Table 4.8 The cell viabilitys of 40 blinded test substance substances and solvents used (continued)

			Lab.								
			1			2			3		
Chemical	Conc.	Round	Cell Viability	MEAN	Solvent	Cell Viability	MEAN	Solvent	Cell Viability	MEAN	Solvent
c25	High	1	59.5	51.0	Saline	95.9	87.5	Saline	.	.	.
		2	51.4			84.2			.		
		3	42.2			82.4			.		
	Low	1	91.3	96.5		107.0	109.5		.	.	
		2	98.2			107.5			.		
		3	99.9			114.1			.		
c26	High	1	3.2	5.4	Saline	21.1	18.5	Saline	.	.	.
		2	6.5			17.4			.		
		3	6.7			17.0			.		
	Low	1	93.1	97.9		100.4	94.8		.	.	
		2	101.5			92.3			.		
		3	98.9			91.6			.		
c27	High	1	3.4	3.4	Mineral	3.0	4.4	Mineral	0.0	0.6	Mineral
		2	2.4			5.8			1.8		
		3	4.6			4.3			0.1		
	Low	1	92.4	95.4		100.1	87.1		69.8	72.3	
		2	91.5			85.4			73.3		
		3	102.3			75.9			73.9		
c28	High	1	2.0	1.5	Mineral	36.1	44.4	Mineral	0.0	4.8	Mineral
		2	2.4			51.0			4.6		
		3	0.0			46.0			9.9		
	Low	1	104.8	100.0		110.1	105.1		70.6	77.1	
		2	93.6			101.2			72.9		
		3	101.8			104.1			88.0		
c29	High	1	.	.	.	91.1	93.7	Saline	102.6	101.2	Saline
		2	.			97.3			99.8		
		3	.			92.5			101.3		
	Low	1	.	.		91.6	92.4		91.3	95.6	
		2	.			94.8			92.1		
		3	.			90.9			103.6		
c30	High	1	9.7	8.5	Saline	.	.	.	6 2	6.44	Saline
		2	7.8			.			8.0		
		3	7.9			.			5.1		
	Low	1	100.4	92.4		.	.		91.6	97.9	
		2	84.2			.			102.4		
		3	92.6			.			99.7		
c31	High	1	4.9	5.1	5% DMSO	.	.	.	1.6	3.1	5% DMSO
		2	5.5			.			5.3		
		3	4.8			.			2.2		
	Low	1	97.2	96.3		.	.		97.8	98.2	
		2	97.7			.			96.1		
		3	94.1			.			100.7		
c32	High	1	0.0	0.0	Saline	0.6	0.8	Saline	0.3	0.6	Saline
		2	0.0			0.0			1.4		
		3	0.0			1.7			0.2		
	Low	1	101.2	100.3		98.6	101.6		95.9	100.5	
		2	94.3			106.6			104.2		
		3	105.4			99.6			101.4		

Table 4.8 The cell viabilitys of 40 blinded test substance substances and solvents used (continued)

			Lab.								
			1			2			3		
Chemical	Conc.	Round	Cell Viability	MEAN	Solvent	Cell Viability	MEAN	Solvent	Cell Viability	MEAN	Solvent
c33	High	1	105.6	102.2	Saline	65.1	76.0	Saline	.	.	.
		2	104.0			73.2			.		
		3	97.2			89.6			.		
	Low	1	89.6	92.6		76.2	84.6		.	.	
		2	91.6			82.1			.		
		3	96.6			95.5			.		
c34	High	1	84.7	86.4	Saline	.	.	.	77.1	84.4	Saline
		2	88.0			.			84.4		
		3	86.6			.			91.7		
	Low	1	98.1	97.6		.	.		91.8	95.4	
		2	96.1			.			92.0		
		3	98.4			.			102.3		
c35	High	1	98.4	104.7	Mineral	.	.	.	75.8	75.3	Mineral
		2	110.9			.			68.9		
		3	104.8			.			81.1		
	Low	1	95.4	97.5		.	.		85.9	84.8	
		2	101.6			.			83.2		
		3	95.3			.			85.3		
c36	High	1	.	.	.	87.7	94.4	Saline	95.5	95.28	Saline
		2	.			95.0			94.5		
		3	.			100.6			95.9		
	Low	1	.	.		96.2	95.7		95.0	97.76	
		2	.			97.2			95.9		
		3	.			93.7			102.4		
c37	High	1	0.0	0.4	Mineral	2.7	4.7	Mineral	2.0	1.7	Mineral
		2	1.1			6.3			3.0		
		3	0.0			5.0			0.0		
	Low	1	90.4	93.7		101.7	98.4		76.9	79.3	
		2	89.3			99.5			88.4		
		3	101.5			94.0			72.8		
c38	High	1	.	.	.	0.0	0.1	Saline	1.5	1.8	Saline
		2	.			0.3			2.2		
		3	.			0.1			1.8		
	Low	1	.	.		94.1	98.3		99.5	99.4	
		2	.			91.1			95.1		
		3	.			109.7			103.6		
c39	High	1	5.3	4.6	Saline	8.8	9.0	Saline	.	.	.
		2	4.4			8.9			.		
		3	4.0			9.4			.		
	Low	1	89.6	91.9		101.2	102.0		.	.	
		2	92.5			108.3			.		
		3	93.7			96.4			.		
c40	High	1	3.7	3.9	Mineral	9.7	8.8	Mineral	5.8	5.5	Saline
		2	5.7			14.8			6.1		
		3	2.4			1.8			4.7		
	Low	1	92.3	99.8		84.1	89.7		95.0	98.6	
		2	100.2			94.0			97.2		
		3	107.1			91.0			103.6		

