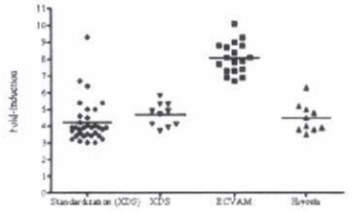
Interlaboratory Comparison of E2 Fold-Induction

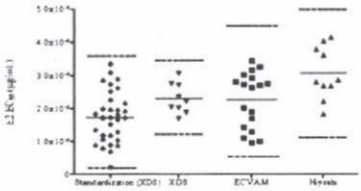


Data points represent E2 fold-induction values from plates tested in the protocol standardization and Phase I studies. Solid horizontal lines represent the E2 fold-induction value for each data set. Plates are rejected if the fold-induction for the maximum E2 response is less than three.

	Fold-Induction			
	N[1]	Mean[2]	SD[2]	CV
XDS	10	4.7	0.70	15%
ECVAM	18	8.1	0.93	11%
Hiyoshi	10	4.5	0.86	19%
Standardization	33	4.2	1.30	30%

[1]Number of plates tested
[2]Units are expressed as maximum E2 fold-induction

Interlaboratory Comparison of E2 EC_{50}

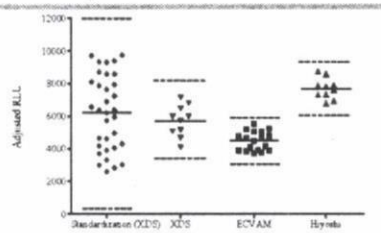


Data points represent E2 EC_{50} values from plates tested in the protocol standardization and Phase I studies. Solid horizontal lines represent the mean E2 EC_{50} value for each data set. Dashed lines indicate the mean E2 EC_{50} value plus and minus 2.5 times the standard deviation from the mean.

	E2 EC_{50}			
	N[1]	Mean[2]	SD[2]	CV
XDS	9*	2.3×10^{-9}	4.5×10^{-10}	20%
ECVAM	18	2.3×10^{-9}	8.5×10^{-10}	37%
Hiyoshi	10	3.1×10^{-9}	7.9×10^{-10}	26%
Standardization	33	1.7×10^{-9}	7.6×10^{-10}	44%

*A single EC_{50} value was excluded from analysis after failing the Q-test for outliers
[1]Number of plates tested
[2]Units are expressed as pg/mL

Interlaboratory Comparison of Methoxychlor



Data points represent methoxychlor control values in adjusted RLU from plates tested in the protocol standardization and Phase I studies. Solid horizontal lines represent the mean methoxychlor control value for each data set. Dashed lines represent the mean plus and minus 2.5 times the standard deviation from the mean.

	Methoxychlor Control			
	N[1]	Mean[2]	SD[2]	CV
XDS	10	5709	974	17%
ECVAM	18	4494	590	13%
Hiyoshi	10	7692	633	8%
Standardization	33	6218	2299	37%

[1]Number of plates tested
[2]Units are expressed as adjusted relative light units

Agonist ANOVA Results for Interlaboratory Comparison of Reference Standard and Controls

	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 – 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.

Newman-Keuls Results for Interlaboratory Comparison of Agonist Reference Standard and Controls

	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*	*-3.9*	*<0.001*	*0.3×10^{-7}*	*>0.05*	*1213*	*<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	*4.9*	*<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 ug/mL
[6]Presented in adjusted relative light units

Dunnett's Results for Agonist Interlaboratory Comparison of Reference Standard and Controls

Lab vs Standardization Study	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	-296.5	<0.01	-0.8	>0.05	-5.7×10^{-7}	>0.05	509	>0.05
ECVAM	-1057	>0.05	*-3.8*	*<0.01*	*-5.3×10^{-7}*	>0.05	*1724*	*<0.01*
Hiyoshi	-1577	<0.05	0.2	>0.05	*-13.4×10^{-7}*	<0.01	*-1474*	*<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-induction
[5]Presented in ug/mL
[6]Presented in adjusted relative light units

Agonist Historical Database Values Established for Phase IIa Acceptance Criteria

	XDS				
	Units	Mean	SD	Mean Plus 2.5 Times SD	Mean Minus 2.5 Times SD
DMSO	RLU	5394	2552	11789	0*
E2 EC_{50}	pg/mL	2.3×10^{-4}	4.5×10^{-5}	3.4×10^{-4}	1.2×10^{-4}
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}	pg/mL	2.7×10^{-4}	8.5×10^{-5}	4.8×10^{-4}	1.9×10^{-4}
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}	pg/mL	3.1×10^{-4}	7.9×10^{-5}	5.1×10^{-4}	1.1×10^{-4}
Methoxychlor	Adjusted RLU	7692	633	9275	6110

*Unadjusted DMSO control values can not be below zero

The LUMI-CELL® ER Assay International Validation Study - Phase I ER Antagonist Testing

Testing of Phase I Antagonist Reference Standards and Controls

- Multiple testing of antagonist reference standards and controls was conducted to:
  - Demonstrate proficiency with the agonist protocol
  - Provide reference standard and control data for an evaluation of intra- and inter-laboratory reproducibility
  - Establish historical databases to be used to develop acceptance criteria for tests to be conducted in Phase IIa

Historical Database for Phase IIa Antagonist Testing

- Acceptance or rejection of antagonist tests to be conducted in Phase IIa will be based on evaluation of test plate reference standard and control results. Results will compared to acceptance criteria derived from the historical databases established from Phase I testing at each laboratory. Antagonist test plate acceptance criteria to be used in Phase IIa are summarized as follows:
  - Plate reduction, as measured by dividing the averaged highest Ral/E2 reference standard RLU value by the averaged lowest Ral/E2 reference standard value, must be greater than three-fold
  - Ral/E2 IC_{50} values must be within 2.5 times the standard deviation of the historical database Ral/E2 IC_{50} value
  - DMSO control RLU values must be within 2.5 times the standard deviation of the historical DMSO control value
  - E2 control RLU values must be within 2.5 times the standard deviation of the historical E2 control value
  - Flavone/E2 control RLU values must be within 2.5 times the standard deviation of the historical flavone/E2 control value

