Endpoints	• EC50 (calculated from the Hill equation) • PC50 • PC10	• IC50 (calculated from the Hill equation) • lin. IC50 • lin. IC30	

6.1. SEEDING CELLS IN A 96-WELL PLATE

Cells to be used for the assay should be grown at 75-90% confluence in a 100 mm cell-culture dish.

(1) Warm the 10% DCC-FBS-EMEM at 37°C in the water bath.

(2) Remove the medium from the dish.

(3) Rinse the cells with 5 mL of PBS (-).

(4) Remove the PBS with a sterile pipette or sucker.

(5) Add 2 mL of Trypsin-EDTA solution, enough to coat the bottom of the culture dish, and then remove the excess.

Note: Be sure that Trypsin-EDTA solution coats the cells in the dish. If cells are not coated with Trypsin-EDTA, cells cannot be detached from the dish.

(6) Allow the Trypsin-treated cell to stand for about three minutes in a 5% CO_2 incubator at 37°C.

(7) (Monitor the cells under microscope. The cells are beginning to detach when they appear rounded.)

(8) Tap the dish gently to detach the cells from the bottom of the dish.

(9) Add 5 mL of 10% DCC-FBS-EMEM to remove the adherent cells and transfer the cell suspension to a centrifuge tube.

Note: Be sure that cells are separated as single cells.

(10) Count the number of cells.

(11) Dilute the cell suspension with 10% DCC-FBS-EMEM to obtain a final cell density of 1×10^5 cells/mL in a sterile reagent tray.

(12) Add 100 μL of cell suspension into each well of a 96-well assay plate (Nunc Catalog# 136102 (flat bottom, tissue culture treated, sterile) or its equivalents) using a multi-channel pipettor (and a 96-well plate for (Corning; Catalog#3595 (flat bottom, tissue culture treated, sterile) or its equivalent) for cytotoxicity testing in the anti-estrogenic assay).

Note: Be sure to re-suspend the cell suspension using the multi-channel pipettor prior to the addition to each well.

(13) Incubate the cells in a 5% CO_2 incubator at 37°C for 3-hr.

6.2. CHEMICAL EXPOSURE FOR ESTROGENIC ASSAY

Do not store the dilutions. Dilution of chemicals should be prepared on the day of assay.

6.2.1. Dilutions of Reference Chemicals in a 1.5 mL Polyprolylene (PP) tube

The stock solution stored at -20°C should be thawed at room temperature and be vortexed prior to use.

☐ **17β-Estradiol (E2)**

(1) Add 10 μL of 10 mM (=10^{-2} M) of E2 stock solution in 90 μL of DMSO to prepare 1 mM (=10^{-3} M) of E2 in a 1.5 mL PP tube.

(2) Add 10 μL of 1 mM of E2 in 90 μL of DMSO to prepare 100 μM (=10^{-4} M) of E2 in a 1.5 mL PP tube.

(3) Add 20 μL of 100 μM of E2 in 180 μL of DMSO to prepare 10 μM (=10^{-5} M) of E2 in a 1.5 mL PP tube.

(4) Add 20 μL of 10 μM of E2 in 180 μL of DMSO to prepare 1 μM (=10^{-6} M) of E2 in a 1.5 mL PP tube.

☐ **17α-Estradiol (α-E2)**

Add 10 μL of 10 mM (=10^{-2} M) of α-E2 stock solution in 90 uL of DMSO to prepare 1 mM (=10^{-3} M) of α-E2.

6.2.2. Preparation of DMSO Dilutions of the test chemicals in a 96-well plate (Fig. 4)

(1) Prepare a PP 96-well plate (Falcon; Catalog# 35-1190 or its equivalent) and a lid (Falcon; Catalog# 35-1191 or its equivalent).

(2) Add 90 μL of DMSO from "B1" – "G4" wells.

(3) Add 100 μL of 10 μM (=10^{-5}M) E2 in "A1" and "A2" wells.

(4) Add 100 μL of 1 mM (=10^{-3} M) α-E2 in "A3" well.

(5) Add 100 μL of 100 mM (=10^{-1} M) of Corticosterone in "A4" well.

(6) Take 10 μL of DMSO solutions in "A" row using multi-channel pipettor to add in corresponding "B" row , and mix well.

(7) Take 10 μL of DMSO solutions in "B" row using multi-channel pipettor to add in corresponding "C" row , and mix well.

(8) Continue these serial dilution steps to "G" row.

(9) Add 100 μL of DMSO in "H1" and "H2" wells.

(10) Add 100 μL of 1 μM E2 in "H3" and "H4" wells.

6.2.3. Dilution in a 96-well deep-well plate "with medium" (Fig. 4)

(1) Warm the 10% DCC-FBS-EMEM at 37°C.

