4.2.2. Preparation of 10%DCC-FBS-EMEM

(1) To prepare 556 mL of 10%DCC-FBS-EMEM, add the following reagents into an appropriate size of a glass flask

- EMEM pre-made powder: 4.7 g
- 7.5w/v% $NaHCO_3$ aq. : 12 mL
- 200 mM *L*-Glutamine aq.: 5.6 mL

(2) Add Milli-Q water to bring the total volume to 500 mL and stir it to dissolve the powder.

(3) Add 56 mL of dextran-coated charcoal (DCC)-treated fetal bovine serum (DCC-FBS) and mix it gently.

(4) Sterilize with a vacuum-driven bottle-top sterilization filter unit (pore size: 0.22 μm).

(5) Store 10%DCC-FBS-EMEM in a refrigerator (4°C) in a sterile glass bottle.

Note: The10%DCC-FBS EMEM can be stored for 1 month.

4.3. Phosphate Buffered Saline without Mg^{2+} and Ca^{2+} (PBS (-))

(1) Dissolve a pack of powder PBS (-) (Cosmobio, Catalog#16232001 or its equivalent) for 1 L to a final volume of 1 L with Milli-Q water.

(2) Sterilize with a vacuum-driven bottle-top sterilization filter unit (pore size: 0.22 μm).

(3) Store at room temperature in a sterile glass bottle.

Note: PBS(-) can be stored for 6 months.

4.4. EDTA-Trypsine

(1) Add 10 mL of Trypsin-EDTA (0.5% Trypsin, 5.3mM EDTA•4Na, phenol-red free (10X), liquid (Gibco; Catalog# 15400-054 or its equivalent)) in a sterile 100 mL glass bottle.

(2) Add 90 mL of PBS(-).

(3) Mix it gently.

(4) Store in a refrigerator (4°C).

Note: The working EDTA-Tripsin can be stored for 1 month.

Note: It is recommended to aliquot Trypsin-EDTA (X10) at 10 mL in a 15 mL conical tube as the stock at -20°C for easy preparation of the working EDTA-Trypsin.

4.5. SOLVENT FOR CHEMICAL STOCK SOLUTIONS

Dimethylsulfoxide (DMSO, >99%) which will be distributed by JaCVAM should be used for the vehicle.

4.6. CONTROL CHEMICALS AND REFERENCE CHEMICALS FOR TASK-1 AND TASK-2

All control and reference chemicals below will be distributed from JaCVAM.

Control chemicals are defined as chemicals that must be tested in each assay plate at a defined concentration.

Reference chemicals are defined as chemicals that must be tested once a day of assay.

All control and reference chemicals below should be dissolved in DMSO.

After making stock solutions in DMSO, aliquot the stock into 4-5 vials such that freezing and thawing of the stock solutions is not repeated. The freezing and thawing cycle of each vial should be recorded.

Control and Reference Chemicals	CAS No.	M.W.
17β-Estradiol (E2)	50-28-2	272.4
17α-Estradiol (αE2)	57-91-0	272.4
Corticosterone (Cor)	50-22-6	346.5
4-Hydroxytamoxifen (OHT)	68047-06-3	387.5
Tamoxifen (TAM)	10540-29-1	371.5
RU486	84371-65-3	429.6
Digitonin (Dig)	11024-24-1	1229.3

4.6.1. 17β-Estradiol (E2)

Prepare 10 mM (=10^{-2}M) DMSO stock solution of E2 and store at -20°C.

This stock solution is used

in the estrogenic assay;

- reference chemical (10^{-14}M ~ 10^{-8} M)
- E2 control (1 nM = 10^{-9}M as final concentration)

in the anti-estrogenic assay

- Spike-in control (25 pM = 25 x 10^{-12} M = 2.5 x 10^{-11} M as final concentration)
- E2 control (1 nM = 10^{-9}M as final concentration)

4.6.2. 17α-Estradiol (αE2)

Prepare 10 mM (=10^{-2}M) DMSO stock solution of αE2 and store at -20°C.

This stock solution is used as a reference chemical (10^{-12} ~ 10^{-6} M) in the estrogenic assay.

4.6.3. Corticosterone (Cor)

Prepare 100 mM (=10^{-1} M) DMSO stock solution of Cor and store at -20°C.

This stock solution is used as a reference chemical (10^{-10} ~ 10^{-4}M) in the estrogenic assay and a reference chemical (10^{-9} ~ 10^{-4}M) in anti-estrogenic assay.