4.5 The STE rank

The STE rank calculated from the mean cell viabilities at 5% and 0.05% are shown in Table 4.9. The STE rank of each test substance calculated from the mean cell viabilities at 5% and 0.05% are shown in Table 4.10.

Table 4.9 the mean cell viability of each test substance

C_CODE	C_CONC	Lab1	Lab2	Lab3	N	MEAN	SD	VAR
c01	High	33.1	70.1	.	2	51.6	26.1	683.7
	Low	83.0	88.5	.	2	85.7	3.9	15.1
c02	High	0.9	.	1.5	2	1.2	0.5	0.2
	Low	58.5	.	77.8	2	68.1	13.7	187.5
c03	High	0.0	0.0	.	2	0.0	0.0	0.0
	Low	0.2	0.0	.	2	0.1	0.1	0.0
c04	High	.	10.4	1.0	2	5.7	6.7	44.5
	Low	.	94.6	82.1	2	88.3	8.8	77.9
c05	High	8.0	9.7	.	2	8.9	1.2	1.4
	Low	92.7	95.6	.	2	94.1	2.0	4.0
c06	High	3.9	7.5	4.0	3	5.2	2.1	4.2
	Low	101.7	106.0	94.9	3	100.9	5.6	31.6
c07	High	104.6	95.8	89.5	3	96.7	7.6	57.9
	Low	102.7	100.2	93.0	3	98.6	5.0	25.4
c08	High	16.9	17.2	.	2	17.0	0.2	0.1
	Low	91.2	102.8	.	2	97.0	8.2	67.3
c09	High	1.2	.	1.7	2	1.5	0.4	0.2
	Low	91.5	.	89.5	2	90.5	1.4	1.9
c10	High	.	7.7	4.8	2	6.3	2.1	4.3
	Low	.	91.8	98.9	2	95.3	5.0	25.3
c11	High	.	46.0	63.2	2	54.6	12.1	147.6
	Low	.	92.1	100.4	2	96.2	5.9	34.4
c12	High	102.0	75.1	.	2	88.6	19.0	362.7
	Low	97.9	93.1	.	2	95.5	3.3	11.2
c13	High	25.8	.	6.7	2	16.2	13.5	181.2
	Low	94.3	.	101.6	2	98.0	5.2	26.9
c14	High	0.5	0.3	1.0	3	0.6	0.4	0.1
	Low	3.8	7.1	1.3	3	4.1	2.9	8.4
c15	High	6.8	7.4	3.7	3	6.0	2.0	3.9
	Low	101.4	95.6	87.7	3	94.9	6.9	47.2
c16	High	.	85.3	73.6	2	79.4	8.3	69.2
	Low	.	106.3	99.3	2	102.8	5.0	24.9
c17	High	99.1	88.6	100.8	3	96.2	6.6	43.6
	Low	95.5	91.4	100.8	3	95.9	4.8	22.6
c18	High	.	6.5	3.0	2	4.7	2.5	6.3
	Low	.	79.9	90.1	2	85.0	7.2	52.0
c19	High	2.7	.	3.4	2	3.0	0.5	0.3
	Low	97.1	.	97.4	2	97.3	0.2	0.0
c20	High	101.2	.	90.0	2	95.6	7.9	62.7
	Low	102.2	.	100.4	2	101.3	1.3	1.7

