Phase-2 study

47. The aims of the Phase-2 study were to provide the supplemental data according to previous Peer review comments of this assay and to evaluate the assay performance (within/between-laboratory reproducibility and predictive capacity) by testing 10 coded chemicals (five each for agonist and antagonist activity).

Study design

48. The Phase-2 study was conducted with all four laboratories who passed the performance criteria in the Phase-1 study.

49. The Phase-2 study was performed with 5 test chemicals for the agonist assay and 5 test chemicals for the antagonist assay. Each laboratory tested the Phase-2 chemicals in triplicate at appropriate concentration ranges of each chemical for the assessment of their activity to evaluate the assay performance (within/between-laboratory reproducibility and predictive capacity).

50. All 10 chemicals for Phase-2 study were provided by Chemical Distribution Management in a coded manner. Each laboratory tested these chemicals according to the assay protocol up to the maximum concentration decided according to the diagram for the solubility test.

51. Every runs of the Phase-2 study required a simultaneous test of the reference chemicals used in Phase-1 study.

52. The plate assignments for Phase-2 study complied with the assay protocol (ANNEX 1).

53. All assay results were stored and locked in the Specified work sheet previously provided by CERI. Each laboratory then submitted at least three sets of assay results that met the all performance criteria shown in Table 10 including newly decided criteria for Mestanolone to the Project Coordinator.

54. All results in the validation study were analyzed in NIHS to evaluate the performance of this assay. The inter-laboratory concordance of judgment (positive/negative) of coded test chemicals was required to be more than 80% for each of the agonist and antagonist assays as acceptance criteria.

Agonist assay

55. Phase-2 study was started with the solubility test, and all laboratories decided the maximum dose according to the schema for the solubility test in the assay protocol.

56. The results of the solubility test were shown in Table 12. Consequently, the dose range for the all test chemicals for in Phase-2 were decided to be 10^{-12}- 10^{-6} M or 10^{-11}- 10^{-5}M.

Table 12 Test concentration range decided by solubility test in agonist assay

Test chemical	Test concentration range (M)			
	CERI	Sumitomo	Hokkaido	NiFDS
Testosterone	10^{-12}- 10^{-6}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}
17β-estradiol	10^{-12}- 10^{-6}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}
Medroxyprogesterone 17-acetate	10^{-12}- 10^{-6}	10^{-12}- 10^{-6}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}
17α-ethinyl estradiol	10^{-12}- 10^{-6}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}
Butylbenzyl phthalate	10^{-12}- 10^{-6}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}

57. All laboratories passed all reference criteria in the first three runs. The results for reference chemicals were shown in Table 13.

58. The LogPC10(M) and LogPC50(M) for DHT ranged from -10.54 to -10.82 and from -9.56 to -10.04, respectively. The LogPC10(M) and LogPC50(M) for Mestanolone were ranged from -10.47 to -10.88 and from -9.49 and -10.02, respectively. The CV% LogPC10(M) and LogPC50(M) for each parameter was less than 2%.

59. For the test chemicals, the positive candidate chemicals, Testosterone, 17β-estradiol and Medroxyprogesterone 17-acetate, tested positive in all runs of all laboratories, and the negative candidate chemicals, 17α-ethinyl estradiol and Butylbenzyl phthalate, tested negative in all runs of all laboratories. In addition, the CV% of LogPC10(M) and LogPC50(M) for each chemical were less than 5% (Table 14).

60. The results of the two-by-two table analysis with the candidate effects are shown in Table 15. The Accuracy, Sensitivity and Specificity of the assay were all calculated to be 100% in each laboratory. Accuracy, Sensitivity and Specificity across all four laboratories were also 100%.

