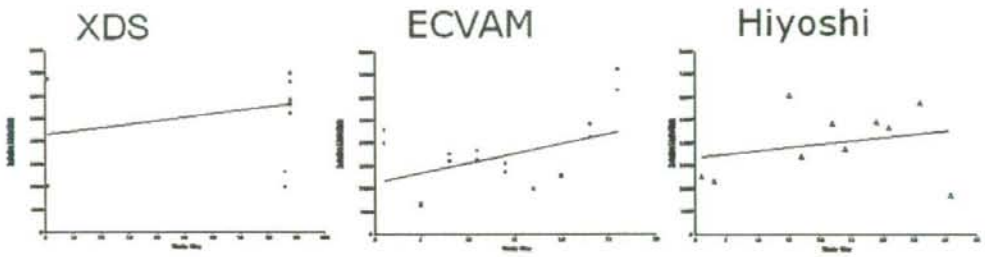
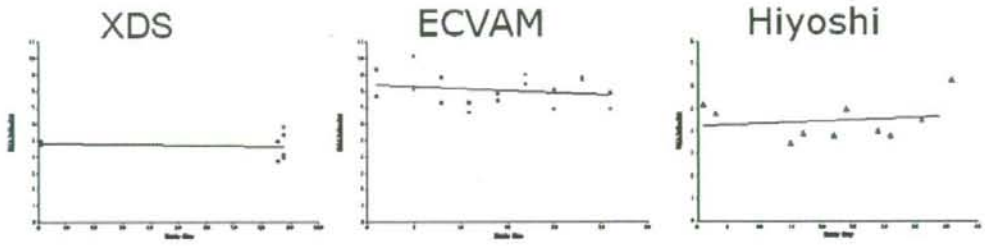
表 2-10　Phase Ⅱb 被検化合物の EC_{50}（3 回繰返し）結果一覧

Code	1st.	2nd.	3rd.	Ave.	S.D.	C.V.
H0009	0.83	0.99	0.43	0.75	0.29	38
H0010	0.27	0.26	0.21	0.25	0.032	13
H0011	3.0E-06	1.9E-06	2.6E-06	2.5E-06	5.9E-07	24
H0012	0.49	0.79	0.41	0.56	0.20	36
H0013	11	18	25	18	7.3	40
H0014	0.051	0.16	0.14	0.12	0.058	49
H0015	positive	negative	negative	-	-	-
H0016	1.4	1.3	8.1	3.6	3.95	110
H0017	positive	negative	positive	-	-	-
H0018	positive	negative	positive	-	-	-
H0019	negative	negative	negative	-	-	-
H0020	negative	positive	positive	-	-	-
H0021	550	28	18	200	300	150
H0022	25	26	27	26	0.9	3.5
H0023	negative	negative	negative	-	-	-
H0024	positive	positive	positive	-	-	-