Adjustment and Normalization of Antagonist Assay Luminescence Measurements

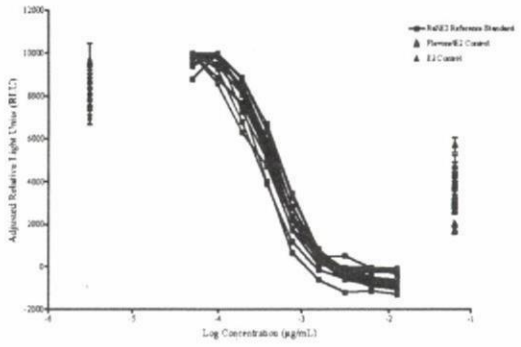
- Luminescence measurements from the assay are adjusted and normalized by:
  - Subtracting the averaged RLU values from DMSO control wells from RLU values from wells containing Ral/E2 reference standard, E2 control, flavone/E2 control, or test substance
  - Luminescence measurements are further adjusted (normalized) by scaling RLU values to the highest RLU value from Ral/E2 reference standard, which is assigned an RLU value of 10,000

Testing of Antagonist Reference Standards and Controls at XDS, ECVAM, and Hiyoshi

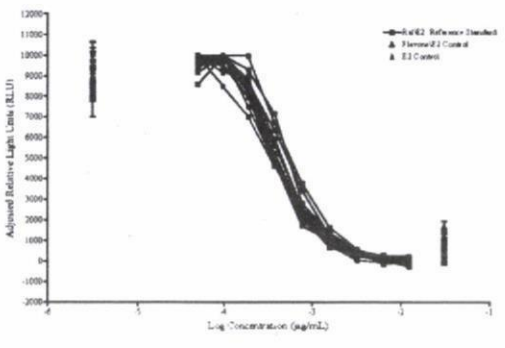
- At XDS, reference standard and controls were tested in 15 separate plates on 7 separate days (2 plates each on 4 separate days, 3 plates each on 2 separate days, and 1 plate on another day [**note**: 1 plate was contaminated and was not used in analysis of data])
- At ECVAM, reference standard and controls were tested in 18 separate plates on 9 separate days (2 plates each on 9 separate days)
- At Hiyoshi, reference standard and controls were tested in 12 separate plates on 12 separate days

The LUMI-CELL® ER Assay International Validation Study - Phase I ER Antagonist Intralaboratory Analyses

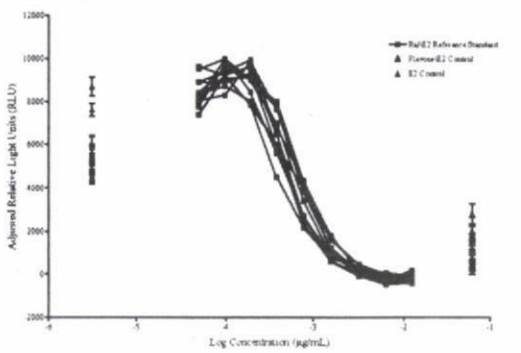
Test Plate Results from Antagonist Testing at XDS



Test Plate Results from Antagonist Testing at ECVAM



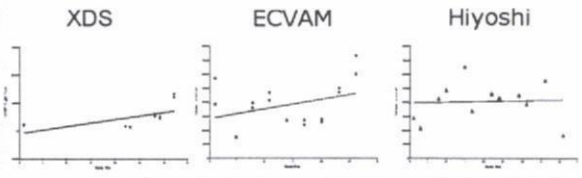
Test Plate Results from Antagonist Testing Hiyoshi



Intralaboratory Reproducibility of Antagonist Reference Standards and Controls

- Intralaboratory reproducibility of the RLU values associated with the DMSO control wells, the fold-reduction of Ral/E2 at its maximum response, the calculated Ral/E2 IC_{50} values, and the adjusted and normalized RLU values associated with the E2 control and flavone/E2 weak positive control wells were statistically analyzed.
  - A linear regression analysis was conducted to assess intralaboratory reproducibility over time for each laboratory
  - At XDS and ECVAM, reference standards and controls were tested in multiple plates on four or more separate days so within-day and across-day variability was analyzed using an ANOVA

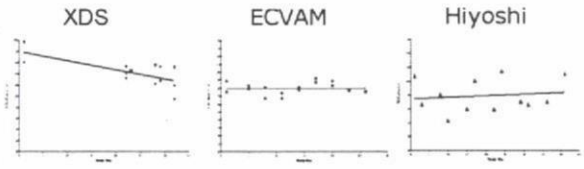
Antagonist DMSO Linear Regression Analysis



	N[1]	Intercept[2]	Slope	p-value (Slope)[3,4]
XDS	*14*	*-91*	*25.4*	*0.027*
ECVAM	18	2809	70.2	0.142
Hiyoshi	12	3934	5.8	0.867

[1]Number of plates tested.
[2]Intercept values are reported as relative light units.
[3]Statistically significant from zero at $p<0.05$.
[4]Values in italics have p values that are less than 0.05.

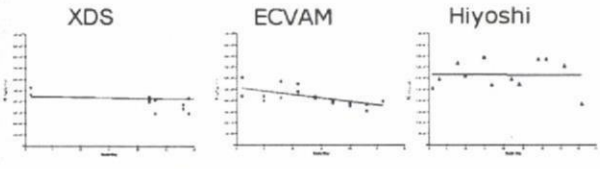
Ral/E2 Fold-Reduction Linear Regression Analysis



	N[1]	Intercept[2]	Slope	p-value (Slope)[3,4]
XDS	*14*	*18.0*	*-0.17*	*0.005*
ECVAM	18	7.9	0.003	0.890
Hiyoshi	12	7.4	0.02	0.674

[1]Number of plates tested.
[2]Intercept values are reported as fold-reduction.
[3]Statistically significant from zero at $p<0.05$.
[4]Values in italics have p values that are less than 0.05.