Table 13 Results for the reference chemicals in agonist assay

	Run No.	FI	FI VC mean+2SD	FI of PC10	DHT Log PC10 (M)	DHT Log PC50 (M)	Mestanolone Log PC10 (M)	Mestanolone Log PC50 (M)
CERI	1	8.19	1.05	1.72	-10.69	-9.70	-10.69	-9.65
		8.92	1.11	1.79				
	2	8.18	1.13	1.72	-10.78	-9.84	-10.72	-9.72
		8.23	1.15	1.72				
	3	8.14	1.04	1.71	-10.71	-9.71	-10.71	-9.66
		7.61	1.12	1.66				
	Mean	8.21	1.10	1.72	-10.72	-9.75	-10.71	-9.68
	SD	0.42	0.04	0.04	0.05	0.08	0.02	0.04
	CV%	5.07%	4.09%	2.42%	0.46%	0.82%	0.15%	0.41%
Sumitomo	1	7.47	1.06	1.65	-10.74	-9.75	-10.62	-9.56
		7.33	1.07	1.63				
	2	7.27	1.08	1.63	-10.73	-9.76	-10.59	-9.55
		7.34	1.04	1.63				
	3	7.56	1.07	1.66	-10.76	-9.77	-10.66	-9.59
		7.15	1.12	1.61				
	Mean	7.35	1.07	1.64	-10.75	-9.76	-10.62	-9.57
	SD	0.15	0.03	0.01	0.01	0.01	0.04	0.02
	CV%	1.97%	2.45%	0.89%	0.13%	0.11%	0.35%	0.23%
Hokkaido	1	7.41	1.11	1.64	-10.82	-10.04	-10.85	-10.02
		6.96	1.09	1.60				
	2	7.49	1.09	1.65	-10.78	-9.87	-10.74	-9.77
		7.22	1.07	1.62				
	3	7.35	1.09	1.64	-10.82	-9.97	-10.82	-9.99
		7.88	1.12	1.69				
	Mean	7.39	1.09	1.64	-10.81	-9.96	-10.80	-9.93
	SD	0.31	0.02	0.03	0.02	0.09	0.05	0.14
	CV%	4.13%	1.64%	1.86%	0.22%	0.89%	0.50%	1.39%
NiFDS	1	7.42	1.04	1.64	-10.54	-9.56	-10.47	-9.49
		7.17	1.04	1.62				
	2	7.51	1.06	1.65	-10.70	-9.71	-10.61	-9.57
		7.84	1.05	1.68				
	3	7.34	1.06	1.63	-10.76	-9.73	-10.88	-9.89
		6.62	1.05	1.56				
	Mean	7.32	1.05	1.63	-10.66	-9.67	-10.66	-9.65
	SD	0.41	0.01	0.04	0.11	0.09	0.21	0.21
	CV%	5.59%	0.67%	2.51%	1.08%	0.93%	1.94%	2.22%
For four labs.								
	Mean	7.57	1.08	1.66	-10.73	-9.78	-10.70	-9.71
	SD	0.49	0.03	0.05	0.08	0.13	0.12	0.18
	CV%	6.53%	2.95%	2.98%	0.71%	1.32%	1.10%	1.84%
	Max	8.92	1.15	1.79	-10.54	-9.56	-10.47	-9.49
	Min	6.62	1.04	1.56	-10.82	-10.04	-10.88	-10.02

Table 14 Summary of the results for test chemicals in agonist assay

	Lab	Run No.	Log IC30 (M)	Mean SD CV%	Log IC50 (M)	Mean SD CV%	Decision
17a-ethinyl estradiol CAS:57-63-6	CERI	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Sumitomo	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Hokkaido	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	NiFDS	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	For 4 labs	Mean	ND		ND		Negative
		SD					
		CV%					
17β-estradiol CAS:50-28-2	CERI	1	-7.63	-7.63	ND		Positive
		2	-7.67	0.03	ND		
		3	-7.60	0.43%	ND		
	Sumitomo	1	-7.24	-7.23	ND		Positive
		2	-7.19	0.04	ND		
		3	-7.27	0.58%	ND		
	Hokkaido	1	-7.74	-7.72	-5.33	-5.27	Positive
		2	-7.73	0.02	-5.34	0.12	
		3	-7.70	0.30%	-5.13	2.29%	
	NiFDS	1	-7.05	-6.96	-4.93	-4.99	Positive
		2	-7.08	0.19	-4.88	0.15	
		3	-6.75	2.67%	-5.15	2.94%	
	For 4 labs	Mean	-7.39		-5.13		Positive
		SD	0.33		0.19		
		CV%	4.50%		3.80%		
Butylbenzyl phthalate CAS:85-68-7	CERI	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Sumitomo	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Hokkaido	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	NiFDS	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	For 4 labs	Mean	ND		ND		Negative
		SD					
		CV%					

Table 14 (continued)

