4.4 Preparation of cells

Assay plate will be prepared according to the support protocol No.5

4.5 Reagents for luciferase assay

Commercial luciferase assay reagent, Steady-Glo Luciferase Assay System (Promega, E2510 and its equivalents) or standard luciferase assay system (Promega, E1500 and its equivalents) will be used in this study. A bottle of Luciferase Assay Substrate is dissolved with the Luciferase Assay Buffer. Dissolved substrate should be used immediately or stored below -20°C.

In the case of using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531) should be used before adding the substrate.

4.7 Chemical exposure

Each test chemical diluted in DMSO will be added to the wells to final concentrations of 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-11}-10^{-5}M) for test in quadruplicate.

To achieve the above test condition, each chemical stock solution should be serially diluted in common ratio of 1:10 with DMSO to obtain 10 μM, 10 μM, 1 μM, 100 nM, 10 nM and 1 nM working solutions. Exact 1.5 μl of 10 mM chemical stock and 6 working solutions will dilute in serum-free EMEM (500 μl) supplemented with 75pM of E2.

Then 50μl of the diluted test samples will add to each well of assay plate according to the assignment table shown in Figure 1.

Reference control wells (n=4) treated with 25pM of E2 without any other chemicals and vehicle control wells (n=4) treated with DMSO alone will be prepared on every assay plate. After adding the chemicals, the assay plates will be incubated in as CO_2 incubator for 20-24 h to induce the reporter gene product.

Figure 1 Typical assignment of assay plate for antagonist assay

	Chemical 1				Chemical 2				Chemical 3			
	1	2	3	4	5	6	7	8	9	10	11	12
A	10 μM	→	→	→	→	→	→	→	→	→	→	→
B	1 μM	→	→	→	→	→	→	→	→	→	→	→
C	100 nM	→	→	→	→	→	→	→	→	→	→	→
D	10 nM	→	→	→	→	→	→	→	→	→	→	→
E	1 nM	→	→	→	→	→	→	→	→	→	→	→
F	100 pM	→	→	→	→	→	→	→	→	→	→	→

G	10 pM	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	→	→	→	RC	→	→	→	→	→

VC: Vehicle control (DMSO only), RC: Reference control (25pM E2 only)

In the case that the anti-estrogenic like effect or downward trends in transcriptional activity are noted, cytotoxicity of chemicals should be examined by using HeLa-9903 control cell. Cytotoxicity of chemicals will be evaluated by luciferase activity under existence of test chemicals. The assay will be performed in the same manner to the above mentioned assay procedure except using HeLa-9903 control cell. The plate format should be according to Figure 2.

Figure 2 Typical assignment of assay plate for cytotoxicity

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 μM	→	→	→	→	→	→	→	→	→	→	→
B	1 μM	→	→	→	→	→	→	→	→	→	→	→
C	100 nM	→	→	→	→	→	→	→	→	→	→	→
D	10 nM	→	→	→	→	→	→	→	→	→	→	→
E	1 nM	→	→	→	→	→	→	→	→	→	→	→
F	100 pM	→	→	→	→	→	→	→	→	→	→	→
G	10 pM	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	→	→	→	→	→	→	→	→	→

VC: Vehicle control (DMSO only)

4.8 Luciferase assay (See support protocol No. 6)

Luciferase activity will be measured with the luciferase assay reagent and a luminometer according to the manufacturer's instructions.

5. Analysis of data

The luminescence signal data was processed, and the average and standard deviation for the vehicle control wells will be calculated. The integrated value for each test well will be divided by the average integrated value of the vehicle control wells to obtain individual relative transcriptional activity. Then the average transcriptional activity will be calculated for each concentration of the test chemical. Then 50% inhibitory concentration against mean transcriptional activity induced by reference wells (25pM E2), were calculated, and used for evaluating anti-estrogenic activity of chemicals.

Calculation described above was made by the commercial software with the Hill's logistic equation showing below;

Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

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

In the cytotoxicity test, the luminescence signal data was also processed, and the average will be calculated. The integrated value for each test well will be divided by the average integrated value of the vehicle control wells to obtain individual relative transcriptional activity. When transcriptional activity are reduced less than 80% of the mean transcriptional activity of vehicle control wells, the concentration should be regarded as cytotoxic concentration and excluded for evaluation of anti-estrogenic effect.

6/8/2005

SUPPORT PROTOCOLS

No.1 Preparation of medium

Reagents

- Eagle's Minimal Essential medium without Neutral red (Nissui Pharmaceutical Co.)

