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0. ACRONYMS

αE2	17α-Estradiol
ATG	Antagonist
CCK-8	Cell Counting Kit-8
CERI	Chemicals Evaluation and Research Institute (Japan)
Cor.	Corticosterone
CV	Coefficient of Variation
Cytotox.	Cytotoxicity
DCC-FBS	Dextran-Coated Charcoal-treated Fetal Bovine Serum
Dig.	Digitonin
DMSO	Dimethylsulfoxide
E2	17β-Estradiol
EC50	The molar concentration of a compound which produces 50% of the maximum possible response for that compound
EDTA	Ethylenediamine-*N,N,N',N'*-tetraacetic acid
EMEM	Eagle's Minimum Essential Medium
ER	Estrogen Receptor
ERE	Estrogen Responsive Element
Flu.	Flutamide
HeLa9903	hERα-*HeLa*-9903
linIC30/linIC50	The concentration of chemical estimated to cause 30% or 50% inhibition of the spiked-in (25 pM of E2) response, respectively, on a plate by plate basis.
JaCVAM	Japanese Center for the Validation of Alternative Methods
JCRB	Japanese Collection of Research Bioresources
M.W.	Molecular Weight
$NaHCO_3$	Sodium bicarbonate
OHT	4-Hydroxytamoxifen
PBS (-)	Phosphate Buffered Saline without Mg^{2+} and Ca^{2+}
PBS (+)	Phosphate Buffered Saline with Mg^{2+}
PC50/PC10	The concentration of chemical estimated to cause 50% or 10%, respectively, of activity of the positive control response on a plate by plate basis.
PP	Polypropylene
RTA	Relative transcriptional activation

SD	Standard Deviation
SE	Standard Error
SOP	Standard Operating Procedure
STTA	Stably transfected transcriptional activation
TA	Transcriptional Activation
TAM	Tamoxifen
WST	Water soluble tetrazolium
10%DCC-FBS-EMEM	EMEM containing 10%DCC-FBS

1. PROVISIONAL OVERVIEW OF THE EXPERIMENTS FOR THE MULTI-LABORATORY VALIDATION STUDY

<table>
<tr><th>Tasks</th><th>Purpose</th><th>Procedures in brief</th><th>Draft Schedule</th></tr>
<tr><td colspan="3">Start chemical distribution from JaCVAM</td><td>2008.5</td></tr>
<tr><td rowspan="2">Task-1:</td><td>Confirm the edge effects to establish the plate layout for further testing</td><td>(1) Expose 1 nM of E2 to all wells in a 96-well plate
(2) Check if the value of coefficient of variation (CV) value among all wells of luminescence intensity is less than 10%.
If yes, no edge effects are expected and all wells of 96-well plate can be used.
If no, edge effects are expected and the wells on the edge should not be used for further evaluation.</td><td rowspan="2">2008. 6
Data should be submitted until the end of 2008.6.</td></tr>
<tr><td>Test system setup</td><td>(1) Naïve laboratories to test “agonistic” activities of 3 chemicals to confirm the test performance
(2) Check if the performance criteria (see 7.3.) can be fully met.
<table><tr><td>Agonist</td><td>•E2</td></tr><tr><td></td><td>•17α-Estradiol</td></tr><tr><td></td><td>•Corticosterone</td></tr></table></td></tr>
<tr><td>Task-2:</td><td>Confirm Lab performance for antagonist (ATG) assay
(including range finding test, cytotoxicity (cytotox.) test)</td><td>(1) Test “anti-estrogenic” activities of 4 chemicals.
<table><tr><td>Antagonist</td><td>•4-Hydroxytamoxifen</td><td>Strongly anti-estrogenic</td></tr><tr><td></td><td>•Tamoxifen</td><td>Moderately anti-estrogenic</td></tr><tr><td></td><td>•RU-486</td><td>Weakly anti-estrogenic, cytotoxic</td></tr><tr><td></td><td>•Negatives</td><td>Negative, cytotoxic</td></tr></table></td><td>Beginning of 2008.8 – End. of 2008.8
Data should be submitted until the Mid. of 2008.9.</td></tr>
<tr><td>Task-3:</td><td>Test coded chemicals</td><td>(1) Test “anti-estrogenic” activities of coded X chemicals</td><td>End of 2008.9 - End of 2008.11
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</table>

2. PURPOSE OF THE ASSAY

The "Stably Transfected Transcriptional Activation Assay Using hERα-*HeLa*-9903 (HeLa9903) the potential to inhibit the estrogenic response induced by a natural estrogen ligand, 17β-Estradiol (E2).