Table 4.9 the mean cell viability of each test substance (continued)

C_CODE	C_CONC	Lab1	Lab2	Lab3	N	MEAN	SD	VAR
c21	High	.	19.1	4.4	2	11.7	10.4	107.7
	Low	.	80.9	98.0	2	89.4	12.1	145.4
c22	High	88.6	95.9	.	2	92.3	5.2	26.9
	Low	85.6	98.6	.	2	92.1	9.2	84.6
c23	High	5.9	.	1.2	2	3.6	3.3	11.1
	Low	95.9	.	99.3	2	97.6	2.4	5.9
c24	High	.	88.7	93.4	2	91.0	3.3	10.8
	Low	.	95.7	100.7	2	98.2	3.6	12.7
c25	High	51.0	87.5	.	2	69.3	25.8	666.1
	Low	96.5	109.5	.	2	103.0	9.3	85.7
c26	High	5.4	18.5	.	2	12.0	9.2	85.4
	Low	97.9	94.8	.	2	96.3	2.2	4.8
c27	High	3.4	4.4	0.6	3	2.8	2.0	3.8
	Low	95.4	87.1	72.3	3	85.0	11.7	136.6
c28	High	1.5	44.4	4.8	3	16.9	23.8	568.4
	Low	100.0	105.1	77.1	3	94.1	14.9	222.5
c29	High	.	93.7	101.2	2	97.4	5.4	28.7
	Low	.	92.4	95.6	2	94.0	2.3	5.2
c30	High	8.5	.	6.4	2	7.4	1.4	2.0
	Low	92.4	.	97.9	2	95.1	3.9	15.4
c31	High	5.1	.	3.1	2	4.1	1.4	2.0
	Low	96.3	.	98.2	2	97.2	1.3	1.8
c32	High	0.0	0.8	0.6	3	0.5	0.4	0.2
	Low	100.3	101.6	100.5	3	100.8	0.7	0.5
c33	High	102.2	76.0	.	2	89.1	18.6	344.5
	Low	92.6	84.6	.	2	88.6	5.7	32.0
c34	High	86.4	.	84.4	2	85.4	1.4	2.0
	Low	97.6	.	95.4	2	96.5	1.5	2.4
c35	High	104.68	.	75.3	2	90.0	20.8	432.6
	Low	97.451	.	84.8	2	91.1	8.9	79.7
c36	High	.	94.4	95.3	2	94.9	0.6	0.4
	Low	.	95.7	97.8	2	96.7	1.5	2.2
c37	High	0.4	4.7	1.7	3	2.2	2.2	4.9
	Low	93.7	98.4	79.3	3	90.5	9.9	98.7
c38	High	.	0.1	1.8	2	1.0	1.2	1.4
	Low	.	98.3	99.4	2	98.9	0.8	0.6
c39	High	4.6	9.0	.	2	6.8	3.2	9.9
	Low	91.9	102.0	.	2	96.9	7.1	50.3
c40	High	3.9	8.8	5.5	3	6.1	2.5	6.1
	Low	99.8	89.7	98.6	3	96.0	5.5	30.7

4.6 Inter-laboratory reproducibility

As shown in Table 4.11, there are the different results with test substances C01, and C25 between the STE classification and GHS classification at two laboratories. As shown in Table 4.12, there are the different results with test substances C01, C02 and C25 between the STE rank and GHS category at two laboratories. From only three miss categories of test substances per forty, we consider the Inter-laboratory repeatability of the STE test is high. The difference classification determined not to be used for predictivity by the VMT. To be clear the reason of difference results between laboratories, however, the VMT indicated an additional study to each laboratory. Therefore, three test substances (C01, C02 and C25) were re-evaluated by three laboratories (see session 4.6)

On the other hand, we confirmed same STE ranks of common two test substances (C07: 2-ethylhexyl

p-dimethyl-amino benzoate and C37: 1-octanol) with the 1st phase and 2nd phase validation study on all laboratories. From these data, we consider the Inter-laboratory repeatability of the STE test is high additionally.