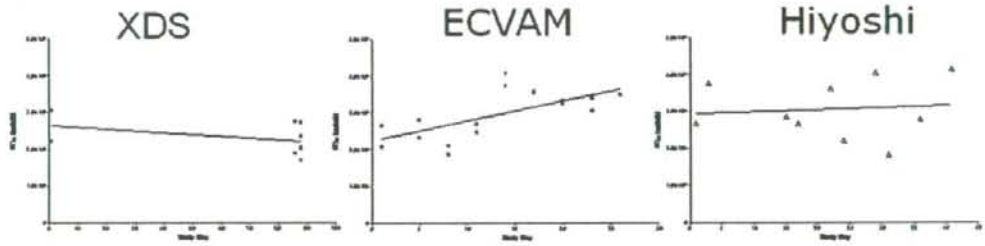
DMSO



E2 Fold-induction



E2 EC50



Methoxychlor control

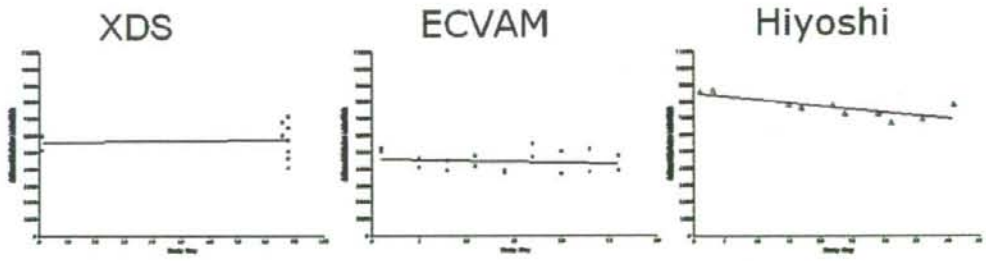
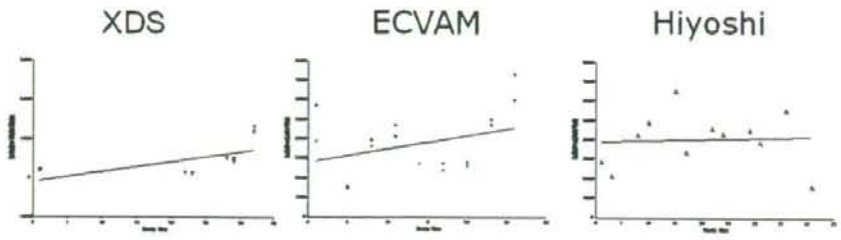
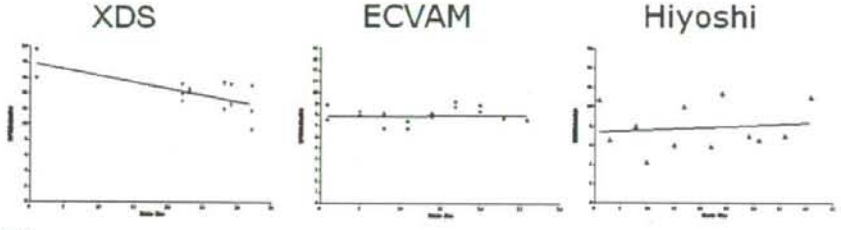