To ensure the reliability and sensitivity of the assay, "Control chemicals" must be tested at a defined concentration in each assay plate and "Reference chemicals" must be tested once per day of assay.

This validation study for the detection of anti-estrogenic activities of chemicals using HeLa9903 cell line consists of the following three tasks,

[Task-1]: Set up the test system and demonstrate the basic skill of participating lab by testing three reference chemicals (17β-Estradiol (E2), 17α-Estradiol, Corticosterone) in the "estrogenic" assay.

- Selection of lab for further testing.

[Task-2]: Test un-coded chemicals in the anti-estrogenic assay.

- Selection of cytotoxicity testing
- Re-define the performance criteria, if necessary.
- Selection of lab for further testing.

[Task-3]: Test coded chemicals provided.

3. EQUIPMENTS

3.1. EQUIPMENTS FOR THE STUDY

- ☐ Lumiometer
- ☐ Plate reader (with 450 nm filter if CCK-8 assay is used as the cell viability testing.)
- ☐ Class II biological safety cabinet for cell handling
- ☐ CO_2 incubator that can keep 37±1 °C and CO_2 5±0.1%
- ☐ Liquid N_2 tank for cell stock
- ☐ -80°C freezer
- ☐ -20 °C freezer
- ☐ 4°C refrigerator
- ☐ Autoclave
- ☐ Balance, analytical
- ☐ pH Meter with Tris-Compatible Electrode with traceable standards (pH: 4, 7, and 9)
- ☐ Ultra-pure water system
- ☐ Pipettes:
  - 0.5 to 2 µL
  - 2 to 20 µL
  - 20 to 100 µL
  - 40 to 200 µL
  - 200 to 1000 µL
- ☐ Multi-Channel micropipettor for eight wells
  - 0.5 to 10 µL
  - 10 to 50 µL
  - 50 to 200 µL
- ☐ Multi-channel dispenser

4. MATERIALS

4.1. CELL LINES

The hERα-*HeLa*-9903 cell line (HeLa9903) (provided from Sumitomo Chemical Co.) should be used for the assay.

Cells provided by the lead laboratory should be stored in liquid nitrogen.

4.2. CELL MEDIUM

4.2.1. Reagent

☐ Eagle's Minimum Essential Medium (EMEM) pre-made powder without phenol red (Nissui Pharmaceutical Co., Catalog# 05901)

- Store at 4°C

Note: Kanamycin is contained in this pre-made powder EMEM as the antibiotic.

☐ 7.5w/v% Sodium bicarbonate ($NaHCO_3$) aq.

- Dissolve 7.5 g of $NaHCO_3$ (Nacalai tesque, Catalog# 31213-15, > 99% or equivalent) to a final volume of 100 mL with Milli-Q water.
- Sterilize using a vacuum-driven bottle-top sterilization filter unit (pore size: 0.22 μm).
- Store at room temperature (This solution can be stored for 1 month).

Note: Commercially available equivalent product can be used (7.5w/v% Sodium bicarbonate aq., Gibco, Catalog# 25080-094 or equivalent).

☐ 200 mM *L*-Glutamine aq.

- Dissolve 2.92 g of *L*-glutamine (Wako, Catalog# 074-00522, > 99% or equivalent) to a final volume of 100 mL with Milli-Q water.
- Sterilize using vacuum-driven bottle-top sterilization filter unit (pore size: 0.22 μm).
- Dispense 12.5 mL of 200 mM *L*-glutamine in a 15 mL conical tube.
- Store under -20°C. (This solution can be stored for 6 months.)

Note: Commercially available equivalent product can be used (200 mM L-Glutamine aq., Gibco, Catalog# 25030-081 or equivalent).

