5. CELL CULTURING

Prior to conducting an experiment, cells to be used for the estrogenic or anti-estrogenic assay should be first cultured for more than one passage from the frozen stock in the conditioned media and should not be cultured more than 3 months (less than 30-40 passages).

It is recommended to expand the cells obtained from Sumitomo Chemical Co. in the conditioned media and to prepare frozen stock for Task-1, 2 and 3 testing.

Note:

- Once cells are conditioned with the medium used at each laboratory (the source of FBS can probably differ amongst laboratories), cells which are split more than once from the frozen stock can be used for the assay.
- Cell should not be continuously cultured for more than 3 months (30-40 passages). It is advised to grow cells at each Task from the frozen stock.
- It should be noted that even if a new HeLa9903 obtained from Sumitomo Chemical Co. is properly reconstituted from the frozen stock, cells might not adhere to the cell dish the following day (day-2) in the non-conditioned medium. In such cases, check the cell condition for the following two days (day-2, 3) and if the cells are attached to the dish, the medium can be changed. If not, contact the lead laboratory directly.

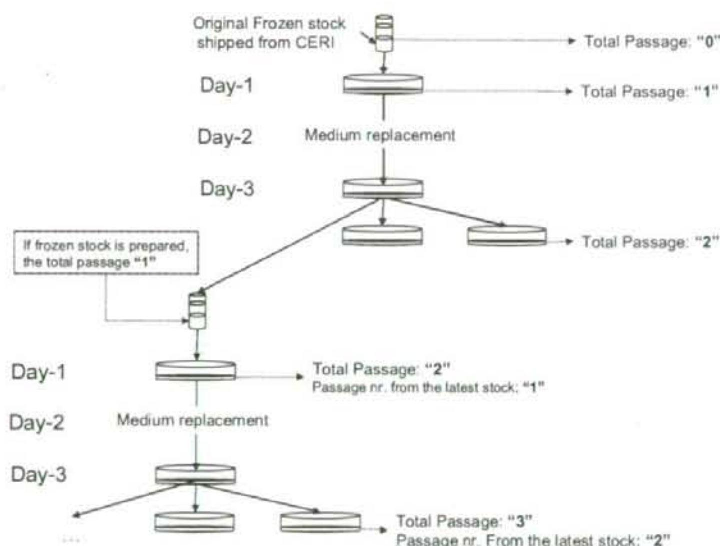


Fig. 1 How to count the cell passage

5.1. Reconstitution of Cells from the Frozen Stock

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

(2) Remove the vial from the liquid nitrogen or the freezer and immediately thaw the cells in a 37°C water bath with gentle agitation.

(3) After the cells are thawed, transfer the cell stock into 5 mL of 10% DCC-FBS-EMEM in a 15 mL conical tube and pipette well.

(4) Centrifuge the tube at 1,100 rpm (200-300 x g) for 5-min at 4°C.

(5) Remove the supernatant carefully not to take cells.

(6) Re-suspend the cell with 10 mL of 10% DCC-FBS-EMEM and place it in a 100 mm cell-culture dish (area: 58.95 cm^2, BD Falcon, Catalog#353003 or its equivalent).

(7) Incubate the cells in a 5% CO_2 incubator at 37°C.

Note: Cell-culture dish or flask can be used for cell culturing.

5.2. Medium Replacement

The medium should be replaced at least once for 2-3 days.

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

(2) Check the cell condition

(3) Remove the medium from the culture dish with a sterile pipette or sucker

(4) Add 10 mL of 10% DCC-FBS-EMEM

5.3. Cell Propagation

Cells should be passaged on reaching 75-90% confluence. Usually, cells need to be expanded 2-3 times a week.

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

(2) Check the cell condition

(3) Remove the medium from the cell-culture dish with a sterile pipette or sucker.

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

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

(6) Add 2 mL of Trypsin-EDTA solution enough to coat the bottom of the 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.