図 2-6 各施設ごとのアゴニスト系の評価基準項目の経時変化

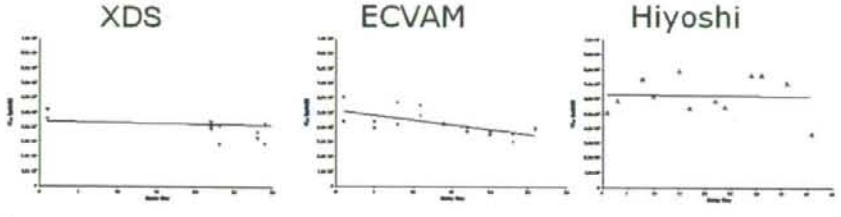
DMSO



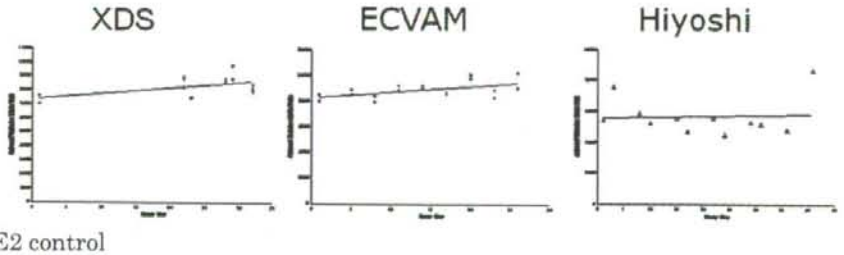
Ral/E2 fold induction



Ral/E2 IC50



E2 control



Flavone/E2 control

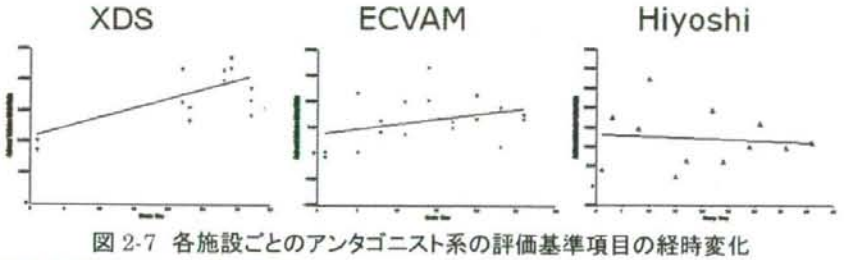


図 2-7 各施設ごとのアンタゴニスト系の評価基準項目の経時変化

アゴニスト系基準値の ANOVA 解析結果

	p-Value[1,2,3]	F Value[4]
DMSO	*0.045*	*3.4*
E2 Maximum Fold-Induction	*<0.001*	*88.5*
E2 EC_{50}	*<0.001*	*8.4*
Methoxychlor	*<0.001*	*63.8*

[1]Variability is statistically significant at p<0.05.
[2]ANOVA analyzed values from the three participating laboratories. Standardization data is not included in this analysis.
[3]Values in italics have p values that are less than 0.05.
[4]F = ratio of between-day variability to within-day variability Ŝ a ratio of 1.0 indicates that the within-day variability to between-day variability is equal and a ratio of zero indicates that all means are equal.

アゴニスト系基準値の Newman-Keuls 解析結果

	DMSO		E2 Fold-Induction		E2 EC_{50}		Methoxychlor	
	Mean Difference[1]	p-value[2,3]	Mean Difference[4]	p-value[2,3]	Mean Difference[5]	p-value[2,3]	Mean Difference[6]	p-value[2,3]
XDS vs ECVAM	*1908*	*<0.05*	*-3.0*	*<0.001*	0.3×10^7	>0.05	*1215*	*<0.001*
XDS vs Hiyoshi	1388	>0.05	*1.0*	*<0.05*	-7.8×10^7	*<0.05*	*-1983*	*<0.001*
ECVAM vs Hiyoshi	-520	>0.05	*4.0*	*<0.001*	-8.1×10^7	*<0.05*	*-3198*	*<0.001*

[1]Presented in relative light units
[2]Variability is statistically significant at p<0.05.
[3]Values in italics have p values that are less than 0.05.
[4]Presented in fold-induction.
[5]Presented in μg/mL.
[6]Presented in adjusted relative light units.

アンタゴニスト系基準値の ANOVA 解析結果

	p-Value[1,2,3]	F Value[4]
DMSO	*<0.001*	*34.1*
Reduction	*0.001*	*8.1*
Ral/E2 IC_{50}	*<0.001*	*18.5*
E2	*<0.001*	*50.1*
Flavone/E2	*<0.001*	*59.9*

[1]Variability is statistically significant at p<0.05.
[2]ANOVA analyzed values from the three participating laboratories. Standardization data is not included in this analysis.
[3]Values in italics have p values that are less than 0.05.
[4]F = ratio of between-day variability to within-day variability Ŝ a ratio of 1.0 indicates that the within-day variability to between-day variability is equal and a ratio of zero indicates that all means are equal.

アンタゴニスト系基準値の Newman-Keuls 解析結果

	DMSO		Fold-Reduction		Ral/E2 IC_{50}		E2 Control		Flavone/E2 Control	
	Mean Difference[1]	p-value[2,3]	Mean Difference[4]	p-value[2,3]	Mean Difference[5]	p-value[2,3]	Mean Difference[6]	p-value[2,3]	Mean Difference[6]	p-value[2,3]
XDS vs ECVAM	*-3286*	*<0.001*	*6.2*	*<0.001*	-4.3 x 10	>0.05	-598	>0.05	*2939*	*<0.001*
XDS vs Hiyshi	*-3551*	*<0.001*	*6.3*	*<0.01*	*-2.0 x 10*	*<0.00*	*2556*	*<0.00*	*2357*	*<0.001*
ECVAM vs Hiyshi	-265	>0.05	0.1	>0.05	*-2.0 x 10*	*<0.00*	*3153*	*<0.00*	-582	>0.05

[1]Presented in relative light units
[2]Variability is statistically significant at p<0.05.
[3]Values in italics have p values that are less than 0.05.
[4]Presented in fold-reduction.
[5]Presented in μg/mL.
[6]Presented in adjusted relative light units.