	Lab	ID	Log IC30 (M)	Mean SD CV%	Log IC50 (M)	Mean SD CV%	Decision
Medroxyprogesterone 17-acetate CAS:71-58-9	CERI	1	-8.94	-8.93	-8.45	-8.46	Positive
		2	-8.93	0.02	-8.50	0.03	
		3	-8.90	0.23%	-8.44	0.38%	
	Sumitomo	1	-8.92	-8.91	-8.44	-8.42	Positive
		2	-8.91	0.02	-8.45	0.04	
		3	-8.89	0.18%	-8.37	0.51%	
	Hokkaido	1	-9.64	-9.38	-8.77	-8.71	Positive
		2	-8.98	0.35	-8.62	0.08	
		3	-9.52	3.76%	-8.72	0.89%	
	NiFDS	1	-8.95	-9.11	-8.51	-8.57	Positive
		2	-9.00	0.24	-8.58	0.06	
		3	-9.39	2.63%	-8.63	0.69%	
	For 4 labs	Mean	-9.08		-8.54		Positive
		SD	0.27		0.13		
		CV%	2.96%		1.47%		
Testosterone CAS:58-22-0	CERI	1	-9.83	-9.89	-9.28	-9.30	Positive
		2	-9.98	0.08	-9.35	0.04	
		3	-9.85	0.82%	-9.28	0.41%	
	Sumitomo	1	-9.85	-9.84	-9.24	-9.23	Positive
		2	-9.84	0.00	-9.20	0.02	
		3	-9.84	0.03%	-9.24	0.24%	
	Hokkaido	1	-10.42	-10.32	-9.46	-9.41	Positive
		2	-10.17	0.13	-9.37	0.05	
		3	-10.36	1.24%	-9.39	0.54%	
	NiFDS	1	-9.77	-9.75	-9.13	-9.07	Positive
		2	-9.75	0.02	-9.10	0.09	
		3	-9.73	0.24%	-8.96	0.99%	
	For 4 labs	Mean	-9.95		-9.25		Positive
		SD	0.24		0.14		
		CV%	2.37%		1.50%		

Table 15 Positive/negative outcomes in agonist assay and results of two-by-two table analysis

	Candidate effect	CERI	Sumitomo	Hokkaido	NiFDS	4 Lab
Testosterone						
17β-estradiol						
Medroxyprogesterone 17-acetate						
17α-ethinyl estradiol	N	N	N	N	N	N
Butylbenzyl phthalate	N	N	N	N	N	N
P:Positive						
N:Negative	Accuracy	100%	100%	100%	100%	100%
	Sensitivity	100%	100%	100%	100%	100%
	Specificity	100%	100%	100%	100%	100%

Antagonist assay

61. Phase-2 study was started with the solubility test, and all laboratories decided the maximum dose according to the schema for the solubility test shown in the assay protocol.

62. The results of the solubility test were shown in Table 16. Consequently, the dose range for Flutamide, Atrazine, Vinclozolin and Prochloraz in Phase-2 were decided as 10^{-11}- 10^{-6} M or 10^{-10}- 10^{-5}M. The dose range for the 6-Propyl-2-thiouracil was decided as 10^{-9}- 10^{-4} M or 10^{-10}- 10^{-5}M.

Table 16 Test concentration range decided by solubility test in antagonist assay

Test chemical	Test concentration range(M)			
	CERI	Sumitomo	Hokkaido	NiFDS
Flutamide	10^{-11}- 10^{-6}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}
Atrazine	10^{-11}- 10^{-6}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}
Vinclozolin	10^{-11}- 10^{-6}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}
Prochloraz	10^{-11}- 10^{-6}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}
6-Propyl-2-thiouracil	10^{-9}- 10^{-4}	10^{-9}- 10^{-4}	10^{-9}- 10^{-4}	10^{-10}- 10^{-5}

63. All laboratories passed all reference criteria in the first three runs. Tthe results for reference chemicals were shown in Table 17.

64. The LogIC30(M) and LogIC50(M) for HF were ranged from -7.11 to -7.81 and from -6.73 to -7.40, respectively. The LogIC30(M) and LogIC50(M) for BisA were ranged from -5.55 to -6.20 and from -5.28 to -5.75, respectively. The CV% for LogIC30(M) and LogIC50(M) was less than 4%.

65. Among the positive candidate chemicals, Flutamide and Vinclozolin, tested positive in all runs of all laboratories, and the negative candidate chemicals, Atrazine and 6-Propyl-2-thiouracil, tested negative in all runs of all laboratories. In addition, the CV% of LogIC10(M) and LogIC50(M) for each chemicals were less than 4% (Table 18).

66. Meanwhile, one of the positive candidate chemicals, Prochloraz, was tested positive in three laboratories, and tested negative in one laboratory in first three runs.