(7) Incubate the dish for 3-4 min. in a 5% CO_2 incubator at 37°C.

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

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

(10) Add 5 mL of 10% DCC-FBS-EMEM and pipette the medium several times in the dish to completely detach the cells.

(11) Count the number of cells

(12) Dilute the cell suspension with 10% DCC-FBS-EMEM to prepare 0.4-1.0 x 10^5 cells/mL.

(13) Place 10 mL of cell suspension in a 100 mm culture dish.

(14) Incubate the dish in a 5% CO_2 incubator at 37°C.

5.4. Preparation of frozen stock

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

(2) Check the cell condition

(3) Remove the medium from the culture dish with a sterile pipette or sucker.

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

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

(6) 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.

(7) Allow the Trypsin-treated cell to stand for 3-4 min. in a 5% CO_2 incubator at 37°C.

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

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

(10) Add 5 mL of 10% DCC-FBS-EMEM and pipette the medium several times in the dish to completely detach the cells.

(11) Count the number of cells.

(12) Centrifuge the cell suspension in a 15 mL conical tube at 1100 rpm (200-300 x g) for five minutes, and remove the supernatant carefully.

(13) Add Cell-Banker* (Juji Field Inc. or its equivalent) and resuspend the cell at density of ca. 2 x 10^6 cells/mL.

(14) Make 0.5 mL aliquots of cell stock (Caryogenic vial (sterile, 1.5 mL) Nalgene, Catalog#, 5000-1020 or its equivalent).

(15) Freeze and store the cell stock below -80°C.**

* A conventional freeze medium (90% FBS / 10% DMSO) can be used in place of Cell-Banker.

** Storage in liquid nitrogen would be preferable for long-term storage (longer than three months).

6. PROCEDURE FOR THE ER-STTA ASSAY

The scheme for the ER-STTA assay for estrogenic and anti-estrogenic assays is shown in **Fig. 2** and **Fig. 3**, respectively, and the summary of assay is provided in Table 1.

There are only two differences between the estrogenic and anti-estrogenic assays as below;

- Plate layout (see **Fig. 2** and **Fig. 3**)
- The anti-estrogenic assay requires concurrent cytotoxicity testing.

Thus, with the exceptions of both the chemical exposure procedure and the requirement for cytotoxicity testing, the assay procedure for both the estrogenic and anti-estrogenic assays are identical.

The duration of the assay, from seeding the cells in a 96-well plate, to luminescence measurement is 2-days.