Ral/E2 IC_{50} Linear Regression Analysis

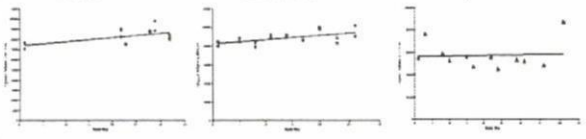


	N[1]	Intercept[2]	Slope	p-value (Slope)[3,4]
XDS	14	4.5×10^{-4}	9.4×10^{-7}	0.718
ECVAM	*18*	*4.6×10^{-4}*	*-6.3×10^{-6}*	*0.001*
Hiyoshi	12	6.4×10^{-4}	-3.6×10^{-7}	0.924

[1]Number of plates tested.
[2]Intercept units are reported as µg/mL.
[3]Statistically significant from zero at $p<0.05$.
[4]Values in italics have p values that are less than 0.05.

E2 Control Linear Regression Analysis

XDS ECVAM Hiyoshi

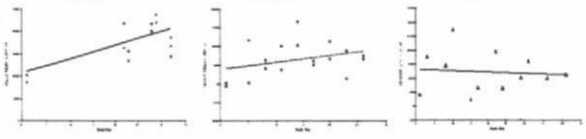


	N[1]	Intercept[2]	Slope	p-value (Slope)[3,4]
XDS	*14*	*7355*	*40.1*	*0.043*
ECVAM	*18*	*8249*	*45.5*	*0.012*
Hiyoshi	12	5597	6.7	0.827

[1]Number of plates tested.
[2]Intercept units are reported as adjusted relative light units.
[3]Statistically significant from zero at p<0.05.
[4]Values in italics have p values that are less than 0.05.

Flavone\E2 Control Linear Regression Analysis

XDS ECVAM Hiyoshi



	N[1]	Intercept[2]	Slope	p-value (Slope)[2,3]
XDS	*14*	*2159*	*61.5*	*0.032*
ECVAM	18	384.6	18.7	0.178
Hiyoshi	12	1324	-4.0	0.782

[1]Number of plates tested.
[2]Intercept units are reported as adjusted relative light units.
[3]Statistically significant from zero at p<0.05.
[4]Values in italics have p values that are less than 0.05.

Antagonist ANOVA Results for Intralaboratory Comparison of Reference Standard and Controls

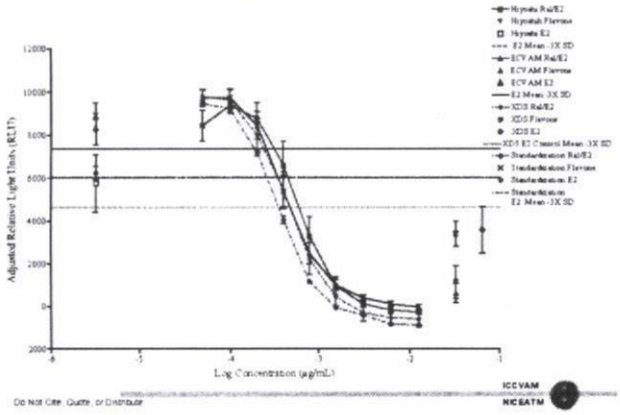
	XDS		ECVAM	
	p-Value[1,2]	F Value[3]	p-Value[1,2]	F Value[3]
DMS O	*<0.001*	*213*	*<0.001*	*15.5*
Ral\E2 Maximum Fold-Reduction	0.22	1.8	0.107	2.4
Ral\E2 IC_{50}	*0.02*	*5.2*	0.078	2.7
E 2	*0.004*	*8.5*	*0.012*	*5.7*
Flavone\E2	*0.02*	*5.1*	0.252	1.6

[1]Variability is statistically significant at p<0.05
[2]Values in italics have p values that are less than 0.05
[3]F = ratio of between-day variability to within-day variability – 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

At XDS and ECVAM, reference standards and controls were tested in 10 or more plates on four or more separate days. The within-day and across-day variability of the RLU values associated with the DMSO wells, the fold-reduction of Ral\E2, the Ral\E2 IC_{50} values, and the adjusted RLU values associated with the E2 and Flavone\E2 controls were analyzed using an ANOVA. Results from the analysis indicate that with-in day variability was statistically different for DMSO control, Ral\E2 IC_{50}, E2 control, and flavone/E2 control values at XDS, and for DMSO and E2 control values at ECVAM.

The LUMI-CELL® ER Assay International Validation Study - Phase I ER Antagonist Interlaboratory Analyses

Comparison of Antagonist Historical Databases



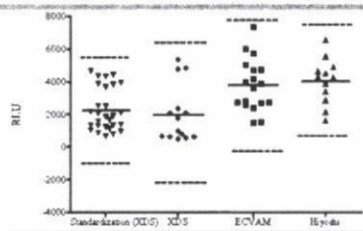
Interlaboratory Reproducibility of Reference Standard and Controls (1)

- Interlaboratory reproducibility of the RLU values associated with the DMSO control wells, the fold-reduction of Ral/E2 at its maximum response, the calculated Ral/E2 IC_{50} values, and the adjusted and normalized RLU values associated with the E2 control and the flavone/E2 weak positive control wells was evaluated:
  - Means, standard deviations and coefficients of variation of reference standard and control values were compared
  - Variability of reference standard and control values across laboratories was evaluated by conducting an analysis of variance (ANOVA)

Interlaboratory Reproducibility of Reference Standard and Controls (2)

- If a significant p-value was obtained for the ANOVA, a Newman-Keuls post-test was used to test for significant differences in reference standard and control values between pairs of laboratories.
- To test for significant differences between the reference standard and control values obtained in each laboratory versus the corresponding endpoint values obtained in the protocol standardization study, a Dunnett's analysis was conducted.