67. Accordingly, in the results of the two-by-two table analysis in first three runs (Table 19), the Accuracy, Sensitivity and Specificity for all four laboratories were calculated to be 95%, 92% and 100%, respectively.

68. For the discordant chemical, Prochloraz, the concentration range tested by CERI (10^{-11}- 10^{-6}M) in which the chemical was negative, was higher than that of other three laboratories (10^{-10}- 10^{-5}M). This was based on the results of the solubility test conducted previously.

69. The decision on the concentration range was made by the study director in CERI based on the occurrence of precipitation rather than cell viability.

70. To confirm the cause of this discordant result for Prochloraz, an additional trial was conducted by CERI using the same concentration range (10^{-10}- 10^{-5}M) as the other three laboratories.

71. The results of the additional trial by CERI, showed that Prochloraz gave clear positive results in the antagonist assay (Fig. 2). This results showed that the discordant results for Prochloraz were caused by the different concentration range selected by the solubility test rather than technical issues.

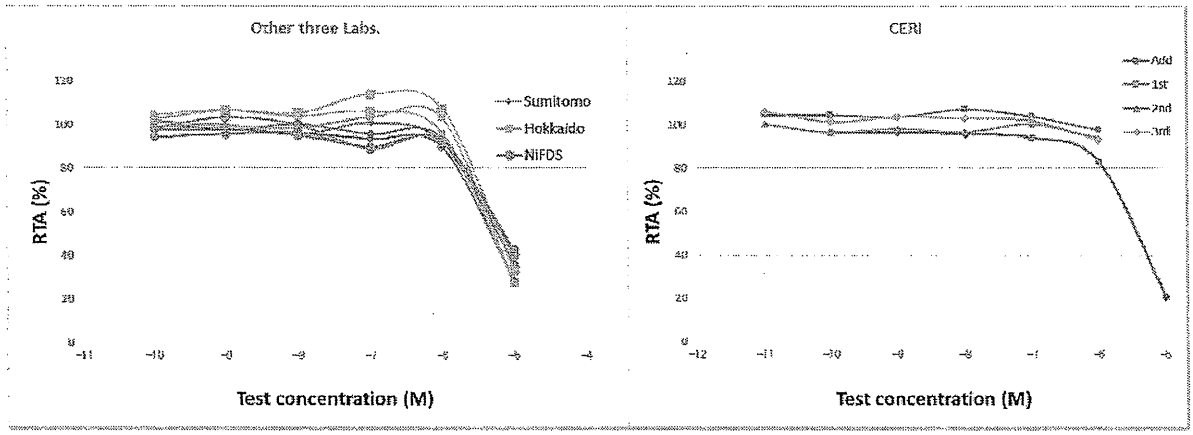

Fig. 2 Comparison of dose response curve for Prochloraz in each laboratory

72. Consequently with the additional trial, all the positive candidate chemicals tested positive in all laboratories, and the negative candidate chemicals also tested negative in all laboratories. In this case, the CV% of LogIC30(M) and LogIC50(M) for each positive chemicals were also less than 4% (Fig. 2, Table 18).

73. The results of the two-by-two table analysis containing the additional trial are shown in Table 20. The Accuracy, Sensitivity and Specificity of the assay was calculated to be all 100% in all laboratories. The Accuracy, Sensitivity and Specificity for all four laboratories were also 100%.