Table 4.10 The STE rank of each test substance

	Lab.								
	1			2			3		
Chemical	Score for high	Score for low	STE rank	Score for high	Score for low	STE rank	Score for high	Score for low	STE rank
c01	1	1	2	0	1	1	.	.	.
c02	1	2	3	.	.	.	1	1	2
c03	1	2	3	1	2	3	.	.	.
c04	.	.	.	1	1	2	1	1	2
c05	1	1	2	1	1	2	.	.	.
c06	1	1	2	1	1	2	1	1	2
c07	0	1	1	0	1	1	0	1	1
c08	1	1	2	1	1	2	.	.	.
c09	1	1	2	.	.	.	1	1	2
c10	.	.	.	1	1	2	1	1	2
c11	.	.	.	1	1	2	1	1	2
c12	0	1	1	0	1	1	.	.	.
c13	1	1	2	.	.	.	1	1	2
c14	1	2	3	1	2	3	1	2	3
c15	1	1	2	1	1	2	1	1	2
c16	.	.	.	0	1	1	0	1	1
c17	0	1	1	0	1	1	0	1	1
c18	.	.	.	1	1	2	1	1	2
c19	1	1	2	.	.	.	1	1	2
c20	0	1	1	.	.	.	0	1	1
c21	.	.	.	1	1	2	1	1	2
c22	0	1	1	0	1	1	.	.	.
c23	1	1	2	.	.	.	1	1	2
c24	.	.	.	0	1	1	0	1	1
c25	1	1	2	0	1	1	.	.	.
c26	1	1	2	1	1	2	.	.	.
c27	1	1	2	1	1	2	1	1	2
c28	1	1	2	1	1	2	1	1	2
c29	.	.	.	0	1	1	0	1	1
c30	1	1	2	.	.	.	1	1	2
c31	1	1	2	.	.	.	1	1	2
c32	1	1	2	1	1	2	1	1	2
c33	0	1	1	0	1	1	.	.	.
c34	0	1	1	.	.	.	0	1	1
c35	0	1	1	.	.	.	0	1	1
c36	.	.	.	0	1	1	0	1	1
c37	1	1	2	1	1	2	1	1	2
c38	.	.	.	1	1	2	1	1	2
c39	1	1	2	1	1	2	.	.	.
c40	1	1	2	1	1	2	1	1	2

Table 4.11 The GHS classification and mean cell viability at 5%, and STE classification

		Lab.					
		1		2		3	
Chemical	GHS	Mean CV for high	Score for high	Mean CV for high	Score for high	Mean CV for high	Score for high
c01	I	33.1	1	70.1	0	.	.
c02	I	0.9	1	.	.	1.6	1
c03	I	0.0	1	0.0	1	.	.
c04	I	.	.	10.4	1	1.0	1
c05	I	8.0	1	9.7	1	.	.
c06	I	3.9	1	7.5	1	4.0	1
c07	NI	104.7	0	95.8	0	89.5	0
c08	NI	16.9	1	17.2	1	.	.
c09	I	1.2	1	.	.	1.7	1
c10	I	.	.	7.7	1	4.8	1
c11	I	.	.	46.0	1	63.2	1
c12	I	102.0	0	75.1	0	.	.
c13	I	25.8	1	.	.	6.7	1
c14	I	0.5	1	0.3	1	1.0	1
c15	I	6.8	1	7.4	1	3.7	1
c16	I	.	.	85.3	0	73.6	0
c17	NI	99.1	0	88.6	0	100.8	0
c18	I	.	.	6.5	1	3.0	1
c19	I	2.7	1	.	.	3.4	1
c20	I	101.2	0	.	.	90.0	0
c21	I	.	.	19.1	1	4.4	1
c22	NI	88.6	0	95.9	0	.	.
c23	I	5.9	1	.	.	1.2	1
c24	I	.	.	88.7	0	93.4	0
c25	I	51.0	1	87.5	0	.	.
c26	I	5.4	1	18.5	1	.	.
c27	I	3.5	1	4.4	1	0.6	1
c28	I	1.5	1	44	1	4.8	1
c29	I	.	.	93.7	0	101.2	0
c30	NI	8.5	1	.	.	6.4	1
c31	I	5.1	1	.	.	3.1	1
c32	I	0.0	1	0.8	1	0.6	1
c33	I	102.2	0	76.0	0	.	.
c34	I	86.4	0	.	.	84.4	0
c35	I	104.7	0	.	.	75.3	0
c36	I	.	.	94.4	0	95.3	0
c37	I	0.4	1	4.7	1	1.7	1
c38	I	.	.	0.1	1	1.8	1
c39	I	4.6	1	9.0	1	.	.
c40	I	3.9	1	8.8	1	5.53	1