4.6.4. 4-Hydroxytamoxifen (OHT)

Prepare 10 mM (=10^{-2} M) DMSO stock solution of OHT and store at -20°C.

This stock solution is used in anti-estrogenic assay as

- OHT control (1 μM=10^{-6} M)
- A reference chemical (10^{-12} ~10^{-7} M)

4.6.5. Tamoxifen (TAM)

Prepare 10 mM DMSO stock solution of TAM and store at -20°C.

This stock solution is used as a reference chemical (10^{-10} ~ 10^{-5} M) in anti-estrogenic assay.

4.6.6. RU486

Prepare 100 mM (=10^{-1}M) DMSO stock solution of RU486 and store at -20°C.

This stock solution is used as a reference chemical (10^{-9} ~ 10^{-4} M) in anti-estrogenic assay.

4.6.7. Digitonin (Dig)

Prepare 100 mM (=10^{-1}M) DMSO stock solution of Dig and store at -20°C.

This stock solution is used as a cytotoxicity control at 100 μM (=10^{-4}M).

4.7. TEST CHEMICALS

All test chemicals for Task -3 will be distributed by JaCVAM as coded chemicals.

All test chemicals for Task-3 should be dissolved in dimethylsulfoxide (DMSO) to prepare 1 M DMSO stock solution. If 1 M DMSO stock solution cannot be prepared due to lack of solubility, prepare 100 mM ($=10^{-1}$M) DMSO stock solution. If not, prepare 10 mM ($=10^{-2}$M) DMSO stock solution.

The DMSO stock solution should be aliquoted into 4-5 vials and be stored at -20°C. The freezing and thawing cycle of the solution should be recorded.

4.8. LUCIFERASE ASSAY REAGENT

A commercial luciferase assay reagent (Steady-Glo Luciferase Assay System (Promega; Catalog# E2510 or its equivalents [glo type]) or a standard luciferase assay system (Promega, E1500 or its equivalents [flush type]) can be used. If the flush type of luciferase reagent is used, Cell Culture Lysis Reagent (Promega, E1531 or its equivalents) should be used before adding the substrate.

Preparation of luciferase reagent should be followed the manufacturer's instruction.

If Steady-Glo Luciferase Assay System ((Promega, Catalog# E2510) is used, a bottle of Luciferase Assay Substrate is dissolved with enclosed Luciferase Assay Buffer as described in manufacturer's protocols. The dissolved substrate should either be used immediately or stored below -20°C. For the storage of the dissolved substrate, it is recommended to make aliquots to avoiding repeated freezing and thawing (eg. More than 2.5 mL is necessary for 1 plate assay).

4.9. PHOSPHATE BUFFERED SALINE WITH Mg^{2+} (PBS (+))

This reagent (PBS (+)) is only necessary if Steady-Glo Luciferase Assay System (Promega) is used as a luciferase reagent

Add 150 µL of 1 M $MgCl_2$ aq. (0.22 µm filter sterilized) in 500 mL PBS(-) to prepare PBS (+) containing 0.3 mM of $MgCl_2$.

Note: 1 M $MgCl_2$ aq. can be prepared by dissolving 20.3 g of $MgCl_2$·6H2O in 100 mL of Milli-Q water. This solution should be sterilized with 0. 22 µm filter.

4.10. REAGENT FOR CYTOTOXICITY (CELL VIABILITY) TESTING

The method for cytotoxicity testing will be the Cell Counting Kit-8 (CCK-8) (Dojindo, Catalog# CK04) assay. (As used during pre-validation).

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.

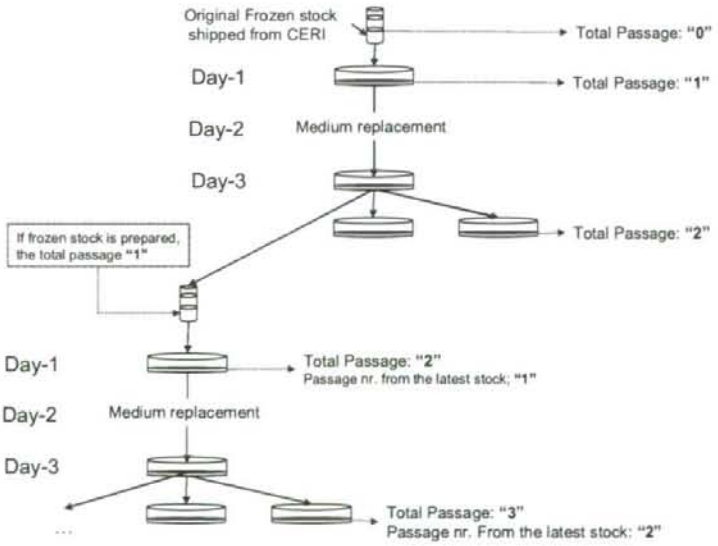

Fig. 1 How to count the cell passage

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.