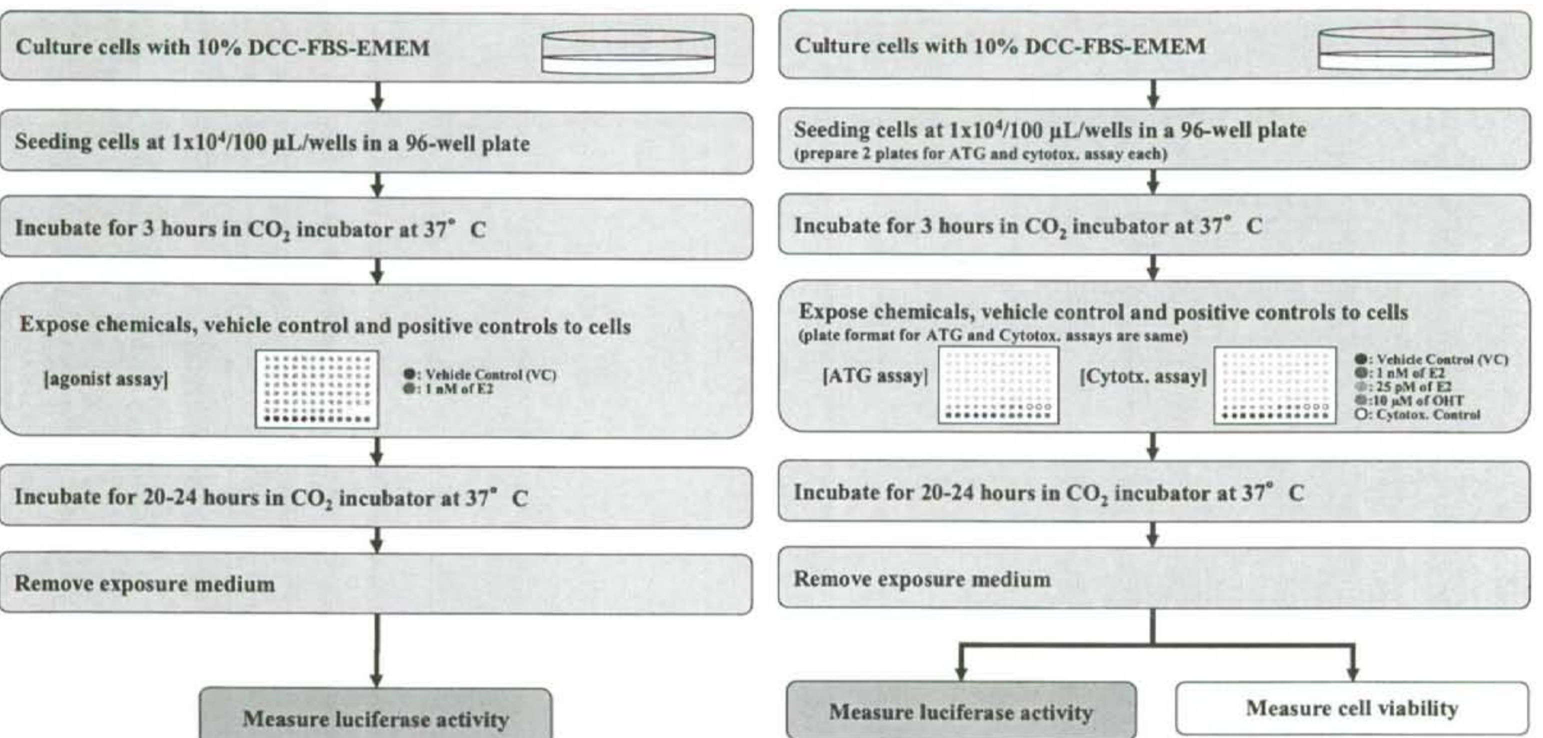


Fig. 2 Schematic Flow for the Estrogenic Assay

Fig. 3 Schematic Flow for the Anti-estrogenic assay

Table 1 Summary of the assay

		For Estrogenic Assay	For Anti-estrogenic Assay	Difference
Cell line		hERα-*HeLa*-9903 stable cell line	hERα-*HeLa*-9903 stable cell line	
Cell medium		Eagle's Minimum Essential Medium (EMEM) without phenol red with 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS)	Eagle's Minimum Essential Medium (EMEM) without phenol red with 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS)	
Cell density in a assay well		10^4 cells/100 μL/well	10^4 cells/100 μL/well	
Total volume of the assay plate		150 μL/well	150 μL/well	
Vehicle		Dimethylsulfoxide (DMSO)	Dimethylsulfoxide (DMSO)	
Final concentration of vehicle		0.1%	0.2%	*
Controls in each plate	Vehicle Control	0.1% of DMSO as a final concentration (6 wells)	0.2% of DMSO as a final concentration (6 wells)	*
	Spike-in Control	None	25 pM of E2 (6 wells)	*
	E2 Control	1 nM of E2 (6wells)	1 nM of E2 (6wells)	
	OHT Control	None	1 μM of 4-Hydroxytamoxifen (OHT) as a antagonist positive controls (3 wells)	*
	Cytotoxicity Control	None	100 μM of Digitonin (Dig.) (3 wells)	*
Concentration range of test chemical		# the concentration of chemicals is provided in Table 2. • 7 concentrations at common ratio of 10.	• 1 mM is a maximum concentration if the precipitating and/or cytotoxicity of test chemical are not observed. • 6 concentrations at common ratio of 10.	*
Reference Chemicals		• 17β-Estradiol (E2) • 17α-Estradiol (αE2) • Corticosterone	• 4-Hydroxytamoxifen • Tamoxifen • RU486 • Flutamide	
Incubation time with test chemicals		20-24 hours	20-24 hours	
Number of run in Task-3 testing		Not applicable	All test chemicals should be assayed 3 runs on the separated days. If the performance criteria are not fully met, the assay needs to be repeated.	