- 10% Sodium bicarbonate ($NaHCO_3$)

  Dissolve 10 grams of $NaHCO_3$ to a final volume of 100 mL with water. Then the solution should be sterilized using vacuum-driven bottle-top sterilization filter unit and stored in room temperature.

- 3% Glutamine

  Dissolve 3 grams of glutamine to a final volume of 100 mL with water. Then the solution should be sterilized using vacuum-driven bottle-top sterilization filter unit. Prepared 3% Glutamine should be stored in aliquots under -20°C.

- Dextran-corted charcoal (DCC)-treated Fetal bovine serum (DCC-FBS)

  Prepared and provided by CERI-Japan.

Preparation of EMEM*

Add following reagent into a 1L conical glass flask and then make to 1 liter with Milli-Q water.

- 9.4 grams of pre-made powder medium
- 18 mL 10% Sodium bicarbonate
- 3% Glutamine 12 mL

Preparation of 10%FBS-EMEM*

Add 56 mL of dextran-corted charcoal (DCC)-treated Fetal bovine serum (DCC-FBS) to 500mL EMEM.

*EMEM and 10%FBS-EMEM should be stored in a refrigerator after sterilized with vacuum-driven bottle-top sterilization filter unit.

6/8/2005

SUPPORT PROTOCOLS

No. 3. Propagation

1. Remove the medium from the culture dish with sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove PBS with sterile pipette or sucker.
4. Add 2mL of Trypsin-EDTA solution (0.25% Trypsin + 0.02%EDTA/PBS) to cover the bottom of the culture dish and then remove the excess.
5. Allow to stand Trypsin treated cell for ca. 3 min in 5% CO_2 incubator at 37°C.
   (Monitor cells under microscope. Cells are beginning to detach when they appear rounded)
6. Tap the dish gently.
7. Wash to remove the adherent cells with 5 mL of 10%FBS-EMEM.
8. Count cell number.
9. Dilute the cell suspension with 10%FBS-EMEM to 0.4-1.0 x 10^5 cells/mL.
10. Place 10 mL of cell suspension to 90 mm culture dish.
11. Incubate the cell in 5% CO_2 incubator at 37°C.

6/8/2005

SUPPORT PROTOCOLS

No. 2. Reconstitute of cell from the frozen stock

1. Remove vial from Liquid Nitrogen or freezer and immediately transfer to 37°C water bath.
2. While holding the tip of the vial, gently agitate the vial.
3. When completely thawed, transfer the cell stock into 5mL pre-warmed 10%FBS-EMEM in 15 mL conical tube.
4. Centrifuge the tube at 1100 rpm (200-300 x g) for 5min, and remove the supernatant carefully.
5. Resuspend the cell with 10 mL of 10%FBS-EMEM and place to 90 mm culture dish.
6. Incubate the cell in 5% CO_2 incubator at 37°C.

6/8/2005

SUPPORT PROTOCOLS

No. 4. Preparation of frozen stock

1. Remove the medium from the culture dish with sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove PBS with sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution to cover the bottom of the culture dish and then remove the excess.
5. Allow to stand Trypsin treated cell for ca. 3 min in 5% CO_2 incubator at 37°C.
   (Monitor cells under microscope. Cells are beginning to detach when they appear rounded)
6. Tap the dish gently.
7. Wash to remove the adherent cells with 5 mL of 10%FBS-EMEM.
8. Count cell number.
9. Centrifuge the tube at 1100 rpm (200-300 x g) for 5min, and remove the supernatant carefully.
10. Add Cell-Banker* (Juji Field Inc.) and resuspend the cell at density of ca 1 x 10^4 cells/mL.
11. Make 1 mL aliquots of cell stock.
12. Freeze and store the cell stock below -80°C**.

*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 (more than 3 months).

5/2/2006

SUPPORT PROTOCOLS

No. 5 Preparation of assay plate

Prepare a dish of cultured hERα-HeLa-9903 cell

1. Remove the medium from the culture dish with sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove PBS with sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution to cover the bottom of the culture dish and then remove the excess.
5. Allow to stand Trypsin treated cell for ca. 3 min in 5% CO_2 incubator at 37°C.
   (Monitor cells under microscope. Cells are beginning to detach when they appear rounded)
6. Tap the dish gently.
7. Wash to remove the adherent cells with 5 mL of 10%FBS-EMEM.
8. Count cell number.
9. Centrifuge the tube at 1100 rpm (200-300 x g) for 5min, and remove the supernatant carefully.
10. Resuspend the cell with 10%FBS-EMEM to obtain a final cell density of 1×10^5 cells/mL.
11. Add 100μL of cell suspension into each well of 96 well assay plate (Nunc #136102 or equivalents).
12. Incubate the cell in 5% CO_2 incubator at 37°C for 3h
13. Proceed to chemical exposure.