Interlaboratory Comparison of Antagonist DMSO Control

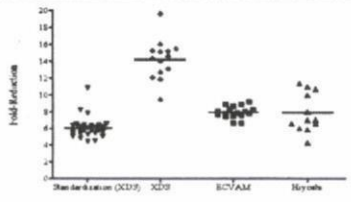


Data points represent DMSO RLU values from plates tested in the protocol standardization and Phase I studies. Solid horizontal lines represent the mean DMSO RLU value for each data set. Dashed lines indicate the mean agonist DMSO value plus and minus 2.5 times the standard deviation from the mean.

	Antagonist DMSO Control			
	N[1]	Mean[2]	SD[2]	CV
XDS	14	1986	1747	88%
ECVAM	18	3783	1587	42%
Hyoshi	12	4048	1386	34%
Standardization	28	2251	1303	58%

[1]Number of plates tested
[2]Units are expressed as relative light units

Interlaboratory Comparison of Ral/E2 Fold-Reduction

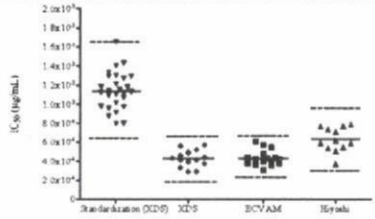


Data points represent Ral/E2 fold-reduction values from plates tested in the protocol standardization and Phase I studies. Solid horizontal lines represent the Ral/E2 fold-reduction value for each data set. Plates are rejected if the fold-reduction for the maximum Ral/E2 response is less than three.

	Fold-Reduction			
	N[1]	Mean[2]	SD[2]	CV
XDS	14	14.2	2.38	17%
ECVAM	18	8.0	0.70	9%
Hyoshi	12	7.9	2.33	30%
Standardization	28	6.1	1.26	21%

[1]Number of plates tested.
[2]Units are expressed as maximum Ral/E2 fold-reduction.

Interlaboratory Comparison of Ral/E2 IC_{50}

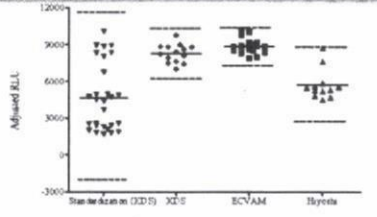


Data points represent Ral/E2 fold-reduction values from plates tested in the protocol standardization and Phase I studies. Solid horizontal lines represent the Ral/E2 fold-reduction value for each data set. Plates are rejected if the fold-reduction for the Ral/E2 response is less than three.

	Ral/E2 IC_{50}			
	N[1]	Mean[2]	SD[2]	CV
XDS	14	4.3×10^{-4}	9.0×10^{-5}	21%
ECVAM	18	4.3×10^{-4}	7.9×10^{-5}	18%
Hyoshi	12	6.3×10^{-4}	12.9×10^{-5}	21%
Standardization	28	11.4×10^{-4}	19.5×10^{-4}	17%

[1]Number of plates tested.
[2]Units are expressed as ug/mL.

Interlaboratory Comparison of E2 Control

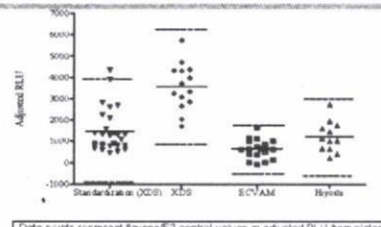


Data points represent E2 control values in adjusted RLU from plates tested in the protocol standardization and Phase I studies. Solid horizontal lines represent the mean E2 control value for each data set. Dashed lines represent the mean plus and minus 2.5 times the standard deviation from the mean.

	E2 Control			
	N[1]	Mean[2]	SD[2]	CV
XDS	14	8284	744	9%
ECVAM	18	8881	640	7%
Hyoshi	12	5728	1221	21%
Standardization	28	4664	2745	59%

[1]Number of plates tested.
[2]Units are expressed as adjusted relative light units.

Interlaboratory Comparison of Flavone/E2



Data points represent flavone/E2 control values in adjusted RLU from plates tested in the protocol standardization and Phase I studies. Solid horizontal lines represent the mean flavone/E2 control value for each data set. Dashed lines represent the mean plus and minus 2.5 times the standard deviation from the mean.

	Flavone/E2			
	N[1]	Mean[2]	SD[2]	CV
XDS	14	1562	1089	30%
ECVAM	18	644	458	71%
Hyoshi	12	1225	722	59%
Standardization	28	1453	1011	70%

[1]Number of plates tested.
[2]Units are expressed as adjusted relative light units.

Antagonist ANOVA Results for Interlaboratory Comparison of Reference Standard and Control Values

	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 – 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.

Newman-Keuls Results for Interlaboratory Comparison of Antagonist Reference Standard and Controls

	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[1]	p-value[2,3]	Mean Difference[5]	p-value[2,3]	Mean Difference[5]	p-value[2,3]
XDS vs ECVAM	*-3286*	*<0.001*	*6.2*	*<0.001*	*-4.3×10^{-4}*	*>0.05*	*-598*	*>0.05*	*2939*	*<0.001*
XDS vs Hiyoshi	*-3551*	*<0.001*	*6.3*	*<0.91*	*-2.0×10^{-4}*	*<0.001*	*2256*	*<0.001*	*2357*	*<0.001*
ECVAM vs Hiyoshi	*265*	*>0.05*	*0.1*	*>0.05*	*-2.0×10^{-4}*	*<0.001*	*3153*	*<0.001*	*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 ug/mL
[6]Presented in adjusted relative light units