☐ Dextran-coated charcoal (DCC)-treated Fetal bovine serum (DCC-FBS)

The DCC-FBS provided by CERI using the procedure provided in Appendix-1 can be used.

Note: Commercially available DCC-FBS can be used if the performance criteria are satisfied (see 7.3.). It is recommended to aliquot DCC-FBS at 28 mL in a 45 mL conical tube as the stock at -20°C for easy preparation. Two tubes of DCC FBS (i.e., 28 mL x 2 = 56 mL) is enough to prepare 556 mL of 10%DCC-FBS-EMEM.

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	84 371-65-3	4 29.6
Flutamide (Flu)	13 311-84-7	2 76.2
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. Flutamide (Flu)

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

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

4.6.8. 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 $MgCl_2 \cdot 6H2O$ 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.

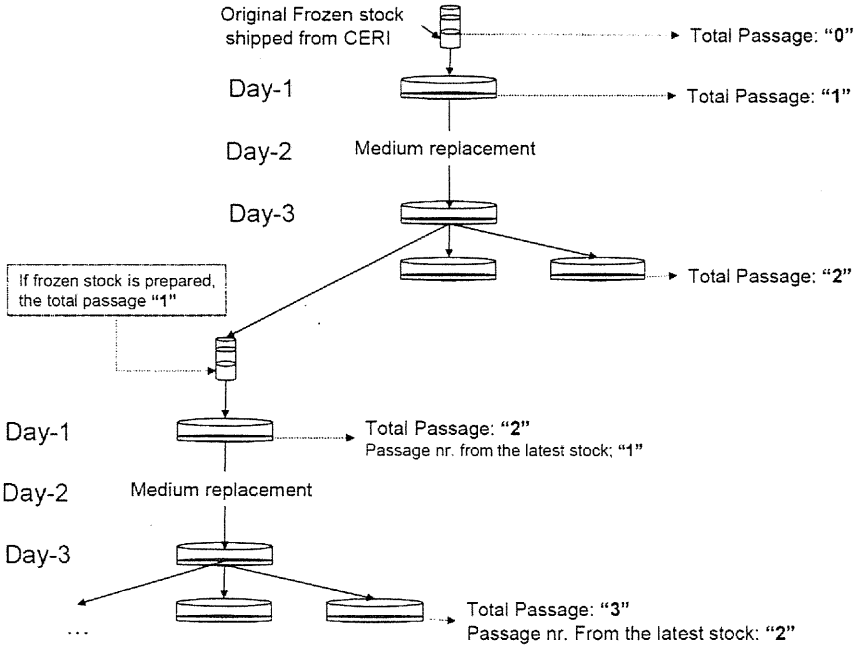

Fig. 1 How to count the cell passage

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. The medium is recommended to be replaced on the next day after the cell propagation and the reconstitution of cells from the 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) 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 about 3 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 about 3 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.