(2) Prepare a 96-well PP deep-well plate (Greiner bio-one; Catalog#786261 or its equivalent).

(3) Dispense 500 μL of 10% DCC-FBS-EMEM in "A1" – "H4" wells using a multi-channel dispenser.

(4) Add 1.5 μL of DMSO dilutions in the corresponding well of the deep-well plate using multi-channel pipettor to prepare 3-fold concentration of the desired final concentration.

Dilutions with DMSO prepared				
	1	2	3	4
	E2	E2	α-E2	Cor.
A	10^{-5}M	10^{-5}M	10^{-3}M	10^{-1}M
B	10^{-6}M	10^{-6}M	10^{-4}M	10^{-2}M
C	10^{-7}M	10^{-7}M	10^{-5}M	10^{-3}M
D	10^{-8}M	10^{-8}M	10^{-6}M	10^{-4}M
E	10^{-9}M	10^{-9}M	10^{-7}M	10^{-5}M
F	10^{-10}M	10^{-10}M	10^{-8}M	10^{-6}M
G	10^{-11}M	10^{-11}M	10^{-9}M	10^{-7}M
H	DMSO	DMSO	1 μM of E2	1 μM of E2

deep-well plate						
	1	2	3	4	11	12
	E2	E2	α-E2	Cor.		
A	$3x10^{-8}$M	$3x10^{-8}$M	$3x10^{-6}$M	$3x10^{-4}$M		
B	$3x10^{-9}$M	$3x10^{-9}$M	$3x10^{-7}$M	$3x10^{-5}$M		
C	$3x10^{-10}$M	$3x10^{-10}$M	$3x10^{-8}$M	$3x10^{-6}$M		
D	$3x10^{-11}$M	$3x10^{-11}$M	$3x10^{-9}$M	$3x10^{-7}$M		
E	$3x10^{-12}$M	$3x10^{-12}$M	$3x10^{-10}$M	$3x10^{-8}$M		
F	$3x10^{-13}$M	$3x10^{-13}$M	$3x10^{-11}$M	$3x10^{-9}$M		
G	$3x10^{-14}$M	$3x10^{-14}$M	$3x10^{-12}$M	$3x10^{-10}$M		
H	0.3% DMSO	0.3% DMSO	3 nM of E2	3 nM of E2		

Fig. 4 Dilution plate layout (left: DMSO dilution, right Medium dilution)

6.2.4. Chemical Exposure to Cells

(1) Mix well the medium in the deep-well plate.

(2) Add 50 μL of the medium solution to the assay plate prepared in **"6.1."** in triplicate.

(3) Incubate for 20-24 hr in CO_2 incubator at 37°C.

	E2			E2			17α-Estradiol			Corticosterone		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-8}M	→	→	10^{-8}M	→	→	10^{-6}M	→	→	10^{-4}M	→	→
B	10^{-9}M	→	→	10^{-9}M	→	→	10^{-7}M	→	→	10^{-5}M	→	→
C	10^{-10}M	→	→	10^{-10}M	→	→	10^{-8}M	→	→	10^{-6}M	→	→
D	10^{-11}M	→	→	10^{-11}M	→	→	10^{-9}M	→	→	10^{-7}M	→	→
E	10^{-12}M	→	→	10^{-12}M	→	→	10^{-10}M	→	→	10^{-8}M	→	→
F	10^{-13}M	→	→	10^{-13}M	→	→	10^{-11}M	→	→	10^{-9}M	→	→
G	10^{-14}M	→	→	10^{-14}M	→	→	10^{-12}M	→	→	10^{-10}M	→	→
H	0.1% DMSO	→	→	0.1% DMSO	→	→	1 nM of E2	→	→	1 nM of E2	→	→

Fig. 5 Assay plate layout for estrogenic assay

6.3. CHEMICAL EXPOSURE FOR ANTI-ESTROGENIC ASSAY

Do not store the dilutions. Dilution of chemicals should be prepared on the day of assay.

The stock solution is stored at -20°C , it should be thawed at room temperature and be vortexed prior to use.

Note: Confirm that the chemicals in DMSO are completely dissolved.

6.3.1. Dilutions of Reference Chemicals and spike-in solution in a 1.5 mL Polyprolylene (PP) tube

☐ **17β-Estradiol (E2)**

(1) Add 10 μL of 10 mM of E2 stock solution in 90 μL of DMSO to prepare 1 mM of E2 in a 1.5 mL PP tube.

(2) Add 10 μL of 1 mM of E2 in 90 μL of DMSO to prepare 100 μM (=10^{-4} M) of E2 in a 1.5 mL PP tube.