Table 17 Results for the reference chemicals in antagonist assay

		FI*	RTA 0.1uM HF	HF Log IC30 (M)	HF Log IC50 (M)	BisA Log IC30 (M)	BisA Log IC50 (M)
CERI	1	6.46	3.25	-7.60	-7.18	-5.85	-5.55
	2	6.18	2.87	-7.37	-6.92	-5.92	-5.59
	3	6.28	2.84	-7.40	-6.98	-5.89	-5.58
	Add	5.46	-0.12	-7.48	-7.06	-5.82	-5.52
	Mean	6.10	2.21	-7.46	-7.03	-5.89	-5.57
	SD	0.44	1.56	0.10	0.13	0.04	0.03
	CV%	7.19%	70.67%	1.39%	1.87%	0.60%	0.45%
Sumitomo	1	5.73	3.47	-7.65	-7.21	-5.85	-5.53
	2	5.94	4.33	-7.37	-6.88	-5.81	-5.48
	3	5.37	3.11	-7.62	-7.23	-5.97	-5.63
	Mean	5.68	3.64	-7.54	-7.11	-5.88	-5.55
	SD	0.29	0.62	0.15	0.20	0.08	0.08
	CV%	5.08%	17.15%	-2.05%	2.78%	1.45%	1.37%
Hokkaido	1	6.40	2.24	-7.19	-6.78	-5.55	-5.28
	2	7.66	4.46	-7.31	-6.84	-5.65	-5.38
	3	7.33	5.26	-7.11	-6.73	-5.57	-5.29
	Mean	7.13	3.99	-7.20	-6.78	-5.59	-5.32
	SD	0.66	1.56	0.10	0.06	0.05	0.06
	CV%	9.22%	39.24%	-1.45%	0.88%	0.97%	1.09%
NiFDS	1	5.69	1.26	-7.81	-7.40	-6.20	-5.75
	2	5.43	1.73	-7.77	-7.36	-5.97	-5.64
	3	5.44	2.30	-7.71	-7.32	-5.92	-5.60
	Mean	5.52	1.77	-7.76	-7.36	-6.03	-5.66
	SD	0.15	0.52	0.05	0.04	0.15	0.08
	CV%	2.67%	29.48%	-0.62%	0.56%	2.53%	1.40%
For four labs.							
	MEAN	6.11	2.85	-7.49	-7.07	-5.84	-5.52
	SD	0.73	1.43	0.22	0.23	0.18	0.14
	CV%	11.93%	50.13%	2.96%	3.26%	3.02%	2.49%
	MAX	7.66	5.26	-7.11	-6.73	-5.55	-5.28
	MIN	5.37	-0.12	-7.81	-7.40	-6.20	-5.75

Table 18 Summary of the results for test chemicals in antagonist assay

	Lab	ID	Log IC30 (M)	Mean SD CV%	Log IC50 (M)	Mean SD CV%	Decision
6-Propyl-2-thiouracil CAS:51-52-5	CERI	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
		Add	ND		ND		Negative
	Sumitomo	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Hokkaido	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	NiFDS	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	For 4 labs	Mean SD CV%	ND		ND		Negative
Atrazine CAS:1912-24-9	CERI	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
		Add	ND		ND		Negative
	Sumitomo	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Hokkaido	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	NiFDS	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	For 4 labs	Mean SD CV%	ND		ND		Negative
Flutamide CAS:13311-84-7	CERI	1	-5.96	-6.14	ND		Positive
		2	-6.13	0.15	ND		
		3	-6.15	2.45%	ND		
		Add	-6.33		-5.82		Positive
	Sumitomo	1	-5.96	-5.97	-5.57	-5.60	Positive
		2	-5.88	0.09	-5.57	0.05	
		3	-6.07	1.57%	-5.66	0.87%	
	Hokkaido	1	-5.71	-5.74	-5.43	-5.47	Positive
		2	-5.81	0.06	-5.53	0.05	
		3	-5.69	1.10%	-5.44	0.96%	
	NiFDS	1	-6.20	-6.04	-5.66	-5.61	Positive
		2	-5.96	0.14	-5.58	0.05	
		3	-5.95	2.31%	-5.58	0.82%	
	For 4 labs	Mean	-5.96	(-5.98)*	-5.56	(-5.58)	Positive
		SD	0.16	(0.19)*	0.08	(0.11)	
		CV%	2.74%	(3.14%)*	1.44%	(1.99%)	

*Values in parenthesis are overall Mean, SD and CV% containing additional trial by CERI.

Table 18 (continued)

