アゴニスト系評価結果

Agonist Test Substance	Laboratory	ER TA Agonist Activity (activities in **Bold** are discordant from ICCVAM meta-data)
Compound A1-1	ICCVAM Meta-data	Positive
	XDS	Positive
	ECVAM	Positive
	Hiyoshi	Positive
Compound A1-2	ICCVAM Meta-data	Positive
	XDS	Positive
	ECVAM	Positive
	Hiyoshi	Positive
Compound A1-3	ICCVAM Meta-data	Negative
	XDS	Negative
	ECVAM	**Positive**
	Hiyoshi	Negative
Compound A1-4	ICCVAM Meta-data	Positive
	XDS	Positive
	ECVAM	Positive
	Hiyoshi	Positive

アンタゴニスト系評価結果

Antagonist Test Substance	Laboratory	ER TA Antagonist Activity (activities in **Bold** are discordant from ICCVAM meta-data)
Compound A2-1	ICCVAM Meta-data	Positive
	XDS	Positive
	ECVAM	Positive
	Hiyoshi	Positive
Compound A2-2	ICCVAM Meta-data	Positive
	XDS	**Negative**
	ECVAM	Positive
	Hiyoshi	Positive
Compound A2-3	ICCVAM Meta-data	Negative
	XDS	**Positive**
	ECVAM	**Positive**
	Hiyoshi	**Positive**
Compound A2-4	ICCVAM Meta-data	Positive
	XDS	Positive
	ECVAM	Positive
	Hiyoshi	Positive

表 2-4 LumiCell Phase Ⅱ a における各施設の評価結果と ICCVAM データとの比較
※バリデーション試験中のため化合物名は、仮の ID で示した

	XDS				
	Units	Mean	SD	Mean Plus 2.5 Times SD	Mean Minus 2.5 Times SD
DMSO	RLU	2258	2454	8393	0*
	ECVAM				
DMSO	RLU	3219	1580	7168	0*
	Hiyoshi				
DMSO	RLU	4273	1538	8119	428

	XDS				
	Units	Mean	SD	Mean Plus 2.5 Times SD	Mean Minus 2.5 Times SD
DMSO	RLU	2258	2454	8393	0*
E2	Adjusted RLU	8415	761	10318	6513
	ECVAM				
DMSO	RLU	3219	1580	7168	0*
E2	Adjusted RLU	8972	675	10659	7284
	Hiyoshi				
DMSO	RLU	4273	1538	8119	428
E2	Adjusted RLU	5866	1013	8399	3333

図 2-7 LumiCell Phase Ⅱb における各施設の品質評価基準値

Agonist Test Substance	Laboratory	ER TA Agonist Activity (activities in **Bold** are discordant from ICCVAM meta-data)
Compound B1-1	ICCVAM Meta-data	Negative
	XDS	Negative (3/3)
	ECVAM	**Positive (3/3)**
	Hiyoshi	Negative (2/3)
Compound B1-2	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)
Compound B1-3	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)
Compound B1-4	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)

Agonist Test Substance	Laboratory	ER TA Agonist Activity (activities in **Bold** are discordant from ICCVAM meta-data)
Compound B1-5	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)
Compound B1-6	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (4/4)
Compound B1-7	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)
Compound B1-8	ICCVAM Meta-data	Negative
	XDS	Negative (3/3)
	ECVAM	**Positive** (3/3)
	Hiyoshi	Negative (3/4)

表 2-5A LumiCell Phase II b アゴニスト測定8化合物の活性判定結果の比較
※バリデーション試験中のため化合物名は、仮の ID で示した

Antagonist Test Substance	Laboratory	ER TA Antagonist Activity (activities in **Bold** are discordant from ICCVAM meta-data)
Compound B2-1	ICCVAM Meta-data	Positive
	XDS	**Negative (3/3)**
	ECVAM	Positive (2/3)
	Hiyoshi	Positive (3/4)
Compound B2-2	ICCVAM Meta-data	Negative
	XDS	Negative (2/3)
	ECVAM	**Positive (3/3)**
	Hiyoshi	**Positive (3/3)**
Compound B2-3	ICCVAM Meta-data	Negative
	XDS	**Positive (3/3)**
	ECVAM	**Positive (2/3)**
	Hiyoshi	**Positive (2/4)**
Compound B2-4	ICCVAM Meta-data	Negative
	XDS	**Positive (3/3)**
	ECVAM	**Positive (3/3)**
	Hiyoshi	**Positive (3/3)**

Antagonist Test Substance	Laboratory	ER TA Antagonist Activity (activities in **Bold** are discordant from ICCVAM meta-data)
Compound B2-5	ICCVAM Meta-data	Positive
	XDS	**Negative (3/3)**
	ECVAM	Positive (2/3)
	Hiyoshi	Positive (2/4)
Compound B2-6	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	**Negative** (4/4)
Compound B2-7	ICCVAM Meta-data	Positive
	XDS	**Negative (2/3)**
	ECVAM	Positive (3/3)
	Hiyoshi	**Negative (3/3)**
Compound B2-8	ICCVAM Meta-data	Positive
	XDS	**Negative (3/3)**
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)

表 2-5B LumiCell Phase Ⅱb アンタゴニスト測定8化合物の活性判定結果の比較
※バリデーション試験中のため化合物名は、仮の ID で示した

添付資料 1

Ver. 2008/12/24

Protocols of Stably Transfected Transcriptional Activation (STTA) Assay Using hERα-*HeLa*-9903 Cell line for Detecting Anti-estrogenic Activities of Chemicals

= For Multi-laboratory Validation Study=

Chemicals Evaluation and Research Institute, Japan

(CERI)

Table of Contents

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
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

Tasks	Purpose	Procedures in brief	Draft Schedule
Start chemical distribution from JaCVAM			2008.5
Task-1:	Confirm the edge effects to establish the plate layout for further testing	(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.	**2008. 6** Data should be submitted **until the end of 2008.6**.
	Test system setup	(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. Agonist: •E2; •17α-Estradiol; •Corticosterone	
Task-2:	Confirm Lab performance for antagonist (ATG) assay (including range finding test, cytotoxicity (cytotox.) test)	(1) Test "anti-estrogenic" activities of 4 chemicals. Antagonist: •4-Hydroxytamoxifen – Strongly anti-estrogenic; •Tamoxifen – Moderately anti-estrogenic; •RU-486 – Weakly anti-estrogenic, cytotoxic; •Negatives – Negative, cytotoxic	Beginning of **2008.8** – End. of **2008.8** Data should be submitted **until the Mid. of 2008.9**.
Task-3:	Test coded chemicals	(1) Test "anti-estrogenic" activities of coded X chemicals	End of **2008.9** - End of **2008.11** Data should be submitted **until the end of 2008.1.**.

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	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).