{\text{Mean RLU of Vehicle control}}$$

$$\text{RTA of } PC_{ANTA} \text{ (\%)} = \frac{\text{Mean RLU of } PC_{ANTA} - \text{Mean RLU of VC}}{\text{Mean RLU of } PC_{AGO2} - \text{Mean RLU of VC}} \times 100$$

- RTA: relative transcriptional activity

Solubility test

16. Test substance stocks are prepared at a maximum concentration of up to 1 M in DMSO or an appropriate solvent. If precipitation occurs, the stock solution should be diluted with solvent at a ratio of 1:10 with DMSO until no precipitation is observed.

CHEMICAL EXPOSURE AND ASSAY PLATE DESIGN

Preliminary test in AR agonist assay

17. The maximal stock concentration of each test substance, determined by the solubility test (see above), should be serially diluted at a ratio of 1:10 in DMSO. The serial dilutions of test substances should be diluted with aqueous media. The recommended

final volume for each well is 100 µL. Triplicate wells are used for each concentration. The final DMSO concentration in the assay well is 0.1%. The reference substances for the AR agonist assay (DHT, mestanolone and DEHP) should be tested in every assay. Wells treated with 10 nM DHT (PC_{AGO1}) and wells treated with 0.1% DMSO alone (VC) should be included in each plate for the AR agonist assay (Table 3). An example of the plate design of test substances is provided in Table 4.

Table 3. Example of plate concentration assignment for the reference substances

	DHT			**Mestanolone**			**DEHP**			**Test Substance**		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6} M	→	→	10^{-6} M	→	→	10^{-5} M	→	→	10^{-3} M	→	→
B	10^{-7} M	→	→	10^{-7} M	→	→	10^{-6} M	→	→	10^{-4} M	→	→
C	10^{-8} M	→	→	10^{-8} M	→	→	10^{-7} M	→	→	10^{-5} M	→	→
D	10^{-9} M	→	→	10^{-9} M	→	→	10^{-8} M	→	→	10^{-6} M	→	→
E	10^{-10} M	→	→	10^{-10} M	→	→	10^{-9} M	→	→	10^{-7} M	→	→
F	10^{-11} M	→	→	10^{-11} M	→	→	10^{-10} M	→	→	10^{-8} M	→	→
G	10^{-12} M	→	→	10^{-12} M	→	→	10^{-11} M	→	→	10^{-9} M	→	→
H	**VC**						**PC_{AGO1}**					

▪ VC: Vehicle control (0.1% DMSO)
▪ PC_{AGO1}: Positive control for AR agonist assay (10 nM DHT)

Table 4. Example of plate concentration assignment for the test substances

	Test Substance 1			**Test Substance 2**			**Test Substance 3**			**Test Substance 4**		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-3} M	→	→	10^{-3} M	→	→	10^{-5} M	→	→	10^{-6} M	→	→
B	10^{-4} M	→	→	10^{-4} M	→	→	10^{-6} M	→	→	10^{-7} M	→	→
C	10^{-5} M	→	→	10^{-5} M	→	→	10^{-7} M	→	→	10^{-8} M	→	→
D	10^{-6} M	→	→	10^{-6} M	→	→	10^{-8} M	→	→	10^{-9} M	→	→
E	10^{-7} M	→	→	10^{-7} M	→	→	10^{-9} M	→	→	10^{-10} M	→	→
F	10^{-8} M	→	→	10^{-8} M	→	→	10^{-10} M	→	→	10^{-11} M	→	→
G	10^{-9} M	→	→	10^{-9} M	→	→	10^{-11} M	→	→	10^{-12} M	→	→
H	**VC**						**PC_{AGO1}**					

▪ VC: Vehicle control (0.1% DMSO)
▪ PC_{AGO1}: Positive control for AR agonist assay (10 nM DHT)

Comprehensive test in AR agonist assay

18. The maximal concentration of the test substance, determined from the concentration response curve generated in the preliminary test, should be serially diluted at a ratio of 1:3 or 1:5 in DMSO. These serially diluted test substances should be added to the wells

of a plate and all concentrations should be tested in triplicate. All tests should be conducted at concentrations where the concentration–response curve can be well characterised. To achieve these conditions, solutions found to contain insoluble solids or concentrations found to induce cytotoxic effects against cell lines should not be included. The recommended final volume for each well is 100 µL. The plate layout for the reference and test substances run in the comprehensive test is the same as for the preliminary test.