Dunnett's Results for Antagonist Interlaboratory Comparison of Reference Standard and Controls

Lab vs. Standardization Study	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[5]	p-value[2,3]	Mean Difference[5]	p-value[2,3]
XDS	*1735*	*<0.01*	*-8.1*	*<0.01*	*-8.93×10^{-4}*	*<0.01*	*-598*	*>0.05*	*-2129*	*<0.01*
ECVAM	*-1512*	*<0.01*	*-1.9*	*>0.05*	*-9.36×10^{-5}*	*<0.01*	*2256*	*<0.001*	*809*	*<0.01*
Hiyoshi	*-1797*	*<0.01*	*-1.9*	*>0.05*	*-2.94×10^{-4}*	*<0.01*	*3153*	*<0.001*	*227*	*>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 ug/mL
[6]Presented in adjusted relative light units

Antagonist Historical Database Values Established for Phase IIa Acceptance Criteria

	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}	ug/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}	ug/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	4043	1384	7503	583
Ral/IC_{50}	ug/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

The LUMI-CELL® ER Assay International Validation Study - Phase I ER Agonist and Antagonist Conclusions

Conclusions (1)

- Statistically significant differences were observed in intra- and inter-laboratory reference standard and control values.
- It was not possible to identify the causes for these differences but some of the contributing factors may be:
  - Lot-to-lot differences in cell culture media and tissue culture supplies (for intra- and inter-lab differences)
  - Differences in luminometers (for inter-lab differences)
- This underscores the importance of developing an historical control database for each individual laboratory.

Conclusions (2)

- Factors supporting reliability of the assay:
  - Assay responds robustly to E2 reference estrogen and raloxifene reference anti-estrogen.
  - Assay consistently responds to weak-acting positive controls at concentrations several orders of magnitude higher than the reference estrogen or anti-estrogen.
  - Assay plate induction or reduction values were consistently greater than three-fold (only 2 of 84 plates tested had values below three-fold)
  - Phase I testing of reference standards and controls established historical databases that produced comparable test plate acceptance criteria for Phase IIa testing

The LUMI-CELL® ER Assay International Validation Study - Phase IIa

Overview of Phase IIa of the Validation Study

- During Phase IIa:
  - Four test substances from the ER minimum list will be tested for agonism in each laboratory on three separate occasions
  - Four test substances from the ER minimum list will be tested for antagonism in each laboratory on three separate occasions
  - Modified range finder and comprehensive plate designs using all 96 wells of test plates will continue to be evaluated
- If there is significant variability in coded substance test results, the SMT will work with participating laboratories to determine cause and recommend appropriate actions to reduce variability

Recommendation to the SMT

- To initiate Phase IIa using the current protocol as modified during Phase I.

平成18年度厚生労働科学研究費補助金(化学物質リスク研究事業)
「化学物質リスク評価法の国際的バリデーションに関する研究(H18-化学-一般-003)」

分担研究報告書

OECD/EDTA validation management 活動との調整

分担研究者　小島　肇　国立医薬品食品衛生研究所

研究要旨

11月にイスプラで開催されたOECD/EDTA validation management Team-Non-Animal(VMT-NA)会議に参加し、現在進めている国際バリデーションの進捗について意見交換した。

EDTA VMT-NAで検討されたた内分泌かく乱物質スクリーニング法であるLumi-cell法、HeLa細胞をベースにしたエストロジェン受容体αに対するレポーターアッセイ試験法(HeLa法)および遺伝毒性試験であるコメットアッセイのOECDガイドライン化を目指し、Standard Project Submission Form(SPSF)を2008年1月までにそれぞれの担当省庁を通してOECDに提出した。

A.　研究目的

動物実験代替法に関しては、化粧品の安全性評価法を中心に、多くの検討が行われている。開発された皮膚腐食性試験や光毒性試験代替法などが、欧米および我が国において大規模なバリデーションと評価が行われ、一部がOECDのガイドラインに取り入れられ、化学物質の評価にも用いられている。しかし、感作性試験や生殖毒性試験など、まだ、開発やOECD基準に則ったバリデーションがなされていないものも多い。一方、内分泌かく乱化学物質の*in vitro*評価法については、無細胞系受容体結合試験、酵母等各種導入受容体結合試験、各種受容体導入レポーター遺伝子転写活性化試験(Lumi-cell法など)、CERIが開発したHeLa細胞をベースにしたエストロジェン受容体αに対するレポーターアッセイ試験法(HeLa法)の他、アロマターゼ活性化試験など、いくつかの方法が開発され、OECD基準に則ったバリデーションが実施されている。DNA損傷性を調べるコメットアッセイについても、*in vitro*および*in vivo*の試験法が開発されているが、データの評価、解釈のみならず方法論に関しても未熟であり、国際的なガイドラインは作成されていない。

本研究はこれら今まで評価が遅れていた化学物質の安全性評価のための試験法をOECDの基準に則ってバリデーションと評価を行い、OECDガイドラインの成立を目指すものである。

B.　研究方法

B-1　OECD/EDTA　VMT-NAでの会合

2007年11月13日～15日にイタリア　イスプラで開催されたOECD/EDTA　VMT-NAに日本から小野敦、小島　肇(以上、国立医薬品食品衛生研究所)、武吉正博、赤堀有美(以上、化学物質評価研究機構)が出席した。各種の内分泌かく乱物質スクリーニングの現状を確認するとともに、日本からも共同研究内容について種々の提案を行った。

B-2　SPSFの作成

内分泌かく乱性スクリーニング法であるLumi-cell法については、提案機関であるICCVAMおよびHeLa法については、開発者である化学物質評価研究機構および遺伝毒性試験であるコメットアッセイについては国立医薬品食品衛生研究所でStandard Project Submission Form(SPSF)の原案が作成された。