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 (=2.5×10^{-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 (=7.5×10^{-11} M) E2 in medium.

(1) Add 60 μL of 25 nM (=2.5×10^{-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*.

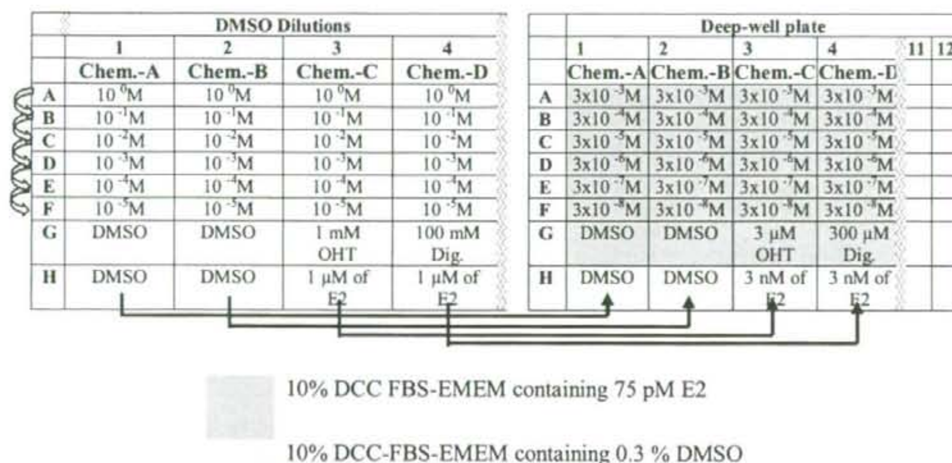
(2) Dispense 500 µL of 10% DCC-FBS-EMEM containing 0.3% DMSO in "H1" – "H4" into the deep-well plate (see Fig. 6).

*: 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		

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.

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.

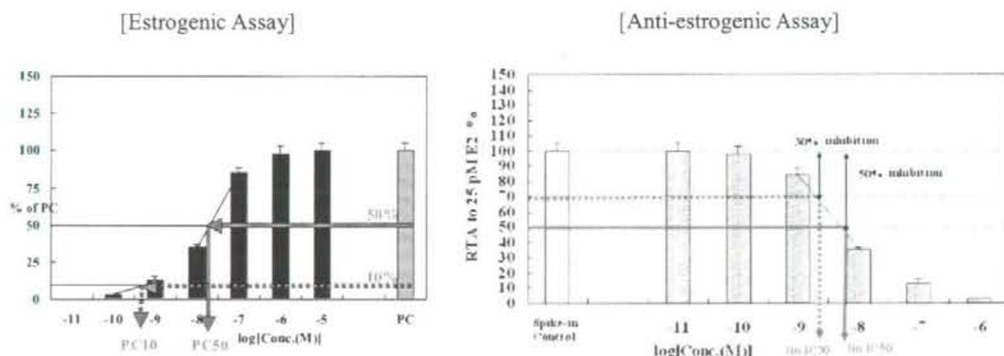


Fig. 8 Description of PC10, PC50 lin IC30 and IC50

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	=< 39.4%
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	-9.62 ~ -8.73	-9.46 ~ -8.16	-9.32 ~ -8.20
TAM	-7.55 ~ -6.84	-7.08 ~ -6.26	-7.02 ~ -6.32
RU486	-6.18 ~ -5.41	-5.61 ~ -5.08	-5.53 ~ -4.86
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).