Preliminary test in AR antagonist assay

19. The maximal concentration of each test substance, determined by solubility limits, should be serially diluted at a ratio of 1:10 in DMSO. Serially diluted test substance should be added to the wells of an assay plate and all concentrations should be tested in triplicate. The recommended final volume for each well is 100 µL. The AR antagonist assay reference substances (bicalutamide, bisphenol A and DEHP) should be tested in every assay. A positive AR agonist control (PC_{AGO2}; 800 pM DHT), a positive AR antagonist control (PC_{ANTA}; 800 pM DHT and 1 µM bicalutamide) and cytotoxic positive control (PC_{CT}; 1 mM SDS) should be prepared for the AR antagonist assay (Table 5). The plate design of test substances is provided in Table 6.

Table 5. Example of plate concentration assignment for the reference substances

	Bicalutamide			**Bisphenol A**			**DEHP**			**Test Substance 1**		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-4} M	→	→	10^{-5} M	→	→	10^{-5} M	→	→	10^{-3} M	→	→
B	10^{-5} M	→	→	10^{-6} M	→	→	10^{-6} M	→	→	10^{-4} M	→	→
C	10^{-6} M	→	→	10^{-7} M	→	→	10^{-7} M	→	→	10^{-5} M	→	→
D	10^{-7} M	→	→	10^{-8} M	→	→	10^{-8} M	→	→	10^{-6} M	→	→
E	10^{-8} M	→	→	10^{-9} M	→	→	10^{-9} M	→	→	10^{-7} M	→	→
F	10^{-9} M	→	→	10^{-10} M	→	→	10^{-10} M	→	→	10^{-8} M	→	→
G	10^{-10} M	→	→	10^{-11} M	→	→	10^{-11} M	→	→	10^{-9} M	→	→
H	**VC**			**PC_{AGO2}**			**PC_{ANTA}**			**PC_{CT}**		

- VC: Vehicle control (0.1% DMSO)
- PC_{AGO2}: Agonist positive control for AR antagonist assay (800 pM DHT)
- PC_{ANTA}: Antagonist positive control (1 µM bicalutamide)
- PC_{CT}: Cytotoxic positive control (1 mM SDS)
- Grey wells include 800 pM DHT

Table 6. Example of plate concentration assignment for test substances

	Test Substance 1			Test Substance 2			Test Substance 3			Test Substance 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-3} M	→	→	10^{-3} M	→	→	10^{-5} M	→	→	10^{-3} M	→	→
B	10^{-4} M	→	→	10^{-4} M	→	→	10^{-6} M	→	→	10^{-4} M	→	→
C	10^{-5} M	→	→	10^{-5} M	→	→	10^{-7} M	→	→	10^{-5} M	→	→
D	10^{-6} M	→	→	10^{-6} M	→	→	10^{-8} M	→	→	10^{-6} M	→	→
E	10^{-7} M	→	→	10^{-7} M	→	→	10^{-9} M	→	→	10^{-7} M	→	→
F	10^{-8} M	→	→	10^{-8} M	→	→	10^{-10} M	→	→	10^{-8} M	→	→
G	10^{-9} M	→	→	10^{-9} M	→	→	10^{-11} M	→	→	10^{-9} M	→	→
H	**VC**			**PC_{AGO2}**			**PC_{ANTA}**			**PC_{CT}**		

- VC: Vehicle control (0.1% DMSO)
- PC_{AGO2}: Agonist positive control for AR antagonist assay (800 pM DHT)
- PC_{ANTA}: Antagonist positive control (1 µM bicalutamide)
- PC_{CT}: Cytotoxic positive control (1 mM SDS)
- Grey wells include 800 pM DHT

Comprehensive test in AR antagonist assay

20. The maximal concentration of the test substance, determined from the concentration–response curves generated in the preliminary test, should be serially diluted at a ratio of 1:3 or 1:5 in DMSO. Seven different concentrations of serially-diluted test substance should be added to the wells of an assay plate and all concentrations should be tested in triplicate. The recommended final volume for each well is 100 µL. The plate layout for the reference and test substances for the comprehensive test is the same as for the preliminary test (Tables 5 and 6).

Specificity control assay

21. To ensure clarity for the identification of AR antagonist among test substances that are determined to be positive in the preliminary test and comprehensive test, a specificity control assay is conducted using 800 pM DHT and 100 nM DHT. The experimental procedure is the same as for the comprehensive test. A positive AR agonist control (PC_{AGO2}; 800 pM DHT), a positive AR antagonist control (PC_{ANTA}; 800 pM DHT and 1 µM bicalutamide) and cytotoxic positive control (PC_{CT}; 1 mM SDS) should be prepared for the AR antagonist assay. The plate layout is given in Table 7.