SUPPORT PROTOCOLS

No. 6-1. Chemiluminescence Detection with standard luciferase reagent

Reagents

Cell lysis reagent (4.5x): Dilute 10 mL of 5×Cell Culture Lysis Reagent (CCLR, #E1531) with 45 mL of distilled water.

Luciferase Assay Reagent: Add 1 vial (105 mL) of Luciferase Assay buffer (Promega, #E4550) into a vial containing Luciferase Assay Substrate (Promega, #E4550), and dissolve the substrate thoroughly. Store the substrate below -20□ if necessary.

Chemiluminescence Detection

1. Flick and drain off the contents of the assay plate.
2. Add 100μl of PBS to the well to wash the plate.
3. Flick and drain off the contents of the assay plate.
4. Add 100μl of PBS to the well to wash the plate again.
5. Flick and drain off the contents of the assay plate.
6. Add 15uL of Cell lysis reagent (4.5x) to wells.
7. Incubate for 10 min at room temperature.
8. Add 50uL of Luciferase Assay Reagent to wells.
9. Read plates on a Chemiluminescence plate reader.

SUPPORT PROTOCOLS

No. 6-2. Chemiluminescence Detection with luciferase reagent using Steady-Glo Luciferase Assay System

Reagents

Luciferase Assay Reagent: Add 1 vial (100 mL) of Luciferase Assay buffer into a vial containing Luciferase Assay Substrate (Promega, #E2520), and dissolve the substrate thoroughly. Store the substrate below -20□ if necessary.

Chemiluminescence Detection

1. Remove 50μL of assay medium from all wells of assay plate.
2. Add 100μL of Luciferase Assay Reagent to wells.
3. Allowed to stand for 5 min.
4. Read plates on a Chemiluminescence plate reader

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Detection of estrogenic activity using reporter gene assay

Description: This document provides a methodology for detecting anti-estrogenic activity of chemicals by reporter gene assay technique using hER-HeLa-9903 cell line.

Materials and methods

1. Test chemicals

Test chemicals should be dissolved in in dimethylsulfoxide (DMSO) at a concentration of 10 mM.

2. Competitive substance

17β-Estradiol (E2)

3. Vehicle for chemical stock solutions

Dimethylsulfoxide (DMSO) should be used for the vehicle.

4. Materials

4.1 Cell lines

hERα-*HeLa*-9903 stable cell line (Sumitomo Chemicals Co.) will be used for the assay.

4.2 Cell culture (See support protocols No.1 – No. 4)

Cells should be maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with 10% dextran-coated-charcoal (DCC)-treated fetal bovine serum (DCC-FBS), in a CO_2 incubator (5% CO_2) at 37°C.

4.3 Preparation of chemicals

All chemicals will be dissolved in DMSO at a concentration of 10 mM, and the solutions will be serially diluted with the same solvent at a common ratio of 1:10 to prepare stock solutions with concentrations of 1 mM, 100 μM, 10 μM, 1 μM, 100 nM and 10 nM. In the case of positive control substance (E2), stock solutions will be prepared at concentrations of 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, 1 nM and 100 pM.

4.4 Preparation of cells

Assay plate will be prepared according to the support protocol No.5

4.5 Reagents for luciferase assay

Commercial luciferase assay reagent, Steady-Glo Luciferase Assay System (Promega, E2510 and its equivalents) or standard luciferase assay system (Promega, E1500 and its equivalents) will be used in this study. A bottle of Luciferase Assay Substrate is dissolved with the Luciferase Assay Buffer. Dissolved substrate should be used immediately or stored below -20°C.

In the case of using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531) should be used before adding the substrate.

4.7 Chemical exposure

Each test chemical diluted in DMSO will be added to the wells to final concentrations of 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-11}-10^{-5}M) for test in quadruplicate.