(3) Add 10 μL of 100 μM of E2 in 90 μL of DMSO to prepare 10 μM (=10^{-5} M) of E2 in a 1.5 mL PP tube.

(4) Add 30 μL of 10 μM of E2 in 270 μL of DMSO to prepare 1 μM (=10^{-6} M) of E2 in a 1.5 mL PP tube.

(5) Add 10 μL of 1 μM of E2 in 90 μL of DMSO to prepare 100 nM (=10^{-7} M) of E2 in a 1.5 mL PP tube.

(6) Add 25 μL of 100 nM of E2 in 75 μL of DMSO to prepare 25 nM (=2.5x10^{-8} M) of E2 in a 1.5 mL PP tube*.

*: The volumes provided are for 1 set of plate (for anti-estrogenic and cytotoxicity testing). If more than 2 set of plates are tested, the volume should be adjusted as appropriate. 60 μL of 25 nM E2 is required for each set of plates.

☐ **4-Hydroxytamoxifen (OHT)**

(1) Add 20 μL of 10 mM (=10^{-2} M) of OHT stock solution in 180 uL to prepare 1 mM (=10^{-3} M) of OHT.

(2) Add 10 μL of 1 mM (=10^{-3} M) of OHT in 90 uL of DMSO to prepare 100 μM (=10^{-4} M) of OHT.

6.3.2. Preparation of DMSO Dilution of the test chemicals in a 96-well plate (Fig. 6)

(1) Prepare a PP 96-well plate (Falcon; Catalog# 35-1190 or its equivalent) and a lid (Falcon; Catalog# 35-1191 or its equivalent).

(2) Add 90 μL of DMSO from "B1" – "F4" wells.

(3) Add 100 μL of the stock of test chemical in wells of "A" row.

In case reference chemicals are used, add them according to the following procedure.

Add 100 μL of 100 μM (=10^{-4} M) of OHT in "A1" well.

Add 100 μL of 10 mM (=10^{-2} M) of TAM in "A2" well.

Add 100 μL of 100 mM (=10^{-1} M) of RU486 in "A3" well.

Add 100 μL of 100 mM (=10^{-1} M) of Flu. in "A4" well.

(4) Take 10 μL of DMSO solutions in "A" row using multi-channel pipettor to add in corresponding "B" row, and mix well.

(5) Take 10 μL of DMSO solutions in "B" row using multi-channel pipettor to add in corresponding "C" row, and mix well.

(6) Continue these serial dilution steps to row. "F"

(7) Add 100 μL of DMSO in "G1", "G2", "H1" and "H2" wells

(8) Add 100 μL of 1 μM E2 in "H3" and "H4" wells.

(9) Add 100 μL of 1 mM OHT in "G3" well.

(10) Add 100 μL of 100 mM Dig. In "G4" well.

6.3.3. 10% DCC-FBS-EMEM containing 75 pM E2

The following procedure provides the preparation of 75 pM (=7.5x10^{-11} M) E2 in medium.

(1) Add 60 μL of 25 nM (=2.5x10^{-8} M) of E2 in 20 mL of pre-warmed 10% DCC-FBS-EMEM in a sterile reagent tray and mix well to prepare 75 pM of E2 in pre-warmed medium at 37°C*.

(2) Dispense 500 μL of 75 pM of E2 in medium in "A1" – "G4" of a deep-well plate (Greiner bio-one, , 96-well 0.5 mL, Catalog# 786261 or 96-well 1 mL, Catalog#) (see Fig. 6).

*: The volumes provided are for 1 set of plate (for anti-estrogenic and cytotoxicity testing). If more than 2-plates are tested, the volume should be adjusted as appropriate. 20 mL of 10% DCC-FBS-EMEM allows comfortable handling to prepare each set of plates.

6.3.4. 10% DCC-FBS-EMEM containing 0.3% DMSO

This medium is used to prepare the vehicle control and E2 control (1 nM=10^{-9} M).

(1) Add 12 μL of DMSO in 4 mL of pre-warmed 10% DCC-FBS-EMEM at 37°C in a

sterile reagent tray and mix well*.

(2) Dispense 500 μL of 10% DCC-FBS-EMEM containing 0.3% DMSO in "H1" – "H4" into the deep-well plate (see Fig. 6).

*: The volumes provided are for 1 set of plate (for anti-estrogenic and cytotoxicity testing). If more than 2-plates are tested, the volume should be adjusted as appropriate. 20 mL of 10% DCC-FBS-EMEM allows comfortable handling to prepare each set of plates.

6.3.5. Dilution in a 96-well deep-well plate "with medium" (Fig. 6)

Add 1.5 µL of DMSO dilutions from the DMSO dilution plate in the corresponding well of the deep-well plate using multi-channel pipettor to prepare 3-fold concentration of the desired final concentration.