A.　結果

C-1　OECD/EDTA　VMT-NAでの会合

OECD/EDTA　VMT-NAで検討が進められている各種の内分泌かく乱物質スクリーニングの進捗について報告があり、各国の代表とその内容について意見交換した。特に、日本で開発されたHeLa法については、8月にOECDよりガイドライン化のために、プロトコールを修正することに加え、アンタゴニストのバリデーション研究を追加指示する提案を受けた。本会議では、この提案を受け、日本主導でバリデーションの準備を進めていると報告し、その内容にEDTA　VMG-NAメンバーから助言を頂くとともに、協力を要請した。その後、アンタゴニストのバリデーション研究を2008年度早々に実施すべく、プロトコールの見直しを行うとともに、計画を立案した(詳細は、分担研究者小野　敦の報告書を参照されたい)。

OECDからのコメントを添付資料1に、議事録を添付資料2として示した。

C-2　SPSF の提出

内分泌かく乱性スクリーニング法である Lumi-cell 法については、FDA および HeLa 法については、経済産業省および遺伝毒性試験であるコメットアッセイについては厚生労働省の担当官から Standard Project Submission Form (SPSF) が OECD に提出された。

それらを添付資料 3～5 として示した。

D. 考察

本研究班のテーマである「化学物質リスク評価法の国際的バリデーション」の目的は、安全性評価に有用な新規試験法を公定化することである。その代表が OECD ガイドラインであることから、このガイドライン化を目指して SPSF が提出され、今後本格的な国際議論が巻き起こることを期待するものである。

E. 結論

内分泌かく乱性については Lumi-cell 法および HeLa 法、遺伝毒性についてはコメットアッセイの OECD ガイドライン化を目指し、SPSF を 2008 年 1 月までにそれぞれの担当省庁を通して OECD に提出した。

F. 健康危険情報

なし

G. 研究発表

G-1）論文発表

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H. 知的財産権の出願、登録状況

なし

I.　　添付資料

添付資料 1 ：FOLLOW UP TO THE PEER REVIEW OF THE STABLY TRANSFECTED TRANSCRIPTIONAL ACTIVATION (STTA) ASSAY AND TECHNICAL ISSUES TO BE ADDRESSED BY THE VMG NA 5

添付資料2：Draft Report of the 5th meeting of the validation management group for non-animal testing (vmg-na)

添付資料3：SPSF Stably transfected Transcriptional Activation (TA) assay for detection of anti-estrogenic activity of chemicals

添付資料4：SPSF Stably transfected Transcriptional Activation (TA) assay for detection of androgenic and anti-androgenic activity of chemicals

添付資料5：SPSF IN vivo Comet Assay in Genotoxicity Testing

OECD ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

ENVIRONMENT DIRECTORATE
Environment, Health and Safety Division

ENV/EHS/PA/jh/2007.13

Paris, 20 July 2007

To: Working Group of National Co-ordinators of the Test Guidelines Programme (WNT)

FOLLOW UP TO THE PEER REVIEW OF THE STABLY TRANSFECTED TRANSCRIPTIONAL ACTIVATION (STTA) ASSAY AND TECHNICAL ISSUES TO BE ADDRESSED BY THE VMG NA 5

Dear Madam/Sir,

The prevalidation results of the Stably Transfected Transcriptional Activation (STTA) Assay were presented at the 1st meeting of the VMG NA in 2002 after which CERI, Japan, took the assay into a multi-laboratory validation study. At the 2nd VMG NA the multi-laboratory approach was approved and the validation report and SOP were sent to the OECD. The 3rd VMG NA agreed to proceed with the study and to arrange for a Preliminary Validation Assessment Panel (PVAP) to assist the Japanese and to check whether the assay would be ready for a final peer review. The report of the PVAP gave a clear indication that the test method was suitable for an official Peer Review. Japan led the peer review and followed the same procedures that were applied to the Herschberger and the 407 reviews.

A Peer Review Panel (PRP) was established in November 2006 to provide an independent review of the validation study of the STTA assay. The assay is intended to be used for identifying and prioritizing substances that have the potential to act as estrogen receptor (ER) agonists binding to ERα. The work of the PRP was coordinated by an external consultant. The panel members were requested to address specific issues as well as to consider the 8 validation criteria outlined in OECD Guidance Document No.34.

The preliminary draft Test Guideline and the Validation Report are available on the public website at the following URL: [http://www.oecd.org/document/62/0,3343,en_2649_34377_2348606_1_1_1_1,00.html]
The PRP Report [ENV/JM/TG/RD(2007)5] was presented to the WNT19 and is available on the password-protected website of the meeting. As agreed at the last WNT, the PRP Report will be sent to the Joint Meeting for declassification once the WNT agrees on a paragraph to be attached to the PRP Report regarding the development of an OECD Test Guideline (please see a draft paragraph attached to this letter as annex 1).

The PRP identified some areas where the eight validation criteria where not completely met and additional information should be provided. These included:

- Criteria for positive responses were unclear and needs to be further elaborated;
- Guidance on the criteria for acceptable test performance was insufficient, and,
- The STTA assay can at this point only be used for estrogen agonist testing and further studies would be needed if also estrogen antagonists could be tested.

National Co-ordinators are kindly requested to comment, **by 31 August 2007 at the latest**, on the paragraph to be added to the peer review report (annex 1) and on any other technical issues related to the

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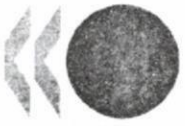

preliminary Test Guideline or PRP report to be addressed by the VMG-NA. A lack of response by this date will be considered as a silent approval of the document. The peer review report and the attached paragraph, revised as appropriate, will be submitted to the Joint Meeting for declassification.

If you have any questions or concerns, please don't hesitate to contact me directly.