Table 7. Example of plate concentration assignment of test substances

	Test substance 1 (log M)						Test substance 2 (log M)					
	1	2	3	4	5	6	7	8	9	10	11	12
A	−5	→	→	−5	→	→	−4	→	→	−4	→	→
B	−5.7	→	→	−5.7	→	→	−4.7	→	→	−4.7	→	→
C	−6.4	→	→	−6.4	→	→	−5.4	→	→	−5.4	→	→
D	−7.1	→	→	−7.1	→	→	−6.1	→	→	−6.1	→	→
E	−7.8	→	→	−7.8	→	→	−6.8	→	→	−6.8	→	→
F	−8.5	→	→	−8.5	→	→	−7.5	→	→	−7.5	→	→
G	−9.2	→	→	−9.2	→	→	−8.2	→	→	−8.2	→	→
H	**VC**			**PC_{AGO2}**			**PC_{ANTA}**			**PC_{CT}**		

- VC: Vehicle control (DMSO);
- PC_{AGO2}: Positive control (800 pM of DHT);
- PC_{ANTA}: Positive control (1 μM of bicalutamide);
- PC_{CT}: Cytotoxicity control (1 mM of SDS);
- Grey wells are spiked with 800 pM DHT
- Dark grey wells are spiked with 100 nM DHT

Endpoint measurements

22. Endpoint measurements are carried out using the Steady-Glo Luciferase assay system for the luciferase assay. The assay reagent is mixed thoroughly with RPMI1640 medium (serum-free) at room temperature (1:1 ratio mixture). In the AR antagonist assay, cytotoxic substances may reduce the AR-mediated transcriptional activation (i.e. luciferase activity); thus, an additional assay is required to exclude false positives. To evaluate the cytotoxicity of the test substances, the live-cell protease detection assay is performed by CellTiter-Fluor™ assay reagent (Promega) in combination with the anti-androgenic activity test.

$$\text{Cell viability (\%)} = \frac{\text{Mean RFU of test substance} - \text{Mean RFU of } PC_{CT}}{\text{Mean RFU of } PC_{AGO2} - \text{Mean RFU of } PC_{CT}} \times 100$$

23. For AR agonist assay:

    1. After incubating cells with chemicals for 20–24 hours, remove the media from each well.
    2. To perform the test, mix well the solution, containing the assay reagent and

RPMI1640 (serum-free) at a ratio of 1:1 at room temperature.

3. Add 100 µL/well of Steady-Glo Luciferase assay reagent to the 96 well plates.
4. Cover the top of each plate with aluminium foil to block out the light, and leave at room temperature for 5–10 min.
5. Measure the luciferase activity using a luminescence reader.

24. For AR antagonist assay

1. Prepare 2 mL of cell viability assay reagent including 10 µL of glycyl phenylalanyl-aminofluorocoumarin (GF-AFC) substrate.
2. Add 20 µL/well of cell viability assay reagent in 96-well plates.
3. Mix the 96-well plate briefly using an orbital shaker.
4. Incubate the plates at 37 °C in a 5% CO_2 incubator for 1–3 hour.
5. Remove plates from incubator and measure resulting fluorescence using a fluorometer (380–400 nm Ex /505 nm Em) for cytotoxicity.
6. After measurement of resulting fluorescence, add 50 µL/well of Steady-Glo Luciferase assay reagent in each well.
7. Cover the top of each plate with aluminium foil to block the light, and leave at room temperature for 5–10 min.
8. Measure the luciferase activity using a luminescence reader.