Note: Check the point of the tip used for addition of 1.5 µL of DMSO dilutions. If precipitation is observed on the point of the tip, it must be recorded. It is advised not to pipette when adding DMSO solution in this stage. Put the points of the tips in the medium and add DMSO solution on the second push of the pipette. When removing the tips from the medium, do not release the second push.

DMSO Dilutions

	1	2	3	4
	Chem.-A	**Chem.-B**	**Chem.-C**	**Chem.-D**
A	10^{0}M	10^{0}M	10^{0}M	10^{0}M
B	10^{-1}M	10^{-1}M	10^{-1}M	10^{-1}M
C	10^{-2}M	10^{-2}M	10^{-2}M	10^{-2}M
D	10^{-3}M	10^{-3}M	10^{-3}M	10^{-3}M
E	10^{-4}M	10^{-4}M	10^{-4}M	10^{-4}M
F	10^{-5}M	10^{-5}M	10^{-5}M	10^{-5}M
G	DMSO	DMSO	1 mM OHT	100 mM Dig.
H	DMSO	DMSO	1 µM of E2	1 µM of E2

Deep-well plate

	1	2	3	4	11	12
	Chem.-A	**Chem.-B**	**Chem.-C**	**Chem.-D**		
A	$3x10^{-3}$M	$3x10^{-3}$M	$3x10^{-3}$M	$3x10^{-3}$M		
B	$3x10^{-4}$M	$3x10^{-4}$M	$3x10^{-4}$M	$3x10^{-4}$M		
C	$3x10^{-5}$M	$3x10^{-5}$M	$3x10^{-5}$M	$3x10^{-5}$M		
D	$3x10^{-6}$M	$3x10^{-6}$M	$3x10^{-6}$M	$3x10^{-6}$M		
E	$3x10^{-7}$M	$3x10^{-7}$M	$3x10^{-7}$M	$3x10^{-7}$M		
F	$3x10^{-8}$M	$3x10^{-8}$M	$3x10^{-8}$M	$3x10^{-8}$M		
G	DMSO	DMSO	3 µM OHT	300 µM Dig.		
H	DMSO	DMSO	3 nM of E2	3 nM of E2		

10% DCC FBS-EMEM containing 75 pM E2

10% DCC-FBS-EMEM containing 0.3 % DMSO

Fig. 6 Plate layouts for dilutions (given concentration of test chemicals are example.)

6.3.6. Chemical Exposure to Cells

(1) Mix well the medium in the deep-well plate.

Note: Confirm that the chemicals in DCC-FBS-EMEM have not precipitated. If precipitation is observed, it must be recorded.

(2) Add 50 µL of the medium solution to both plates for antagonist and cytotoxicity assays prepared in "**6.1.** " in triplicate.

(3) Record if there is precipitation or not in wells by visually comparing the spike-in control wells in the cytotoxicity assay plate using a microscope.

(The plate for cytotoxicity assay has a clear bottom to allow this observation.)

(4) Incubate for 20-24 hr in CO_2 incubator at 37°C.

Note: The same medium dilution should be used to prepare the plate for cytotoxicity testing.

	Chem.-A			Chem.-B			Chem.-C			Chem.-D		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-3}M	→	→	10^{-3}M	→	→	10^{-3}M	→	→	10^{-3}M	→	→
B	10^{-4}M	→	→	10^{-4}M	→	→	10^{-4}M	→	→	10^{-4}M	→	→
C	10^{-5}M	→	→	10^{-5}M	→	→	10^{-5}M	→	→	10^{-5}M	→	→
D	10^{-6}M	→	→	10^{-6}M	→	→	10^{-6}M	→	→	10^{-6}M	→	→
E	10^{-7}M	→	→	10^{-7}M	→	→	10^{-7}M	→	→	10^{-7}M	→	→
F	10^{-8}M	→	→	10^{-8}M	→	→	10^{-8}M	→	→	10^{-8}M	→	→
G	0.1% DMSO	→	→	0.1% DMSO	→	→	1 µM OHT	→	→	100 µM Dig	→	→
H	0.1% DMSO	→	→	0.1% DMSO	→	→	1 nM of E2	→	→	1 nM of E2	→	→

Fig. 7 Assay plate layout for estrogenic assay

Medium to be added: 10% DCC-FBS-EMEM containing 75 pM E2 (= 7.5 x 10^{-11}M), resulting in a final concentration of 25pM E2.
Medium to be added: 10% DCC-FBS-EMEM containing 0.3 % DMSO, resulting in a final DMSO concentration of 0.2%.

6.4. LUCIFERASE ASSAY

After 20-24 hr incubation, luciferase activity can be measured with the luciferase assay reagent and a luminometer in accordance with the manufacturer's instructions.