Yours sincerely,

Patric Amcoff

Administrator
Environment, Health and Safety Division

Cc : European Commission (DG's Environment, Enterprise, Sanco, Science, JRC, ECVAM)
BIAC (including ACC, CEFIC, Croplife International, ECETOC, JCIA)
EEB; ICAPO; ILSI Europe/ North America; IPCS; TUAC
Env Councellors to OECD Permanent delegations

Patric.Amcoff@oecd.org
Paris, 19 December 2007

DRAFT REPORT OF THE 5TH MEETING OF THE VALIDATION MANAGEMENT GROUP FOR NON-ANIMAL TESTING (VMG-NA)

13-15 November 2007, ECVAM-DG JRC, Ispra, Italy

INTRODUCTION

1. The 5th Meeting of the Validation Management Group for Non-Animal tests (VMG-NA) was held in Ispra, Italy on 13th-15th November 2007 at the European Center for the Validation of Alternative Methods (ECVAM) at the Joint Research Center (JRC). The main objective of the VMG-NA is to identify or propose validated or promising non-animal assays for endocrine chemicals testing, and develop and validate tools necessary for the Level 2 (*In vitro* assays providing mechanistic data) of the Conceptual Framework of the Endocrine Disruption Testing and assessment Task Force of the Test Guidelines Programme (EDTA), in addition to report the progress of ongoing co-operations and developments of new tests that was initiated at previous VMG-NA meetings.

2. The list of participants of the Meeting is attached to this report as Annex 1.

3. Patric Amcoff of the Secretariat opened the Meeting and welcomed participants of the VMG NA5 on behalf of the OECD Secretariat and acknowledged ECVAM for hosting the meeting. He explained OECD procedures and introduced Dr. Steve Bradbury (US EPA) and Dr. Daniel Dietrich (Konstanz University, Germany) as the co-chairs of the meeting.

ADOPTION OF THE DRAFT AGENDA

4. The Secretariat introduced the agenda and asked the meeting for some degree of flexibility since the time estimated for several of the agenda items were difficult to foresee. The agenda was adopted by the meeting with the adding of two additional presentations on the morning of the last day on the EU ReproTect project and the EU Cascade Network of Excellence.

OPENING OF THE MEETING

5. The Secretariat explained the background to the establishment of the VMG NA, and the decision by the 6th Meeting of the EDTA of the Test Guideline Programme in 2002 to start a 3rd VMG based on the great importance of, an urgent need for, relatively cheap and quick high-throughput screens and tests not requiring animals. The VMG NA was updated on the latest events of the EDTA and the WNT and that there is an ongoing discussion at the WNT of the exact roles of the EDTA and the three VMG`s.

PRESENTATIONS

6. Masahiro Takeyoshi of CERI gave an update on the current status in Japan for ED non animal tests. The agonist part of the stably transfected Estrogen receptor (ER) transcriptional activation assay (STTA) has gone through validation and peer review and the antagonist part will be subjected to validation in 2008 under JaCVAM lead. (See table from presentation). He informed the meeting that an AR-EcoScreen assay was going through validation and that an SPSF will be submitted to the WNT. Laurence Musset (Secretariat) informed the meeting that the deadline for submission of SPSFs to the WNT20 was 31 January 2008. To not violate the guidance document No.34 rules that states that commercial tests cannot be developed into Test Guidelines unless a generic description and a set of performance standards are

provided, CERI have already asked Sumitomo Chemicals to make the cell line freely available at *e.g.*, the American Type Culture Collection.

7. Atsushi Ono (NIHS) gave an update with an aim for a HTPS assay

8. Hajime Koijima (JaCVAM) gave anUpdate on activities by the Japanese Center for the Validation of Alternative Methods of the MHLW (JaCVAM).

9. Since no representative of the Japanese Ministry of the Environment (MoE) was present to introduce the Detailed Review Paper (DRP) for Fish Receptor assays, the Secretariat gave a short update and asked for input from the meeting. A new session was for more in-depth analysis of the document was scheduled for the last day of the meeting. The Secretariat explained that the Japanese authors needed input on the most promising assays and whether we have any validated tests or can add any other substantial information to the draft DRP.

10. Miriam Jacobs (ECVAM) gave an update on the activities of ECVAM. See presentation. Ray Tice wondered whether cytotoxicity was evaluated in the antagonist assay and Alexius Freyberger explained that it had been mandatory. Ray further stated that different studies have used different limit concentrations, how do you deal with compounds that have been used at different concentrations? How do we handle the data in the future with different levels of activity? The chair Daniel Dietrich informed the meeting that some of these issues raised were already covered by the VMG NA4 meeting in Tokyo and meeting participants should read through the report before the planned discussions for the 2nd day.

11. Miriam described the latest developments of the DRP on Metabolism and that it will be submitted to the Joint Meeting (JM) in December 2007 for declassification. However, due to the high importance of aspects of metabolism for *in vitro* assays the topic will be a standing agenda item for future VMG NA meetings, which is in line with the WNT19 recommendation. Miriam further gave a short update on the most important issues that have been addressed since the last VMG NA meeting and the recommendations for short-, medium- and long-term prospects for metabolism assay developments.

12. Gary Timm (US EPA) gave a presentation of the validation status of the H295 Steroidogenesis assay that expresses all essential components of the steroidogenesis cycle and asked for input from the meeting for what endpoints should be applied, quantitative or qualitative? Expected to be completed by December 2008 when the peer review report will be made available. The validated cell line will be donated to the US National Institutes of Health cell line library.

13. Ray Tice (ICCVAM) presented the pre-validation and standardization work of the LumiCell™ assay. By using the outside wells instead of skipping them due to expected edge effects, they can double their testing of chemicals and will report in late 2008.

14. Shirlee Tan of the US-EPA gave a presentation over the phone on the latest developments for the FWA/CERI protocols for the human receptor ERα assays. The progress was noted and the assays will be validated in 2008 and a validation report is expected to be available by early 2009.