DATA RECORDING AND ANALYSIS

25. The luminescence signal data, as read by the microplate reader in RLU units, is recorded and is transformed as follows:

    - The average for the positive control (AR agonist assay: 10 nM DHT, AR antagonist assay: 800 pM DHT) is set at 100%.
    - The average for vehicle control (0.1% DMSO) is set at 0%

26. For the agonist assay, the data transformation from RLU units is as follows:

Agonistic activity (%) = $\frac{\text{Mean RLU of test substance} - \text{Mean RLU of VC}}{}$ ×100

$$\text{Mean RLU of PC} - \text{Mean RLU of VC}$$

27. For the antagonist assay, the data transformation from RLU units is as follows:

$$\text{Antagonistic activity (\%)} = \frac{\text{Mean RLU of test substance} - \text{Mean RLU of VC}}{\text{Mean RLU of } PC_{AGO2} - \text{Mean RLU of VC}} \times 100$$

28. In the AR agonist assay, the following information should be provided for a positive test substance: the concentrations that induce an effect corresponding to that of a 10% effect for the positive control (log PC_{10}) and, if appropriate, the 50% effect for the positive control (log PC_{50}). Descriptions of log PC_x values, where "x" is a selected response, e.g. 10% or 50% induction, compared to PC_{AGO1}, are provided in Figure 1. Log PC_{10} and log PC_{50} values can be defined as the test substance concentrations estimated to elicit either a 10% or a 50% induction of transcriptional activity by PC_{AGO1} (10 nM of DHT). Each log PC_x 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 PC_x value have the coordinates (a, b) and (c, d) respectively, then the log PC_x value is calculated using the equation below and Figure 1 shows the method for the calculation of $\log[PC_{50}]$:

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

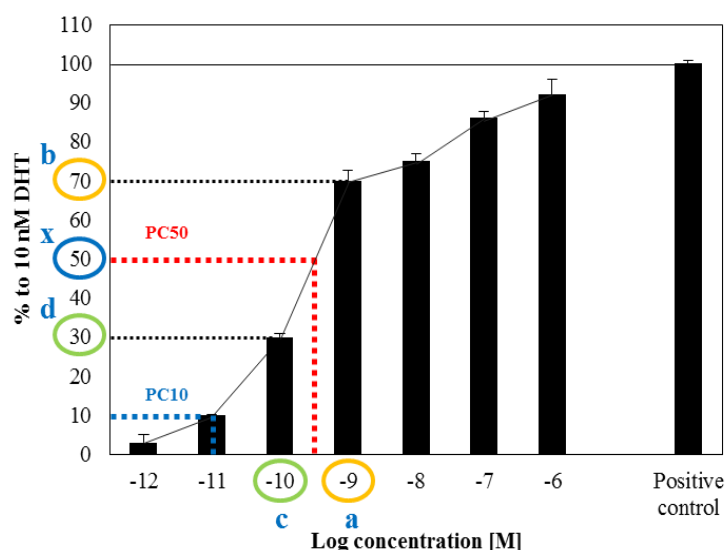

Figure 1. Schematic illustration of the calculation of log PC_x values

▪ The PC_{AGO1} (10 nM of DHT) is included on each assay plate in AR agonist assay.

29. For the AR antagonist assay, the following information should be provided for a positive test substance: the concentrations for 30% inhibition of transcriptional activity induced by 800 pM DHT (log IC_{30}) and, if appropriate, for 50% inhibition of activity by 800 pM DHT (log IC_{50}). Descriptions of log IC_x values, where "x" is a selected response, e.g. 30% or 50% inhibition, compared to PC_{AGO2}, are provided in Figure 2. Log IC_{50} and log IC_{30} values can be defined as the test substance concentrations estimated to elicit either a 50% or a 30% inhibition of transcriptional activity induced by 800 pM DHT. Each log IC_x 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 IC_x value have the coordinates (c, d) and (a, b) respectively, then the log IC_x value is calculated using the equation below and Figure 2 shows an illustration of the calculation of log[IC_{50}]:

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

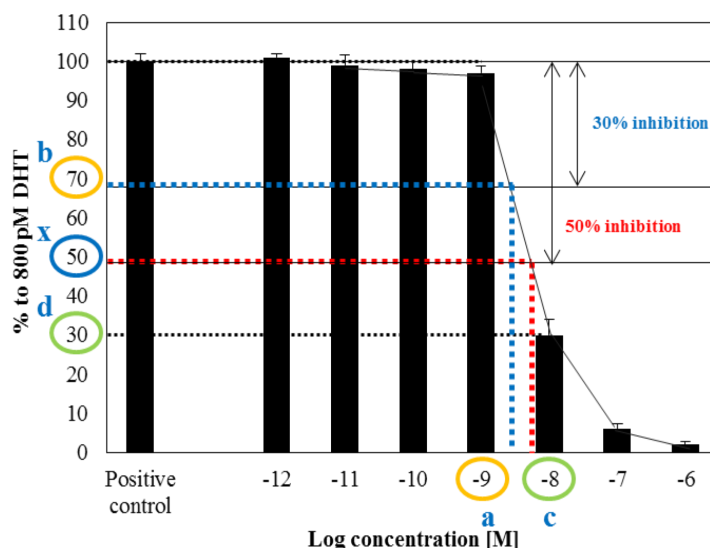

Figure 2. Schematic illustration of the calculation of log IC_x values

▪ The PC_{AGO2} (800 pM DHT) is included on each assay plate in AR antagonist assay.