Table 4.12 The GHS categories, mean cell viability, and STE rank of each test substance

		Lab.		
		1	2	3
Chemical	GHS	STE rank	STE rank	STE rank
c01	1	2	1	.
c02	1	3	.	2
c03	1	3	3	.
c04	2	.	2	2
c05	2	2	2	.
c06	1	2	2	2
c07	NL	1	1	1
c08	NL	2	2	.
c09	1	2	.	2
c10	1	.	2	2
c11	2	.	2	2
c12	2	1	1	.
c13	2	2	.	2
c14	2	3	3	3
c15	2	2	2	2
c16	1	.	1	1
c17	NL	1	1	1
c18	2	.	2	2
c19	2	2	.	2
c20	2	1	.	1
c21	2	.	2	2
c22	NL	1	1	.
c23	2	2	.	2
c24	2	.	1	1
c25	2	2	1	.
c26	2	2	2	.
c27	1	2	2	2
c28	2	2	2	2
c29	2	.	1	1
c30	NL	2	.	2
c31	2	2	.	2
c32	1	2	2	2
c33	2	1	1	.
c34	2	1	.	1
c35	2	1	.	1
c36	1	.	1	1
c37	2	2	2	2
c38	2	.	2	2
c39	1	2	2	.
c40	2	2	2	2

4.7 Corresponding relationships of irritants and non-irritants in separate categories

Table 4.13 shows the corresponding relationships between the GHS classification and the STE classification at the concentration of 5% accepted data at each laboratory, which participated both validation studies. The sensitivity, specificity, and accuracy values include under tables.

The sensitivity values of each laboratory were among 73.9 -77.8 % and the sensitivity of each laboratory were among 73.9 -77.8 % and 86.7-93.8%. The accuracy of each laboratories were among 77.4 -81.1 %.

Table 4.13.1 Relationships between the STE classification and GHS classification at Lab1

STE (5%) / GHS		NI (cell viability ＞70)	I (cell viability ≦70)
		23	30
NI	17	15	2
I (Cat.1, Cat.2)	36	8	28

n= 53

Sensitivity : 77.8 % (28 / 36)

Specificity : 88.2 % (15 / 17)

Accuracy : 81.1 % (43 / 53)

Table 4.13.2 Relationships between the STE classification and GHS classification at Lab2

STE (5%) / GHS		NI (cell viability＞70)	I (cell viability ≦70)
		25	28
NI	16	15	1
I(Cat.1, Cat.2)	37	10	27

n= 53

Sensitivity : 73.0 % (27 / 37)

Specificity : 93.8 % (15 / 16)

Accuracy : 79.2 % (42 / 53)

Table 4.13.3 Relationships between the STE classification and GHS classification at Lab3

GHS \ STE (5%)		NI (cell viability>70)	I (cell viability ≦70)
		23	30
NI	15	13	2
I(Cat.1, Cat.2)	28	10	28

n= 53

Sensitivity : 73.7 % (28 / 38))

Specificity : 86.7 % (13 / 15))

Accuracy : 77.4 % (41 / 53))

4.8 Corresponding relationships between the STE rank and GHS categories

Table 4.14 shows the corresponding relationships between the STE rank and GHS categories. The accuracy values include under tables.

The accuracy values between the STE rank and GHS categories in each laboratory were among 62.3-66%.

Table 4.14.1 Relationships between STE rank and GHS categories at Lab 1

GHS	**STE Rank**			**Total**
	1	**2**	**3**	
I	15	2	0	17
2	8	14	1	23
1	0	7	6	13
Total	23	23	7	53

Accuracy 66.0%(35/53)

Table 4.14.2 Relationships between STE rank and GHS categories at Lab 2

GHS	STE Rank			Total
	1	2	3	
NI	15	1	0	16
2	7	15	1	23
1	3	6	5	14
Total	25	22	6	53

Accuracy 66.0%(35/53)

Table 4.14.3 Relationships between STE rank and GHS categories at Lab 3

GHS	STE Rank			Total
	1	2	3	
NI	13	2	0	15
2	8	16	1	25
1	2	7	4	13
Total	23	25	5	53

Accuracy 62.3%(33/53)

4.9 Additional study

To be clear the reason the different results with test substances C01, C02 and C25 between the STE rank and GHS categories at two laboratories, re-test were performed by three laboratories. According to Lead laboratory, the solvent was unified among laboratories. Using saline and not used 5% DMSO in saline as the solvent, test substance C01 (distearyldimethylammonium chloride) prepared in condition with heat up at 50 C hot water. As the results, three laboratories obtained the similar cell viability and same STE rank as shown in Table 4.15.