If Steady-Glo Luciferase Assay System is used, the following procedure can be used to minimize the use of substrate solution.

(1) Newly prepare or thaw the substrate solution

(2) Mix 3 mL of the substrate solution and 3 mL of PBS (+) in a (for 1-plate use) in a reagent tray.

(3) Remove all the exposure medium.

(4) Add 50 μL of substrate solution with PBS (+) using a multi-channel pipettor.

(5) Leave the plate for 10-min at room temperature in dark area to protect it from light.

(6) Read plates on a Chemiluminescence plate reader.

6.5. CYTOTOXICITY (CELL VIABILITY) ASSAY USING CELL COUNTING KIT-8 (CCK-8)

After 20-24 hr incubation, cell viability can be measured with CCK-8 assay kit.

(1) Mix 16.5 mL of 10% DCC-FBS-EMEM and 880 μL of CCK-8 reagent in a reagent tray (for one plate) [CCK-8 solution].

Note: CCK-8 solution should be prepared for each plate since this solution will increase in colour as time advances.

(2) Remove exposure medium.

(3) Add 100 μL of PBS (-) to each well and remove it to wash cells.

(4) Add 150 μL of CCK-8 solution to each well.

(5) Incubate for 90-min in CO_2 incubator at 37°C.

(6) Measure the absorbance at 450 nm using a microplate reader.

Note: If the bubble is generated, the bubble should be crushed. If the plate bottom is dirty, it should be wiped with 70-80% ethanol before the measurement of the absorbance.

7. DATA ANALYSIS

7.1. DATA ANALYSIS FOR ESTROGENIC AND ANTI-ESTROGENIC ASSAYS

Using the spreadsheet provided, PC10 and PC50 for estrogenic assay and linIC30 and linIC50 for anti-estrogenic assay should be calculated. Additionally EC50 and IC50 for estrogenic and anti-estrogenic assays, respectively, should be calculated with the appropriate nonlinear curve-fitting software (GraphPad Prism (GraphPad Software Inc., San Diego, CA) is recommended.) using the following Hill's logistic equation;

Y=Bottom + (Top-Bottom)/(1+10^((LogE(I)C50-X)*HillSlope))

* Where X is the logarithm of concentration, Y is the response and Y starts at the Bottom and goes to the Top with a sigmoid shape.

[Estrogenic Assay] [Anti-estrogenic Assay]

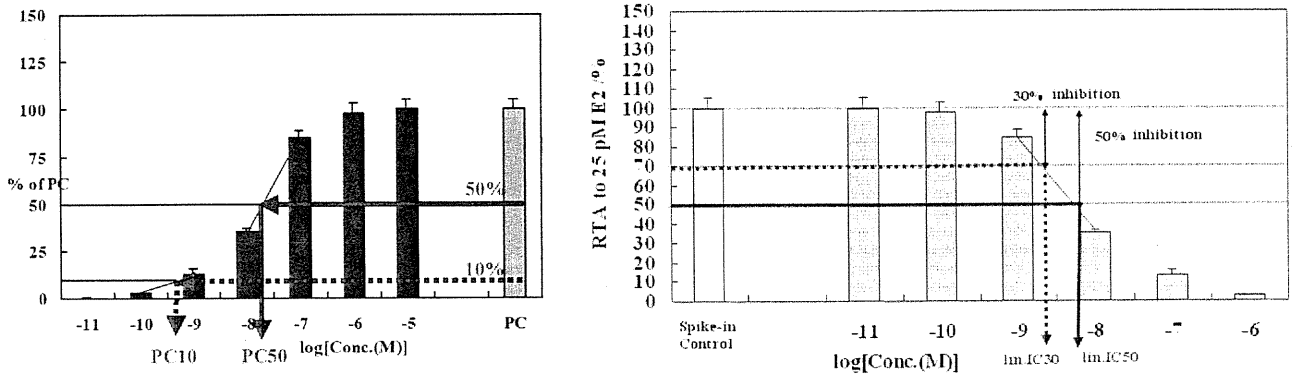

Fig. 8 Description of PC10, PC50 lin IC30 and IC50

7.2. CYTOTOXICITY DATA

In the cytotoxicity test, if the cell viability is less than 80%, the test chemical is cytotoxic at "that" test concentration and such data for evaluating anti-estrogenic assay should be omitted for the calculations of lin.IC30, lin.IC50 or IC50.

Cell viability should be calculated as below;

$$\text{Cell viability (\%)} = \frac{[(\text{Abs.}_{450} \text{ of test well}) - (\text{Mean of Abs.}_{450} \text{ of cytotox. control})]}{[(\text{Mean of Abs.}_{450} \text{ of VC}) - (\text{Mean of Abs.}_{450} \text{ of cytotox. control})]} \times 100$$

7.3. PERFORMANCE CRITERIA

In order to guarantee the assay performance, the performance criteria provided in this section should be fulfilled.