To achieve the above test condition, each chemical stock solution should be serially diluted in common ratio of 1:10 with DMSO to obtain 10 μM, 10 μM, 1 μM, 100 nM, 10 nM and 1 nM working solutions. Exact 1.5 μl of 10 mM chemical stock and 6 working solutions will dilute in serum-free EMEM (500 μl).

Then 50μl of the diluted test samples will add to each well of assay plate according to the assignment table shown in Figure 1.

Positive control wells (n=3) treated with 100pM of E2 and vehicle control wells (n=3) treated with DMSO alone will be prepared on every assay plate. After adding the chemicals, the assay plates will be incubated in as CO_2 incubator for 20-24 h to induce the reporter gene product.

Figure 1 Typical assignment of assay plate for agonist assay

	Test Chemical 1			Test Chemical 2			Test Chemical 3			Test Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10 μM	→	→	→	→	→	→	→	→	→	→	→
B	1 μM	→	→	→	→	→	→	→	→	→	→	→
C	100 nM	→	→	→	→	→	→	→	→	→	→	→
D	10 nM	→	→	→	→	→	→	→	→	→	→	→
E	1 nM	→	→	→	→	→	→	→	→	→	→	→
F	100 pM	→	→	→	→	→	→	→	→	→	→	→
G	10 pM	→	→	→	→	→	→	→	→	→	→	→
H	NC	→	→	BL	→	→	→	→	→	PC	→	→

NC: Negative control (DMSO), BL; Blank, PC: Positive control (1 nM E2)

4.8 Luciferase assay (See support protocol No. 6)

Luciferase activity will be measured with the luciferase assay reagent and a luminometer according to the manufacturer's instructions.

5. Analysis of data

The luminescence signal data will be processed, and the average and standard deviation for the negative control wells were calculated. The integrated value for each test well will be divided by the average integrated value of the negative control wells to obtain individual relative transcriptional activity. Then the average transcriptional activity will be calculated for each concentration of the test chemical. The PC50 and PC10 values will be calculated for each test chemical. These PC values are defined as the concentration of chemical estimated to cause 50% or 10%, respectively, of activity of the positive control response. Calculation described above will be made by the common spread sheet provided from CERI-Japan. If the Hill's logistic equation is applicable to dose response data, EC50 should be calculated by the equation.

Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

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

Descriptions of PC values are provided in Figure 2.

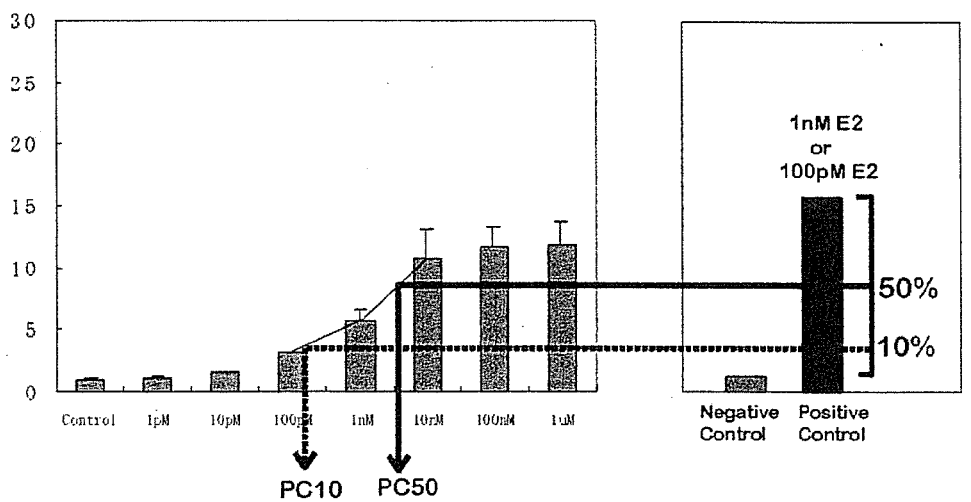

Figure 2 Description of PC10 and PC50

SUPPORT PROTOCOLS

No.1 Preparation of medium

Reagents

- Eagle's Minimal Essential medium without Neutral red (Nissui Pharmaceutical Co.)

- 10% Sodium bicarbonate ($NaHCO_3$)

  Dissolve 10 grams of $NaHCO_3$ to a final volume of 100 mL with water. Then the solution should be sterilized using vacuum-driven bottle-top sterilization filter unit and stored in room temperature.