On the other hand, the results of C02 (promethazine hydrochloride) and C25 (methyl cyanoacetate) were different classification among three laboratories as well as the results of validation study. We can not make it clear that the reason or consideration on different classification of them. We consider relatively few substances exhibited cell viability around 70% in the STE test.

Table 4.15 Results of additional study after validation study

			1				2				3			
NO.	Conc.	Round	cell viability	MEAN ±SD	STE score	Solvent	cell viability	MEAN± SD	STE Score	Solvent	cell viability	MEAN± SD	STE score	Solvent
c01	High	1	32.6	28.2 ±5.0	1	Salin e	33.5	31.2± 3.9	1	Salin e	28.6	25.2 ±3.9	1	Salin e
		2	22.7				33.7				26.1			
		3	29.2				26.8				21.0			
	Low	1	74.8	74.3± 2.0	1		86.0	81.5± 9.4	1		84.5	81.7± 2.8	1	
		2	72.2				87.8				78.9			
		3	76.0				70.7				81.5			
c02	High	1	0.5	0.4±0 .2	1	Salin e	1.0	0.3±0 .6	1	Salin e	3.5	3.1 ±0.6	1	Salin e
		2	0.6				0				2.4			
		3	0.1				0				3.4			
	Low	1	71.7	69.3± 4.0	2		77.2	90.2± 11.7	1		87.2	81.7 ±5.5	1	
		2	71.6				99.9				76.2			
		3	64.7				93.6				81.8			
C25	High	1	52.5	51.0± 6.2	1	Salin e	92.9	97.3± 7.1	0	Salin e	105.4	100.2 ±4.5	0	Salin e
		2	44.2				105.4				97.3			
		3	56.3				93.5				97.9			
	Low	1	93.5	96.8± 5.8	1		100.9	101.7 ±3.8	1		107.4	100.5 ±6.1	1	
		2	103.5				98.4				97.0			
		3	93.4				105.9				96.9			

5. Discussion

5.1 Analysis of results for 40 blinded test substances

In this validation study, we evaluated 40 test substances. In the test substances, common 10 test substances with three laboratories were detected same STE classification. On the other hand, there were the different results with test substances C01 (distearyldimethylammonium chloride), and C25 (methyl cyanoacetate) between STE classification and GHS classification at two laboratories (table 4.11). Furthermore, there are the different results with test substances C01 (distearyldimethylammonium chloride), C02 (promethazine hydrochloride) and C25 (methyl cyanoacetate) between the STE rank and GHS categories at two laboratories (Table 4.12).

To obtain clear reason of the different results with test substances C01 (distearyldimethylammonium chloride), C02 (promethazine hydrochloride) and C25 (methyl cyanoacetate) between the STE rank and GHS categories at two laboratories, re-test were performed by three laboratories. As the results, three laboratories obtained the similar cell viability and same STE rank with C01 as shown in Table 4.15.

On the other hand, the results of C02 (promethazine hydrochloride) and C25 (methyl cyanoacetate) were different classification among three laboratories as well as the results of validation study. We consider relatively few substances exhibited cell viability around 70% in the STE test. It is difficult with evaluating this type of test substances. In this case, it is necessary to compare with other test results according to a tiers testing approach.

5.2 Intra-and inter laboratory reproducibility

In the 1st phase validation study, only one test substance, X (methyl ethyl ketone) showed SD >15. In the 2nd phase validation study, four test substances in five experiments were over SD 15 as shown in Table 4.7. The ratio of invalid data by criterion 4 are 2.8% (6 experiments in total 221 with 65 test substances ,1st phase: 25 test substances X 5 lab+ re-test 1=126, 2nd phase: 40 test substances X 2-3 lab+ re-test 5= 95). From these results, we consider intra-reproducibility is high and its ratio is at 97.2%.