7.3.1. Performance Criteria For Estrogenic Assay for Test System Setup (for Task-1)

Before entering task-2 testing, all laboratories should demonstrate the following performance criteria (Table 2) by using the qualified data that meets the quality criteria in

Table 3 to confirm whether the test system is correctly set up at each laboratory.

Table 2 Performance Criteria for agonistic assay (Acceptable range of reference chemicals)

	Log[EC50]	Log[PC50]	Log[PC10]	Hill slope
17β-Estradiol (E2)	-11.3 ~ -10.1	-11.4 ~ -10.1	<-11	0.7 ~ 1.5
17α-Estradiol	-9.6 ~ -8.4	-9.6 ~ -8.1	-10.7 ~ -9.3	0.9 ~ 2.0
Corticosterone	–	–	–	

Table 3 Quality Criteria for each plate for agonistic assay (control chemical)

Fold-induction[*1] of 1 nM of E2	>= 4
10% fold-induction of 1 nM E2	> 1 ± 2SD of fold-induction of vehicle control

[*1]: Fold-induction = (Mean luminescence intensity of 1 nM of E2) / (Mean luminescence intensity of vehicle control)

7.3.2. Performance Criteria For Anti-estrogenic Assay (for Task-2 and Task-3)

Criteria for each plate with control chemicals (Table 4) and criteria for reference chemicals (Table 5) should be fulfilled.

IMPORTANT NOTE: The final criteria may be modified during the study.

Table 4 Quality Criteria for each plate for anti-estrogenic assay

Fold-induction of Spike-in Control (25 pM of E2) [*1]	>= 4
RTA[*2] of 1 nM E2	>= 100%
RTA of 1 μM OHT	=< 40.6%
RTA of 100 μM Dig.	=< 0%

[*1]: Fold-induction of Spike-in Control = $\frac{\text{(Mean luminescence intensity of Spike-in Control])}}{\text{(Mean luminescence intensity of vehicle control]}}$

[*2]: Relative Transcriptional Activation (RTA)= $\frac{\text{[(luminescence intensity of a well) – (Mean luminescence intensity of vehicle control)]}}{\text{[(Mean luminescence intensity of Spike-in Control) – (Mean of luminescence intensity of vehicle control)]}}$

Table 5 Performance Criteria for anti-estrogenic assay (Acceptable range for reference chemicals)

	log [lin.IC30]	log [lin.IC50]	log [var.IC50]
OHT	-9.58 ~ -8.63	-9.36 ~ -8.09	-9.26 ~ -8.12
TAM	-7.68 ~ -6.37	-7.14 ~ -5.90	-7.21 ~ -5.78
RU486	-6.10 ~ -5.41	-5.57 ~ -5.10	-5.51 ~ -4.91
Flutamide	-	-	-

#: Reference chemicals should be tested in at least one run in each experiment (conducted under the same conditions including the materials, source of cells (passage), and technicians).

Appendix-1

Preparation of Serum treated with Dextran Coated Charcoal (DCC)

The treatment of serum with Dextran-coated charcoal (DCC) is a generally used methodology for the removal of estrogenic compounds from serum. It is added to the cell medium in order to exclude the biased response associated with residual estrogens in serum.

The following materials and equipments will be required;

Materials

- Activated charcoal (Sigma, Catalog# C9157)
- Dextran (MW 64,000~76,000, Sigma, Catalog# D4751)
- Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, Wako, Catalog# 135-00165, >=98% or its equivalent)

  Prepare 1 M $MgCl_2$ aq. by dissolving 20.3 g of $MgCl_2 \cdot 6H_2O$ in 100 mL of Milli-Q and filtering it with sterile filter.
- Sucrose (Wako, Catalog# 196-00015 or its equivalent)
- 1 M HEPES buffer solution (pH 7.4) (Gibco, Catalog# 15630)
- 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.)

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

[Day-1] Prepare 1 litre of dextran coated charcoal suspension by adding the following reagents in the autoclaved glass container and stir it at 4°C, overnight.

• 1 M $MgCl_2$ aq.	1.5 mL
• Sucrose	85.5 g
• Activated charcoal	2.5 g
• Dextrane	0.25 g
• 1 M HEPES	5 mL

[Day-2] Dispense the suspension in 50 mL centrifuge tubes and centrifuge at 10.000 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 fetal bovine serum (FBS) that is thawed at 42°C and left for 30 minutes at 56°C for heat inactivation, and transfer into the 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 10.000 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 at 4°C, overnight.