	Lab	ID	Log IC30 (M)	Mean SD CV%	Log IC50 (M)	Mean SD CV%	Decision
Prochloraz CAS:67747-09-5	CERI	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
		Add	-5.77	-5.77	-5.44	-5.44	Positive
	Sumitomo	1	-5.58	-5.60	-5.22	-5.25	Positive
		2	-5.65	0.05	-5.33	0.06	
		3	-5.56	0.89%	-5.21	1.23%	
	Hokkaido	1	-5.54	-5.53	-5.27	-5.26	Positive
		2	-5.59	0.06	-5.30	0.05	
		3	-5.47	1.14%	-5.20	1.04%	
	NiFDS	1	-5.53	-5.53	-5.15	-5.14	Positive
		2	-5.52	0.01	-5.12	0.02	
		3	-5.54	0.16%	-5.16	0.36%	
	For 4 labs	Mean	-5.55	(-5.57)*	-5.22	(-5.24)	Positive
		SD	0.05	(0.08)*	0.07	(0.10)	
		CV%	0.92%	(1.48%)*	1.36%	(1.87%)	
Vinclozolin CAS:50471-44-8	CERI	1	-6.44	-6.46	-6.07	-6.10	Positive
		2	-6.45	0.03	-6.04	0.05	
		3	-6.46	0.48%	-6.14	0.82%	
		Add	-6.51		-6.14		Positive
	Sumitomo	1	-6.42	-6.38	-5.96	-5.92	Positive
		2	-6.39	0.04	-5.95	0.06	
		3	-6.34	0.62%	-5.85	0.96%	
	Hokkaido	1	-6.46	-6.40	-6.10	-6.07	Positive
		2	-6.42	0.07	-6.12	0.07	
		3	-6.32	1.09%	-6.00	1.09%	
	NiFDS	1	-6.83	-6.70	-6.47	-6.31	Positive
		2	-6.65	0.11	-6.25	0.14	
		3	-6.62	1.67%	-6.21	2.17%	
	For 4 labs	Mean	-6.48	(-6.49)	-6.10	(-6.10)	Positive
		SD	0.17	(0.14)	0.19	(0.16)	
		CV%	2.63%	(2.18%)	3.08%	(2.55%)	

*Values in parenthesis are overall Mean, SD and CV% containing additional trial by CERI.

Table 19 Positive/negative outcomes in antagonist assay and results of two-by-two table analysis

Test chemical	Candidate effect	Result				
		CERI	Sumitomo	Hokkaido	NiFDS	4 Lab
Flutamide	P	P	P	P	P	P
Prochloraz	P	N	P	P	P	P
Vinclozolin	P	P	P	P	P	P
Atrazine	N	N	N	N	N	N
6-Propyl-2-thiouracil	N	N	N	N	N	N
P:Positive						
N:Negative	Accuracy	80%	100%	100%	100%	95%
	Sensitivity	67%	100%	100%	100%	92%
	Specificity	100%	100%	100%	100%	100%

Table 20 Positive/negative outcomes in antagonist assay and results of two-by-two table analysis with consideration of additional trial

Test chemical	Candidate effect	Result				
		CERI	Sumitomo	Hokkaido	NiFDS	4 Lab
Flutamide	P	P	P	P	P	P
Prochloraz	P	P	P	P	P	P
Vinclozolin	P	P	P	P	P	P
Atrazine	N	N	N	N	N	N
6-Propyl-2-thiouracil	N	N	N	N	N	N
P:Positive						
N:Negative	Accuracy	100%	100%	100%	100%	100%
	Sensitivity	100%	100%	100%	100%	100%
	Specificity	100%	100%	100%	100%	100%

7. DISCUSSION

74. The human AR mediated stably transfected TA assay system using AR-EcoScreen™ was developed in Japan, and the assay system consisted of agonist and antagonist assays using a genetically modified stable cell line called AR-EcoScreen™. We have compiled a validation report based on results from the pre-validation study with 40 chemicals and the inter-laboratory validation study performed with the four participating laboratories using same five chemicals for both androgenic and anti-androgenic activities in 2005.

75. The validation report was submitted to OECD in 2010. However the Peer review panel report stated that a dedicated inter-laboratory study should be carried out, using the final test protocol to test substances covering a broad range of activity, especially including non-active substances and weak agonists and antagonists. This was an additional inter-laboratory validation study corresponding to the major Peer review comment for the validation report.

76. The additional validation study was conducted with a total of ten test chemicals covering a broad range of agonist and antagonist activities selected by the chemical selection group consisting of OECD VMG-NA members. The study was conducted with three Japanese and one Korean laboratories.

77. The additional validation study consisted of Phase-1 and Phase-2 studies. The Phase-1 study was to confirm the overall laboratory proficiency by testing the same lots of reference chemicals and to collect data to set reference criteria for mestanolone which was the newly added reference chemical for the agonist study. The Phase-2 study was to provide the supplemental data according to previous Peer review comments on this assay and to evaluate the assay performance (within/between-laboratory reproducibility and predictive capacity) by testing 10 coded chemicals (five each for agonist and antagonist assays).

78. In the Phase-1 study, all laboratories passed the reference criteria within the minimum three runs, and the inexperienced Korean laboratory could yielded successful results for the additional reference chemical for the agonist assay, Mestanolone that met the tentative reference criteria decided based on the results obtained with three Japanese laboratories.