図 2-8 LumiCell アゴニスト、アンタゴニスト測定における評価基準値の施設間比較

Phase II a アゴニスト測定における基準値

	XDS				
	Units	Mean	SD	Mean Plus 2.5 Times SD	Mean Minus 2.5 Times SD
DMSO	RLU	5394	2558	11789	0*
E2 EC_{50}	μg/mL	2.3×10^{-6}	4.5×10^{-7}	3.4×10^{-6}	1.2×10^{-6}
Methoxychlor	Adjusted RLU	5709	974	8144	3274

	ECVAM				
	Units	Mean	SD	Mean Plus 2.5 Times SD	Mean Minus 2.5 Times SD
DMSO	RLU	3486	1582	7441	0*
E2 EC_{50}	μg/mL	2.7×10^{-6}	8.5×10^{-7}	4.8×10^{-6}	1.9×10^{-6}
Methoxychlor	Adjusted RLU	4494	590	5969	3019

	Hiyoshi				
	Units	Mean	SD	Mean Plus 2.5 Times SD	Mean Minus 2.5 Times SD
DMSO	RLU	4006	1500	7756	256
E2 EC_{50}	μg/mL	3.1×10^{-6}	7.9×10^{-7}	5.1×10^{-6}	1.1×10^{-6}
Methoxychlor	Adjusted RLU	7692	633	9275	6110

*Unadjusted DMSO control values can not be below zero

Phase II a アンタゴニスト測定における基準値

	XDS				
	Units	Mean	SD	Mean Plus 2.5 Times SD	Mean Minus 2.5 Times SD
DMSO	RLU	1986	1748	6355	0*
Ral\E2 IC_{50}	μg/mL	4.3×10^{-4}	9.0×10^{-5}	6.5×10^{-4}	2.0×10^{-4}
E2	Adjusted RLU	8284	744	10143	6424
Flavone	Adjusted RLU	3583	1089	6305	860

	ECVAM				
	Units	Mean	SD	Mean Plus 2.5 Times SD	Mean Minus 2.5 Times SD
DMSO	RLU	3783	1587	7752	0*
Ral\E2 IC_{50}	μg/mL	4.3×10^{-4}	7.9×10^{-5}	6.3×10^{-4}	2.3×10^{-4}
E2	Adjusted RLU	8881	640	10480	7282
Flavone	Adjusted RLU	644	458	1789	-501

	Hiyoshi				
	Units	Mean	SD	Mean Plus 2.5 Times SD	Mean Minus 2.5 Times SD
DMSO	RLU	4048	1386	7513	583
Ral\IC_{50}	μg/mL	6.3×10^{-4}	1.3×10^{-4}	9.5×10^{-4}	3.1×10^{-4}
E2	Adjusted RLU	5728	1221	8781	2676
Flavone	Adjusted RLU	1226	724	3036	-584

*Unadjusted DMSO values can not be below zero

図 2-9 各施設の Phase I から計算された LumiCell 評価基準値

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)
BPA	ICCVAM Meta-data	Positive
	XDS	Positive
	ECVAM	Positive
	Hiyoshi	Positive
BPB	ICCVAM Meta-data	Positive
	XDS	Positive
	ECVAM	Positive
	Hiyoshi	Positive
CORT	ICCVAM Meta-data	Negative
	XDS	Negative
	ECVAM	**Positive**
	Hiyoshi	Negative
DES	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)
DBA	ICCVAM Meta-data	Positive
	XDS	Positive
	ECVAM	Positive
	Hiyoshi	Positive
NON	ICCVAM Meta-data	Positive
	XDS	**Negative**
	ECVAM	Positive
	Hiyoshi	Positive
PROG	ICCVAM Meta-data	Negative
	XDS	**Positive**
	ECVAM	**Positive**
	Hiyoshi	**Positive**
TAM	ICCVAM Meta-data	Positive
	XDS	Positive
	ECVAM	Positive
	Hiyoshi	Positive