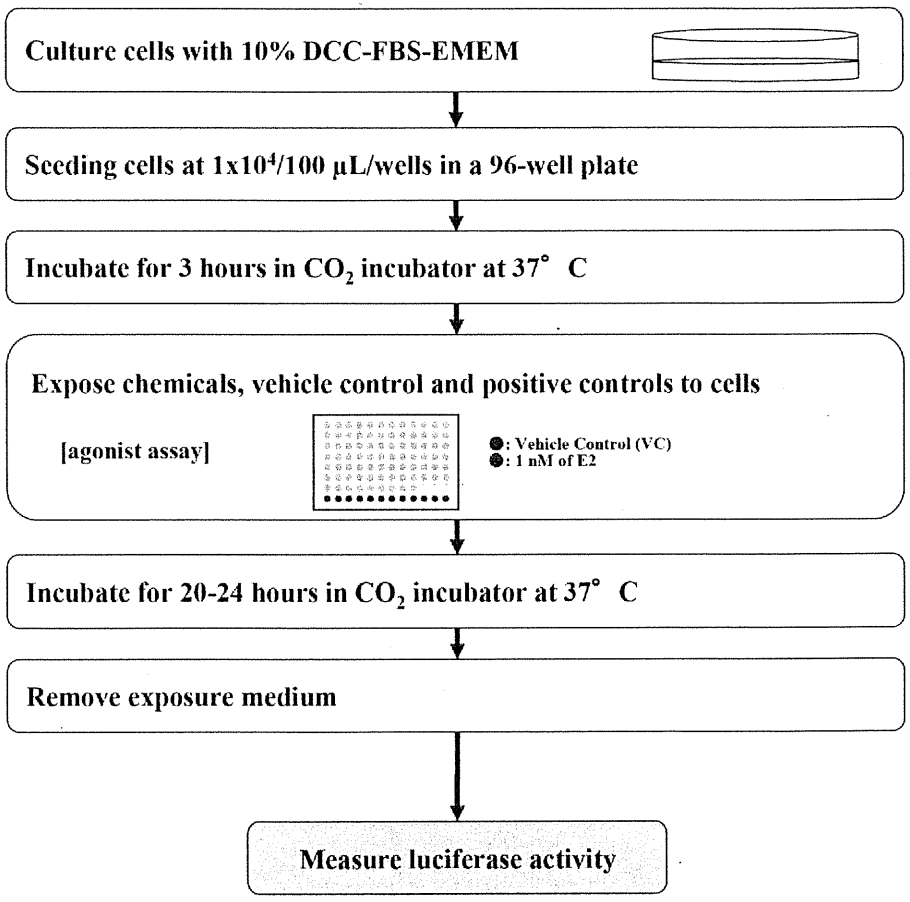

Fig. 2 Schematic Flow for the Estrogenic Assay

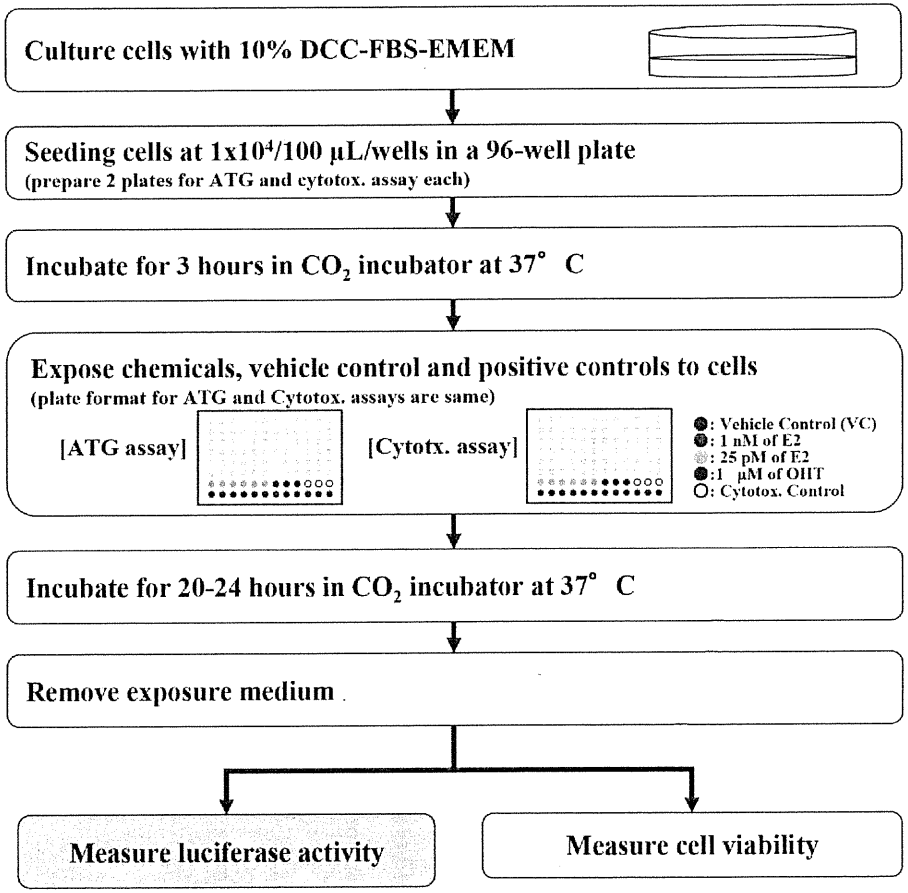

Fig. 3 Schematic Flow for the Anti-estrogenic assay

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.	

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