79. In the Phase-2 agonist study, all laboratories passed the reference criteria within the minimum three run, and all laboratories could yield correct positive/negative outcomes corresponding to the candidate effects. Consequently, the Accuracy, Sensitivity and Specificity of the agonist assay were all calculated to be 100% in all laboratories. In addition, the CV% of LogPC10(M) and LogPC50(M) for positive chemicals were less than 5% and high reproducibility of this

assay was confirmed.

80. In the Phase-2 antagonist study, all laboratories passed the reference criteria within the minimum three runs, and three out of four laboratories could yield correct positive/negative outcomes corresponding to the candidate effects. However, the remaining one laboratory had a false negative result for the positive candidate chemical, Prochloraz.

81. Accordingly, in the results of the two-by-two table analysis in first three runs, the Accuracy, Sensitivity and Specificity for all four laboratories were calculated to be 95%, 92% and 100%, respectively.

82. However the cause of the false negative response for Prochloraz was considered to be a dose-selection issue rather than a technical issue. An additional trial was conducted using same concentration range as the laboratories that achieved a positive response, in order to confirm the cause of the false negative response. The laboratory then yielded a positive result for Prochloraz,

83. Consequently with the additional trial, the all positive candidate chemicals, tested positive in all laboratories, and the Accuracy, Sensitivity and Specificity of the assay was calculated as all 100% in all laboratories. In addition, the CV% of LogIC30(M) and LogIC50(M) for positive chemicals containing additional trial were less than 4%, and high reproducibility of this assay was confirmed.

84. The concordance of positive/negative outcomes of coded test chemicals were more than 80% for each of the agonist and antagonist assays, and the high assay performance of this assay was confirmed.

85. The results of the additional validation study show that the original protocol is well established and robust, however the maximum dose selected by the solubility test described in the original protocol may occasionally affect the sensitivity of the assay. Therefore the following sentence should be including in the section of solubility test in the guideline.

86. "This solubility test is very important step to determine the maximum dose for the assay and it may affect the sensitivity of the assay. The highest concentration should be selected based on the cell viability rather than the avoidance of some precipitation in higher dose range. "

8. CONCLUSIONS

87. Results of the additional inter-laboratory validation study for the human AR mediated stably transfected TA assay system using AR-EcoScreen™ with three Japanese domestic and one Korean laboratories showed the high reproducibility of the assay system and good technical transferability of the assay protocols because the concordance of positive/negative outcomes of coded test chemicals were more than 80% for each of agonist and antagonist assay.

88. Accordingly the assay system is well-established and has been shown to be a well-validated assay for development of an OECD test guideline for the detection of chemicals possessing potential androgenic and anti-androgenic activities through hARα. The assay is a therefore a promising method to use in the prescreening process of an endocrine disruptor screening strategy.

9. RECOMMENDATIONS

89. The original protocol is well established and robust as the results of the validation and additional validation studies demonstrate. However the maximum dose selected by the solubility test described in the original protocol may occasionally affect the sensitivity of the assay. Accordingly, the following sentence should be including in the section of solubility test in the guideline.

90. "This solubility test is very important step to determine the maximum dose for the assay and it may affect the sensitivity of the assay. The highest concentration should be selected based on the cell viability rather than the avoidance of some precipitation in higher dose range. "

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Appendix 1

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Stably Transfected Human Androgen Receptor-α Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals

(Version 2015 Feb.9)

INTRODUCTION

1. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new, Test Guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals comprises five levels, each level corresponding to a different level of biological complexity (1). The Stably Transfected Human Androgen Receptor-α (AR) Transcriptional Activation (TA) Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals (AR-STTA) using the AR-EcoScreen™ cell line (2) is included in level 2 for *"in vitro assays providing data about selected endocrine mechanism(s)/pathway(s) (Mammalian and non mammalian methods)"* (1).

2. *In vitro* TA assays are based upon the production of a reporter gene product induced by a chemical, following binding of the chemical to a specific receptor and subsequent downstream transcriptional activation. TA assays using activation of reporter genes are screening assays that have long been used to evaluate the specific gene expression regulated by specific nuclear receptors, such as the estrogen receptors (ERs) and androgen receptor (AR) (3)(4)(5)(6). They have been proposed for the detection of nuclear receptor mediated transactivation (3)(4)(7).