On the other hand, the STE rank with common two test substances (C07 and C37) in the 1st and 2nd phase validation study was similar in all laboratories as shown in Table 4.12. Furthermore, the correspondence of the STE results revealed that 4 of the 25 substances were different among laboratories in the 1st phase study. The four substances were test substances J (methyl amyl ketone), R (2-ethyl-1-hexanol), W (gluconolactone) and X (methyl ethyl ketone). Similarly, the correspondence of the STE results revealed that 3 of the 40 substances were different among laboratories in the 2nd phase study. The three substances were code C01 (distearyldimethylammonium chloride), C02 (promethazine hydrochloride) and C25 (methyl cyanoacetate). The total different results are seven test substances in 65, its ratio was 10.8%, that is, we consider inter-reproducibility is high and its ratio is at 89.2%.

5.3 Correspondence between the results from the test method to the comparative control (predictive ability)

The corresponding relationships between the GHS classification and the STE classification by accepted data at each laboratory, which participated both validation studies. The sensitivity values of each laboratory were among 73.9 -77.8 % and the sensitivity of each laboratory were among 73.9 -77.8 % and 86.7-93.8%. We consider the accuracy values of each laboratories were sufficient as one of screening assay among 77.4 -81.1 % (Table 4.13). On the other hand, the corresponding relationships between the STE rank and GHS category of each laboratories were

among 62.3 -66% (Table 14.4). We consider the accuracy values between the STE rank and GHS categories in each laboratory are not sufficient as a screening.

From these results, the STE test is recommended as an initial step within a Bottom-Up approach to identify substances that do not require classification for eye irritation (UN GHS No Category) as well as a step within a Top-Down approach to identify severe, moderate or mild irritants and substances that do not require classification for eye irritation (UN GHS No Category) from other toxicity classes, specially for limited types of substances. On the other hand, it is not considered adequately valid for the identification of mild or moderate irritants (ie.UN GHS Categories 2A and 2B) and severe irritants (UN GHS Category 1).

5.4 Substances judged to be false-positive or false-negative

Evaluation of the correspondence between the STE classification and the GHS classification revealed that 15 of the 63 test substances had false-negative results. The 15 substances were test substances E(ethanol),R(2-ethyl-1-hexanol) , S(acetone),X(methyl ethyl ketone), C33(ammonium nitrate), C12(camphen), C16(2,5-dimethyl-2,5-hexanediol), C01(distearyldimethylammonium chloride), C20(hexyl cinnamic aldehyde),C24(isopropyl alcohol), C34(methyl acetate), C35(myristyl alcohol),C25(methyl cyanoacetate), C29(potassium sorbate),and C36(sodium salicylate).

Test substance E (ethanol) and C24 (isopropyl alcohol), is thought to cause cytotoxicity by creating a wide hydrophobic surface in the solution that then affects the cell membrane (lipid layer). We speculate that at the STE sample concentration of 5%, where cell injury is not expressed, viability is high and yields false-negative results, the substance may be too dilute to cause an effect on cell membrane.

In the case of inorganic salts and organic salts (C33: ammonium nitrate, C29: potassium sorbate, C36: sodium salicylate), they are low molecule, easy to soluble and ionization in water and cause high osmotic pressure depend on its trivalent ion. Therefore, we assume low molecule organic acid induce damage to epithelium layer by high osmotic pressure. On the other hand, the STE test is no correlation of osmotic pressure because of treatment of test substances at 5% solution.

In the case of test substance S (acetone), because of its high volatility (vapor pressure: 24.7 kPa (20°C)), cells may have been exposed to a concentration lower than the test concentration of 5%. The reason we speculate is that evaporation can occur between the time of sample preparation and cell exposure, which leads to a lower test concentration than 5%. In addition, since log Pow—i.e., the partition coefficient of octanol/water of acetone—is low at –0.24, it appeared possible that the substance was dissolved in the solvent, physiological saline, and was not released. As a result, cells would not have been sufficiently exposed to the test substance. Since the viability in the evaluation at 5% was 9.6% (Takahashi et al., 2008) and the substance was evaluated as I when mineral oil was used in place of physiological saline, the two aforementioned possibilities require more detailed analysis.