図 2-10 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

アゴニスト測定

Laboratory	Total Number of Plates Tested	Number of Plates Passing Acceptance Criteria	Number of Plates Failing Acceptance Criteria	Failed DMSO Only[1]	Failed E2 EC_{50} Only[2]	Failed Methoxychlor Only[3]	Failed Multiple Acceptance Criteria[4]
XDS	15	7 (47%)	8 (53%)	n.a.	8 (100%)	n.a.	n.a.
ECVAM	30	6 (20%)	24 (80%)[5]	1 (4%)	11 (46%)	3 (12.5%)	9 (37.5%)
Hiyoshi	9	8 (89%)	1 (11%)	n.a.	n.a.	1 (100%)	n.a.
Overall	**54**	**21 (39%)**	**33 (61%)**	**1 (3%)**	**19 (58%)**	**4 (12%)**	**9 (27%)**

アンタゴニスト測定

Laboratory	Total Number of Plates Tested	Number of Plates Passing Acceptance Criteria	Number of Plates Failing Acceptance Criteria	Failed RaIVE2 IC_{50}[1]	Failed E2 Only[2]	Failed Flavone Only[3]	Failed Multiple Acceptance Criteria[4]
XDS	14	8 (57%)	6 (43%)	6 (100%)	n.a.	n.a.	n.a.
ECVAM	14	7 (50%)	7 (50%)[6]	n.a.	4 (57%)	2 (29%)	1 (14%)
Hiyoshi	6	6 (100%)	n.a.	n.a.	n.a.	n.a.	n.a.
Overall	**34**	**21 (62%)**	**13 (38%)**	**6 (46%)**	**4 (31%)**	**2 (15%)**	**1 (8%)**

図 2-11 LumiCell Phase II a における各施設の不採用データとその原因

	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 2-12 LumiCell Phase II b における各施設の測定結果採用基準

Agonist Test Substance	Laboratory	ER TA Agonist Activity (activities in **Bold** are discordant from ICCVAM meta-data)
ATZ	ICCVAM Meta-data	Negative
	XDS	Negative (3/3)
	ECVAM	**Positive (3/3)**
	Hiyoshi	Negative (2/3)
BBP	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)
DDT	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)
EE	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)
FLA	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)
GEN	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (4/4)
NON	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)
VIN	ICCVAM Meta-data	Negative
	XDS	Negative (3/3)
	ECVAM	**Positive** (3/3)
	Hiyoshi	Negative (3/4)

図 2-13 LumiCell Phase Ⅱb アゴニスト測定8化合物の活性判定結果の比較

Antagonist Test Substance	Laboratory	ER TA Antagonist Activity (activities in **Bold** are discordant from ICCVAM meta-data)
API	ICCVAM Meta-data	Positive
	XDS	**Negative (3/3)**
	ECVAM	Positive (2/3)
	Hiyoshi	Positive (3/4)
ATZ	ICCVAM Meta-data	Negative
	XDS	Negative (2/3)
	ECVAM	**Positive (3/3)**
	Hiyoshi	**Positive (3/3)**
BBP	ICCVAM Meta-data	Negative
	XDS	**Positive (3/3)**
	ECVAM	Negative (2/3)
	Hiyoshi	**Positive** (2/4)
CORT	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)
DDT	ICCVAM Meta-data	Positive
	XDS	**Negative (3/3)**
	ECVAM	Positive (2/3)
	Hiyoshi	Positive (2/4)
FLA	ICCVAM Meta-data	Positive
	XDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hiyoshi	**Negative** (4/4)
GEN	ICCVAM Meta-data	Positive
	XDS	**Negative (2/3)**
	ECVAM	Positive (3/3)
	Hiyoshi	**Negative (3/3)**
RES	ICCVAM Meta-data	Positive
	XDS	**Negative (3/3)**
	ECVAM	Positive (3/3)
	Hiyoshi	Positive (3/3)

図 2-14 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

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

<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
Data should be submitted until the end of 2008.1..</td></tr>
</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.