[Day-4] Dispense the suspension for centrifugation at 10.000 rpm at 4°C for 10 minutes and sterilise the supernatant by filtration through 0.22 μm sterile filter. This DCC treated FBS should be stored at -20°C.

添付資料２

XXX

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Stably Transfected Human Androgen Receptor-α Transcriptional Activation Assay for Detection of Androgenic Agonist Agonist/Antagonist Activity of Chemicals

(Version 2010 Nov.16)

INTRODUCTION

1. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new, Test Guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals comprises five levels, each level corresponding to a different level of biological complexity (1). The Transcriptional Activation (TA) assay described in this Test Guideline is a level 2 "*in vitro* assay, providing mechanistic information". The validation study of the Stably Transfected Transactivation Assay (STTA) by Chemicals Evaluation and Research Institute (CERI) in Japan using the AR-EcoScreen™ cell line to detect Androgenic agonist/antagonist activities mediated through human androgen receptor demonstrated the relevance and reliability of the assay for its intended purpose (2).

2. *In vitro* TA assays are based upon the production of a reporter gene product induced by a chemical, following binding of the chemical to a specific receptor and subsequent downstream transcriptional activation. TA assays using activation of reporter genes are screening assays that have long been used to evaluate the specific gene expression regulated by specific nuclear receptors, such as the estrogen receptors (ERs) and androgen receptor (AR) (3)(4)(5)(6). They have been proposed for the detection of nuclear receptor mediated transactivation (1)(3)(4). Several *in vitro* TA and receptor binding assays are currently at validation at national, European and international levels, but are not yet close to completion and full assessment of their validation status. Only the assay "Stably Transfected Transcriptional Activation (STTA) using HeLa-9903 cell line for detecting estrogenic activity of chemicals" has been adopted as OECD test guideline (TG 455) in 2009. Although the need for AR mediated *in vitro* assays are also urgent, at the present time there are no in vitro screening assays for androgenic activity that have been peer reviewed for potential test guideline development, to enable use for OECD regulatory purposes. The aim of this TA assay is to evaluate the ability of a chemical to function as an AR ligand and activate or inhibit androgenic responses, for screening and prioritisation

purposes but can also provide mechanistic information that can be used in a weight of evidence approach.

3. Definitions and abbreviations used in this Test Guideline are described in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. Androgen agonists and antagonist act as ligands for AR, and may activate or inhibit the transcription of androgen responsive genes. This interaction may have the potential to trigger adverse health effects by disrupting androgen-regulated systems. This Test Guideline describes an assay that evaluates transcriptional activation and inhibition of AR mediated responses. This process is considered to be one of the key mechanisms of possible endocrine disruption related health hazards, although there are also other important endocrine disruption mechanisms. These include (i) actions mediated via other nuclear receptors linked to the endocrine system and interactions with steroidogenic enzymes, (ii) metabolic activation or deactivation of hormones, (iii) distribution of hormones to target tissues, and (iv) clearance of hormones from the body. This Test Guideline exclusively addresses transcriptional activation and inhibition of an androgen -regulated reporter gene by agonist binding to the hAR, and therefore it should not be directly extrapolated to the complex *in vivo* situation of androgen regulation of cellular processes.

5. This test method is specifically designed to detect hAR-mediated transcriptional activation and inhibition by measuring chemiluminescence as the endpoint.

6. It is recognized that this assay using the AR-EcoScreen™ cell line is only one of several AR transcriptional activation/inhibition assays currently being developed and validated. It is, therefore the intention that a generic performance based Test Guideline will replace this Test Guideline as soon as such guideline is developed and approved.

PRINCIPLE OF THE TEST

7. The TA assay using a reporter gene technique is an *in vitro* tool that provides mechanistic data. The assay is used to signal binding of the androgen receptor with a ligand. Following ligand binding, the receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer. Luciferase activity can be evaluated quickly and inexpensively with a number of commercially available test kits.

8. The test system provided in this Test Guideline utilises the AR-EcoScreen™ cell line, which is derived from Chinese hamster ovary derived cell line (CHO-K1), with two stably inserted constructs: (i) the human AR expression construct (encoding the full-length human receptor), and (ii) a firefly luciferase reporter construct bearing four tandem repeats of a prostate C3 gene-responsive element driven by a minimal heat shock protein promoter. Where C3 gene derived responsive element is selected to minimize GR mediated responses among known androgen responsive elemnts.

9. Data interpretation for **AR agonist assay** is based upon whether or not the maximum response level induced by a test chemical equals or exceeds an agonist response equal to 10% of that induced by a maximally inducing (10 nM) concentration of the positive control (PC) 5α-dihydrotestosterone (DHT) (*i.e.* the PC10). And that **for AR antagonist assay** is based on 30% inhibitory response against 500 pM DHT by a test chemical. Data analysis and interpretation are discussed in greater detail in paragraphs 37- 52.