3. The AR STTA test method has been validated by collaboration of the Chemicals Evaluation and Research Institute (CERI) and the National Institute of Health Sciences (NIHS) in Japan with support of the study management team from the OECD validation management group for non-animal testing (2). The AR STTA test method provides concentration-response data for substances with in vitro AR agonist or antagonist activity (2), which may be used for screening and prioritization purposes and can also be used as mechanistic information in a weight of evidence approach.

4. Definitions and abbreviations used in this Test Guideline are described in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. Androgen agonists and antagonist act as ligands for AR, and may activate or inhibit the transcription of androgen responsive genes. This interaction may have the potential to trigger adverse health effects by disrupting androgen-regulated systems. This Test Guideline describes an assay that evaluates transcriptional activation and inhibition of AR mediated responses. This process is considered to be one of the key mechanisms of possible endocrine disruption related health hazards, although there are also other important endocrine disruption mechanisms. These include (i) actions mediated via other nuclear receptors linked to the endocrine system and interactions with steroidogenic enzymes, (ii) metabolic activation or deactivation of hormones, (iii) distribution of hormones to target tissues, and (iv) clearance of hormones from the body. This Test Guideline exclusively addresses transcriptional activation and inhibition of an androgen -regulated reporter gene by binding to the human AR, and therefore it should not be directly extrapolated to the complex *in vivo* situation of androgen regulation of cellular processes.

6. This test method is specifically designed to detect human AR-mediated transcriptional activation and inhibition by measuring chemiluminescence as the endpoint.

PRINCIPLE OF THE TEST

7. The TA assay using a reporter gene technique is an *in vitro* tool that provides mechanistic data. The assay is used to signal activation or blocking of the androgen receptor caused by a ligand. Following ligand binding, the receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer. Luciferase activity can be evaluated quickly and inexpensively with a number of commercially available test kits.

8. The test system provided in this Test Guideline utilizes the AR-EcoScreen™ cell line, which is derived from a Chinese hamster ovary cell line (CHO-K1), with two stably inserted constructs: (i) the human AR expression construct (encoding the full-length human receptor), and (ii) a firefly luciferase reporter construct bearing four tandem repeats of a prostate C3 gene-responsive element driven by a minimal heat shock protein promoter. The C3 gene derived responsive element is selected to minimize GR mediated responses among known androgen responsive elements.

9. Data interpretation for an **AR agonistic effect** is based upon the maximum response level

induced by a test chemical. If this response equals or exceeds a response equal to 10% of that induced by a maximally inducing (10 nM) concentration of the positive control (PC) 5α-dihydrotestosterone (DHT) (*i.e.* the log PC10), the test chemical is considered positive. Data interpretation for an **AR antagonistic effect** of a test chemical is based on a cut-off of a 30% inhibitory response against 500 pM DHT. If the response exceeds this 30% AR blocking, then the chemical is considered a positive AR antagonist. Data analysis and interpretation are discussed in greater detail in paragraphs 38- 54.

PROCEDURE

Cell Lines

10. The stably transfected AR-EcoScreen™ cell line should be used for the assay. The cell line can be obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank as a reference No. JCRB1328, upon signing a Material Transfer Agreement (MTA).

11. Only cells characterized as mycoplasma-free should be used in testing. RT PCR (Real Time Polymerase Chain Reaction) is the method of choice for a sensitive detection of mycoplasma infection (8) (9) (10).

Stability of the cell line

12. To monitor the stability of the cell line for the **agonist assay**, DHT, Mestanolone and Di(2-ethylhexyl)phthalate (DEHP) should be used as the reference chemicals and a complete concentration response curve at the test concentration range provided in Table 1-1 should be obtained at least once each time the assay is performed, and the results should be in agreement with the results provided in Table 1-1.

13. To monitor the stability of the cell line for measuring **AR antagonism**, Hydroxyflutamide (HF), Bisphenol A (BisA) and Di(2-ethylhexyl)phthalate (DEHP) should be used as the reference chemicals and a complete concentration response curve at the test concentration range provided in Table 1-2 should be obtained at least once every day the assay is performed, and the results should be in agreement with the results provided in Table 1-2.

Cell Culture and Plating Conditions

14. The following mediums should be prepared;

Medium for dilution: Phenol Red Free D-MEM/F-12.

Medium for cell propagation: Phenol Red Free D-MEM/F-12 supplemented with 5% fetal bovine serum, Zeocin (200 μg/mL), Hygromycin (100 μg/mL), Penicillin (100 units /m L), and