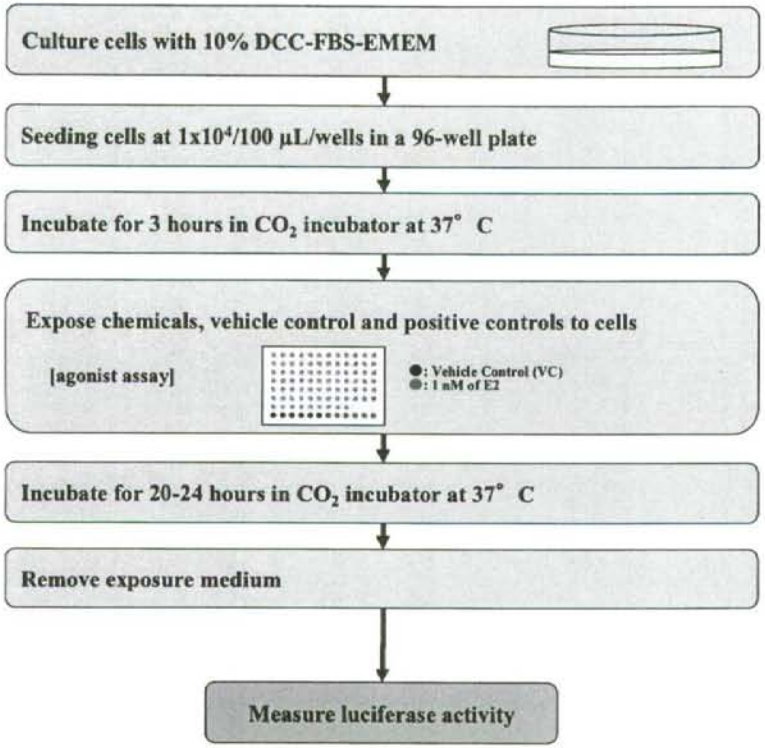

Fig. 2 Schematic Flow for the Estrogenic Assay

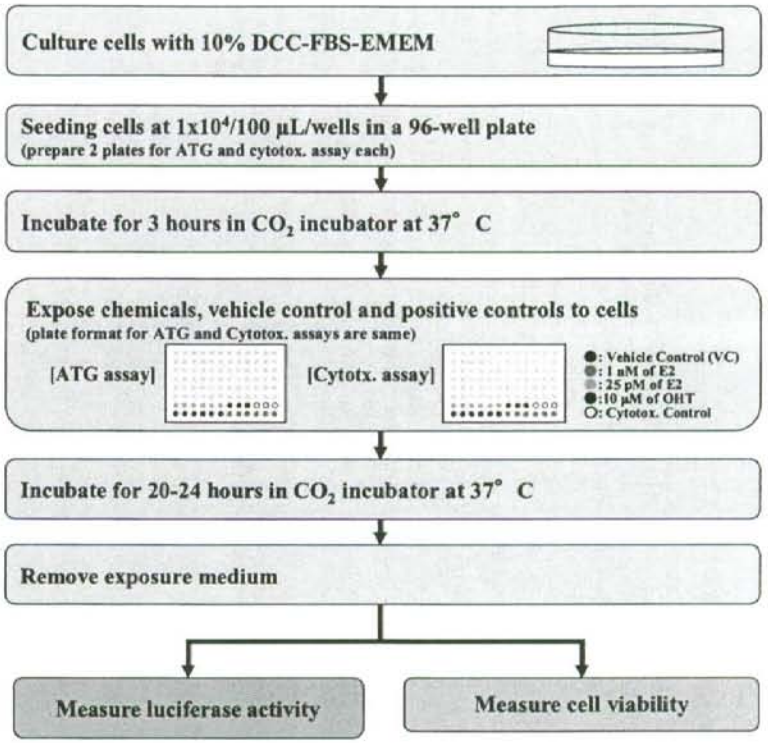

Fig. 3 Schematic Flow for the Anti-estrogenic assay

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		

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.)