30. 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. To evaluate the cytotoxicity of the test substances, the live-cell

protease detection assay was performed by CellTiter-Fluor™ assay reagent (Promega) in combination with the anti-androgenic activity test. Accordingly, AR-mediated transcriptional activity and cytotoxicity should be evaluated simultaneously in the same assay plate. For AR agonists, cytotoxicity can also affect the shape of a concentration response curve. In such case, evaluation of cytotoxicity should be performed or evaluated from the results of antagonist assay conducted for same test substance. Should the results of the cytotoxicity test show that the concentration of the test chemical has reduced CellTiter-Fluor™ protease activity 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.

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ANNEX A. The method to determine the max concentration for the comprehensive test

If test substances are determined to be positive in the preliminary test, comprehensive testing should be conducted to accurately determine the potency of test substances. All test substances classified as positive for AR agonistic activity should have a concentration–response curve consisting of a baseline, and a positive slope; all test substances classified as positive for AR antagonistic activity should have a concentration response curve consisting of a baseline, and a negative slope. If possible, PC_{10}, PC_{50}, IC_{30} and IC_{50} value should be calculated for each positive decision. The comprehensive AR agonist/antagonist assay consists of a seven-point serial dilution (either 1:3 or 1:5 serial dilution) with each concentration tested in triplicate wells of the 96-well plate.