15. Pat Schmieder (US-EPA) gave an update on the work by the ED QSAR group that met before the VMG NA meeting. The primary purpose of the group is to promote exchange of information and increased global collaboration. The purpose of the group is not validation of QSAR's and the group meet and work independently of the VMG NA. The work by Japan and the USA on screening prioritisation and development of inventories with a purpose to prioritize chemicals for screening, generate hypotheses and to identify data gaps was presented. The latest development as to include metabolically active chemicals in the training sets. The USA's expert system for predicting estrogen hormone RBA for inert ingredients used

in food-use pesticides is nearing completion. During the next year it is anticipated that the USA will have the system documented in accordance with OECD's guidance for validating QSARs.

16. David Dix (US EPA) introduced the US EPA ToxCast programme. Problem to be solved: too many chemicals to be tested at a too high cost (www.epa.gov/comptox/toxcast). The Toxcast narrows down the present 90.000 chemicals that need additional assessment data to specific chemical groups (11.000 chemicals). ToxCast will function as a prioritizing tool for further testing across many endpoints (endocrine and non-endocrine) and it is based on pharmaceutical industry experience and drug discovery principles. ToxCast PHASE 1: ToxCast 320 is a subset of pesticides. In total 55 chemicals overlap between the ToxCast 320 and a list of approximately 75 compounds identified by the US-EPA screening program for Tier 1 screening in the US (note: these 75 chemicals were selected based on high exposure potential to humans and the environment only – these chemicals are not presumed *a priori* to have endocrine effects). 10.000 chemicals in >240 HTPS assays are expected to be screened until 2012. Signatures of toxicity in environmental chemicals will be evaluated. A total of 18 people are employed for the whole programme. A chemical library will be available on the website. ToxCast also collaborate with the toxicogenomic working committee at the OECD. The finished ToxCast Programme and derivative results will at the end be compared with existing data, and this will be done in cooperation with other EPA departments.

WEDNESDAY 14 NOVEMBER

Discussions on the STTA Assay

17. The Secretariat opened the meeting and explained that the goal should be to have the agonist STTA Test Guideline submitted to the WNT20 for adoption, which means that the VMG NA need to address all comments from member countries and to develop a performance standard for the assay. Given the short time line a revised draft should be submitted by the latest 2nd week of December to allow for expert commenting in member countries and give the Secretariat a realistic chance to submit the draft TG for approval at the WNT20 in early April 2008. The Secretariat also suggested merging the STTA subcommittee and the PBTG into one group, the STTA sub-committee (STTA-SC).

18. Miriam gave a presentation on the work of the STTA SC. The group agreed that the test should be used as a screen for prioritizing and not as a definite test and the assay response needs to be defined, not its classification properties. The terminology should be slightly changed and the response should be combined with a concentration to define: strong, moderate and negative activity at a given concentration. A number of rather difficult discussions of the assays performance were held. The group discussed why not testing should be done up to maximum solubility, however, since the test was not validated for this application testing up to maximum solubility would not be appropriate and a limit concentration should be set. The use of higher concentrations of DMSO (>1%) than what is outlined in the assay might lead to cytotoxicity and suppression (inactivation) of the reporter luciferase and therefore false-negatives. Ray outlined the three options; (i), use limit dose of xx mmolar; (ii), test until limit of solubility if you don't get a positive; and (iii), start somewhere and go up or down to a maximum concentration. Ray will provide some suggested text on this.

19. The other discussion items involved functional assay conditions such as mycoplasma infection monitoring, fold induction levels and responsive function and quality control.

Metabolism Working Group

20. Establishment of a Metabolism WG (Juliette Legler, Miriam Jacobs(coordinator), Christine Nellemann, Pat Schmieder, Alexius Freyberger, Dan Dietrich, Ray Tice, Gary Timm) that will check with ReproTect about S9-mix uses and other approaches. The group will report to the next VMG NA.

Discussions on PBTG

21. Gary Timm presented some options how performance-based Test Guidelines could be used and described some different scenarios. A lengthy discussion on the benefits and shortcomings of the different options followed and the Secretariat explained that the case with several test methods for the same endpoint are being developed and that me-too tests and performance standards for all of these will have to be developed is a new issue. There is one TG with detailed Performance Standards, and that is TG435, however there have never been any questions about how a TG435 me-too test should be judged, probably because there are no me-too developments for this endpoint. The Secretariat will consult with the OECD legal services if there may be legal problems with some of the options in respect of the Mutual Acceptance of data (MAD).

Discussion on SPSF's

22. Laurence Musset (Secretariat) introduced the SPSF issues and that 31 January 2008 is the deadline for submission of SPSF for the WNT20 meeting. We have already preliminary SOPSFs for the LumiCell, hERalpha and H295R assays that will be posted on the WNT WS. CERI will submit SPSFs for ERTA, ARTA and JaCVAM will hopefully submit an SPSF for the comet assay.

Table 1. Main ongoing projects and their validation status

Receptor Binding Assays				
hrERα	*Protocol 1.* The FWA assay protocol utilizes the Pan Vera hrERα full length ER. *Protocol 2.* CERI protocol utilizes the CERI-ERα, which contains the ligand binding domain of hrERα.	binding	Validation starting in early 2008 in 6 labs. SPSF submitted.	US lead international collaboration study
hrAR	Human recombinant AR assay. Ligand binding domain expressed in E. coli.	binding	Under development. Approximately 900 chemicals have been tested.	METI
	Human recombinant AR assay.	binding	Validation starting in 2008.	ECVAM Lead international collaboration study
hrTR	Human recombinant TR assay. Full-length expressed in E. coli. TRs α1 and β1 binding assays.	binding	Under development. Approximately 60 chemicals have been tested using both receptors.	METI
Transcriptional Activation Assays				
	HeLa-9903 cells with plasmids containing hERα cDNA driven by	Stable, ag/antag	The agonist assay draft TG will be proposed to WNT20 for adoption.	CERI/MHLW