- 3% Glutamine

  Dissolve 3 grams of glutamine to a final volume of 100 mL with water. Then the solution should be sterilized using vacuum-driven bottle-top sterilization filter unit. Prepared 3% Glutamine should be stored in aliquots under -20°C.

- Dextran-corted charcoal (DCC)-treated Fetal bovine serum (DCC-FBS)

  Prepared and provided by CERI-Japan.

Preparation of EMEM*

Add following reagent into a 1L conical glass flask and then make to 1 liter with Milli-Q water.

- 9.4 grams of pre-made powder medium
- 18 mL 10% Sodium bicarbonate
- 3% Glutamine 12 mL

Preparation of 10%FBS-EMEM*

Add 56 mL of dextran-corted charcoal (DCC)-treated Fetal bovine serum (DCC-FBS) to 500mL EMEM.

*EMEM and 10%FBS-EMEM should be stored in a refrigerator after sterilized with vacuum-driven bottle-top sterilization filter unit.

SUPPORT PROTOCOLS

No. 2. Reconstitute of cell from the frozen stock

1. Remove vial from Liquid Nitrogen or freezer and immediately transfer to 37°C water bath.
2. While holding the tip of the vial, gently agitate the vial.
3. When completely thawed, transfer the cell stock into 5mL pre-warmed 10%FBS-EMEM in 15 mL conical tube.
4. Centrifuge the tube at 1100 rpm (200-300 x g) for 5min, and remove the supernatant carefully.
5. Resuspend the cell with 10 mL of 10%FBS-EMEM and place to 90 mm culture dish.
6. Incubate the cell in 5% CO_2 incubator at 37°C.

SUPPORT PROTOCOLS

No. 3. Propagation

1. Remove the medium from the culture dish with sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove PBS with sterile pipette or sucker.
4. Add 2mL of Trypsin-EDTA solution (0.25% Trypsin + 0.02%EDTA/PBS) to cover the bottom of the culture dish and then remove the excess.
5. Allow to stand Trypsin treated cell for ca. 3 min in 5% CO_2 incubator at 37°C.
   (Monitor cells under microscope. Cells are beginning to detach when they appear rounded)
6. Tap the dish gently.
7. Wash to remove the adherent cells with 5 mL of 10%FBS-EMEM.
8. Count cell number.
9. Dilute the cell suspension with 10%FBS-EMEM to 0.4-1.0 x 10^5 cells/mL.
10. Place 10 mL of cell suspension to 90 mm culture dish.
11. Incubate the cell in 5% CO_2 incubator at 37°C.

SUPPORT PROTOCOLS

No. 4. Preparation of frozen stock

1. Remove the medium from the culture dish with sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove PBS with sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution to cover the bottom of the culture dish and then remove the excess.
5. Allow to stand Trypsin treated cell for ca. 3 min in 5% CO_2 incubator at 37°C.
   (Monitor cells under microscope. Cells are beginning to detach when they appear rounded)
6. Tap the dish gently.
7. Wash to remove the adherent cells with 5 mL of 10%FBS-EMEM.
8. Count cell number.
9. Centrifuge the tube at 1100 rpm (200-300 x g) for 5min, and remove the supernatant carefully.
10. Add Cell-Banker* (Juji Field Inc.) and resuspend the cell at density of ca 1 x 10^4 cells/mL.
11. Make 1 mL aliquots of cell stock.
12. Freeze and store the cell stock below -80°C**.

*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 (more than 3 months).

SUPPORT PROTOCOLS

No. 5 Preparation of assay plate

Prepare a dish of cultured hERα-HeLa-9903 cell

1. Remove the medium from the culture dish with sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove PBS with sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution to cover the bottom of the culture dish and then remove the excess.
5. Allow to stand Trypsin treated cell for ca. 3 min in 5% CO_2 incubator at 37°C.
   (Monitor cells under microscope. Cells are beginning to detach when they appear rounded)
6. Tap the dish gently.
7. Wash to remove the adherent cells with 5 mL of 10%FBS-EMEM.
8. Count cell number.
9. Centrifuge the tube at 1100 rpm (200-300 x g) for 5min, and remove the supernatant carefully.
10. Resuspend the cell with 10%FBS-EMEM to obtain a final cell density of 1 x 10^5 cells/mL.
11. Add 100μL of cell suspension into each well of 96 well assay plate (Nunc #136102 or equivalents).
12. Incubate the cell in 5% CO_2 incubator at 37°C for 3h
13. Proceed to chemical exposure.