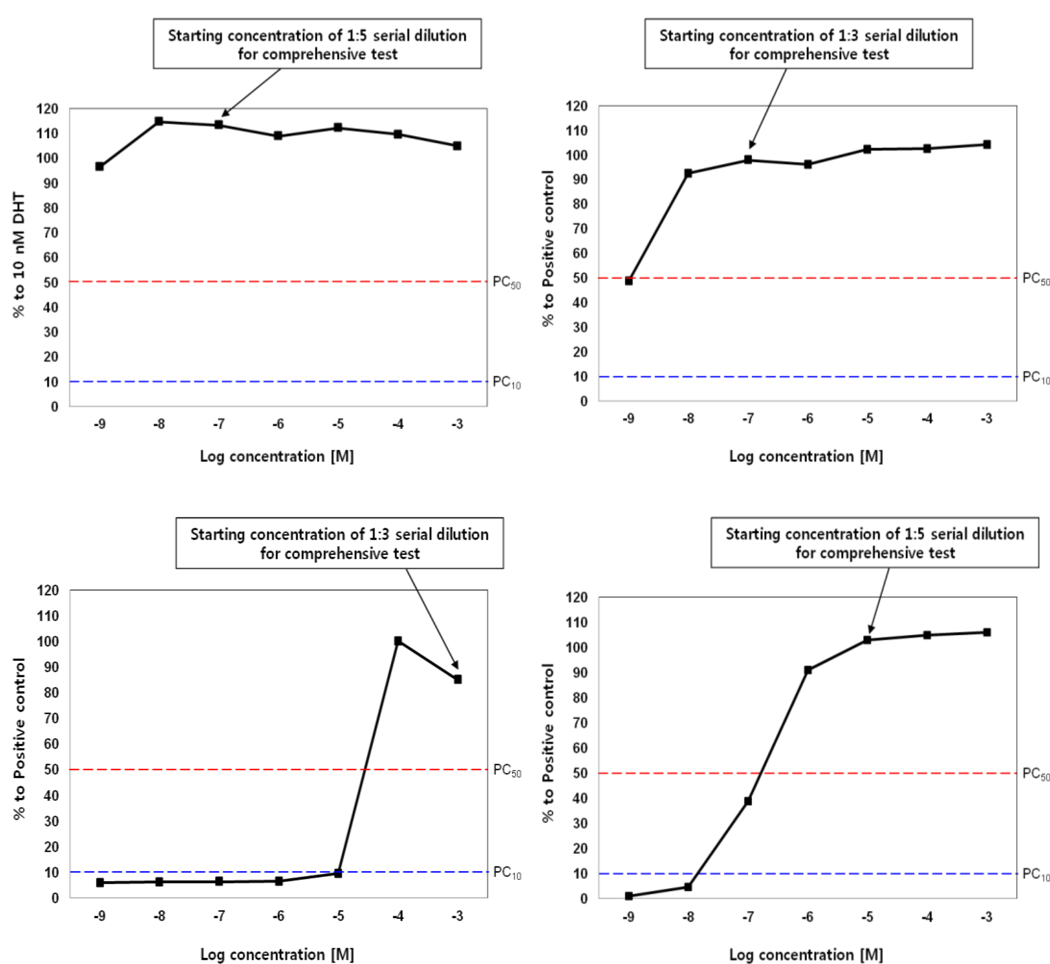

Figure 4. Examples to determine the starting concentration of serial dilution for comprehensive test in AR agonist assay

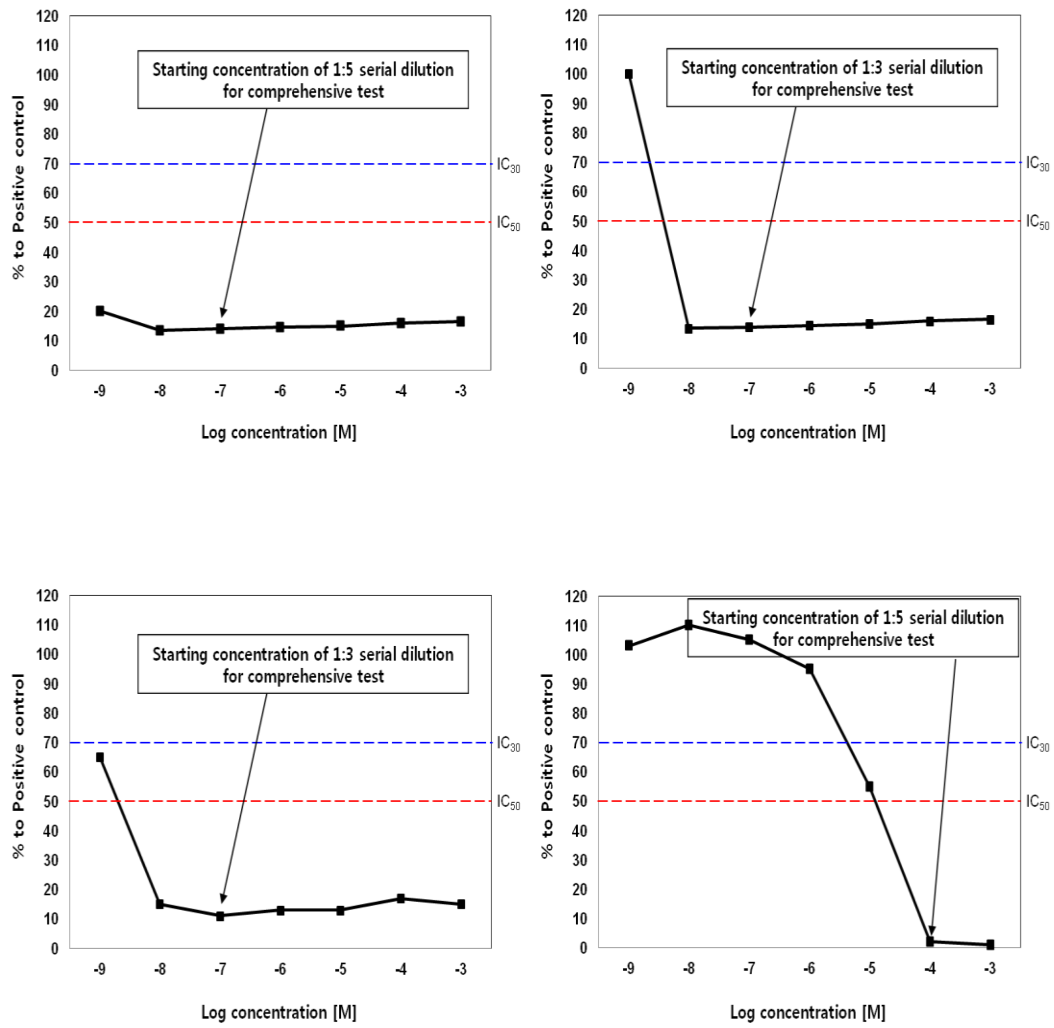

Figure 5. Examples to determine the starting concentration of serial dilution for comprehensive test in the AR antagonist assay