PROCEDURE

Cell Lines

10. The stably transfected AR-EcoScreen™ cell line should be used for the assay. The cell line can be obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank as a reference No. JCRB1328, upon signing a Material Transfer Agreement (MTA).

11. Only cells characterised as mycoplasma-free should be used in testing. RT PCR (Real Time Polymerase Chain Reaction) is the method of choice for a sensitive detection of mycoplasma infection (8) (9) (10).

Stability of the cell line

12. To monitor the stability of the cell line **for agonist assay**, DHT, Methyltrienolone (R1881) and Di(2-ethylhexyl)phthalate (DEHP) should be used as the reference chemicals and a complete concentration-response curve in the test concentration range provided in Table 1 should be measured at least once each time the assay is performed, and the results should be in agreement with the results provided in Table 1-1.

13. To monitor the stability of the cell line **for antagonist assay**, Hydroxyflutamide (HF), Bisphenol A (BisA) and Di(2-ethylhexyl)phthalate (DEHP) should be used as the reference chemicals and a complete concentration response curve in the test concentration range provided in Table 1-2 should be measured in at least one run each day the assay is performed, and the results should be in agreement with the results provided in Table 1-2.

Cell Culture and Plating Conditions

14. Following mediums should be prepared;

Medium for dilution: Phenol Red Free D-MEM/F-12.

Medium for cell propagation: Phenol Red Free D-MEM/F-12 supplemented with 5% charcoal dextran treated fetal bovine serum (DCC-FCS), Zeocin (200 μg/mL), Hygromycin (100 μg/mL) , Penicillin (100 units /m L), and Streptomycin (100 ug/ml).

Medium for the assay plate: Phenol Red Free D-MEM/F-12 supplemented with 5% DCC-FCS, Penicillin (100 units /m L), and Streptomycin (100 ug/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 can be subcultured at 20 mL of 1.5 – 3.0 x 10^4 cells/mL for 75 cm^2 cell culture flask. To prepare the assay plate, cells should be suspended with the Medium for the assay plate and then plated into wells of a microplate at 90 μL/well at a density of 1 x 10^5 cells/mL. Next, the cells should be pre-incubated in a 5% CO_2 incubator at 37°±1°C for 3 hours before the 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 prepared as described in Annex 2, or obtained from commercial sources. The selection of FBS is some time 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 AR agonist assay and Table 1-2 for 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, to ensure minimum variability of EC50, PC50, PC10, linearIC30, linearIC50 and logIC50 values, consistent use of materials for cell culturing is essential. 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 PC10s or linear IC30 of the two positive reference chemicals should fall within the acceptable range, as should the PC50s and EC50s, or linear IC50 and IC50 where they can be calculated (see Table 1).

Table 1-1 Reference chemicals for AR agonist assay

Folf-induction	> = 6.4				
PC10 value	Greater than 1 (fold-induction of VC) +2SD				
Chemical Name [CAS No.]	logPC10	logPC50	logEC50	Hill Slope	Test range
5α-Dehydrotestosterone (DHT) [521-18-6]	-9.87 ~-12.08	-9.00 ~ -11.03	-9.13 ~ -11.02	0.577 ~ 4.358	$10^{-6} \sim 10^{-12}$M
Methyltrienolone (R1881) [965-93-5]	-10.57 ~ -11.07	-9.10 ~ -10.86	-9.37 ~ -10.83	3.996 ~ 0.599	$10^{-5} \sim 10^{-11}$M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	-	-	-	-	$10^{-5} \sim 10^{-10}$M

Table 1-2 Reference chemicals for AR antagonist assay

Fold induction of spike-in [Spike-in of 500 pM DHT] /[Vehicle Control]	> = 5.0				
PC_{ATG} inhibitory ratio	= <0.46				
Chemical Name [CAS No.]	log linearIC30	log linearIC50	logIC50	Hill Slope	Test range
Hydroxyflutamide (HF) [52806-53-8]	-6.41 ~ -8.37	-6.17 ~ -7.80	-6.26 ~ -7.71	-2.503 ~ -0.652	$10^{-5} \sim 10^{-10}$M
Bisphenol A (BisA) [80-05-7]	-4.48 ~ -7.52	-4.29 ~ -7.05	-4.38 ~ -6.89	-2.973 ~ -0.598	$10^{-5} \sim 10^{-10}$M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	-	-	-	-	$10^{-5} \sim 10^{-10}$M

Positive and Vehicle Controls

19. **For 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 antagonist assay**, vehicle control (no spike-in, 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 spike-in control (SPK, 500 pM of DHT, n=12)