Test substances R(2-ethyl-1-hexanol), X(methyl ethyl ketone), C01(distearyldimethylammonium chloride) and C25 (methyl cyanoacetate) were considered the viabilities in the STE test using a 5% solution were as follows: 63.6%, 74.6%, and 72.4% for test substance R in Lab 2; 79.1%, 78.7%, and 83.6% for test substance R in Lab 5; and 73.6%, 69.5%, and 69.9% for test substance X in Lab 1 in the 1st validation study and 65.5%, 81.7% and 62.9% for test substance C01 , 95.9%, 84.2% and 82.4% for test substance C25 (methyl cyanoacetate) in the 2nd validation study. Thus, the cell viability observed was near the cut-off value of 70%. It is possible that substances with a viability of around 70% may yield discrepancies in findings, depending on the laboratory. As a

result, those substances might have been judged differently in certain laboratories and thus generated false-negative results. The reasons of false negative on the other test substance are not clear.

On the other hand, false-positive results were obtained for test substance J (methyl amyl ketone) in one laboratory, Lab 3, and for test substance W (gluconolactone) in Lab 5 in the 1st phase validation study. In Lab 3, test substance J (methyl amyl ketone) was the first substance evaluated using mineral oil as a solvent. Since mineral oil has a higher viscosity than physiological saline, it is possible that cells had been physically scraped off in the process of performing suctioning and plate-washing, and that viability was consequently lower. The false-positive in the case of test substance J (methyl amyl ketone) might thus have been the result of manipulations such as suctioning and washing. The viabilities in test substance W (gluconolactone) in Lab 5 were 67.6%, 66.6%, and 63.1%, and it is possible that substances having a viability of around 70%—like test substance R (2-ethyl-1-hexanol) and test substance X (methyl ethyl ketone), both of which yielded false-negative findings—feature discrepancies in findings, depending on the laboratory. As a result of this, some laboratories may obtain false-positive findings.

Furthermore, in reviewing the correspondence of the STE rank and GHS categories, the discrepancy was by only one rank for all substances except for test substance J (methyl amyl ketone), which was different by two ranks (UN GHS No Category and rank 3 in STE test). The reasons of false positive on test substance C08 (ethyl acetate) and C30 (cyclohexanone) are not clear. For all other substances, correspondence of the STE rank with GHS categories was correct.

5.5 Limitations of the STE test

In the 1st and 2nd phase validation study, a total of 63 substances were evaluated. The results found that the STE test provided an excellent predictive ability. However, relatively few substances exhibited cell viability around 70% in the STE test. In addition, since scattering in the intermediate range (around 20–85%) of mean cell viability was relatively high, the interpretation of STE classification results must be performed carefully. Hence, many more substances with the cell viability near the 70% cut-off point need to be evaluated and added to the databank for future analysis.

In performing the present study, laboratories with experience in the use of SIRC cells were selected. In these laboratories, inter-laboratory reproducibility and predictive ability were excellent despite the short time given to the laboratories to learn the STE test method. On the basis of results of the present study alone, it is unclear whether these excellent results are related to experience with the use of SIRC cells or to the simplicity of the test method. Hence, this study cannot clearly determine whether similar results would be obtained by a laboratory with little experience in handling SIRC cells.

5.6 Proposal of revised criteria and protocol

In this phase validation study, a lot of invalid data were confirmed to not be satisfied with criteria1-3. Especially, the invalid data on positive control of lab2 and lab3 were total 40 data (14 plates), each 19 data (seven plates) and 21 data (seven plates) at Lab 2 and 3. Though these data are checked out the analysis of intra-and inter-laboratory and no effect of the results in this validation study, there are too much. The setting range of

positive control may be re-adjusting them such as not set up the range. If criterion of positive control should set up less than 70% of cell viability (this rate is irritant criterion in the prediction model), invalid data were only 9 data (three plates) out of criterion 3 at Lab 2. In the data given aid to this revised criteria, no data of not be satisfied with criterion 4 (SD>15) confirmed (data no shown).

The STE test requested to select the solvent by step by step in the protocol. After validation study, all solvents are checked in 1st phase and 2nd phase validation study. As a result, DMSO in saline selected one opportunity(C 31) in total 63 test substances. Refer to the previous data by Kao, they used mineral oil and we could obtain the corrective data without DMSO in saline. In future, DMSO in saline should be excluded as one of solvents to revise a simple protocol.

6. Conclusion

The STE test is recommended for use as part of a tiered testing strategy for regulatory classification and labeling. Thus, the STE test is recommended as an initial step within a Bottom-Up approach to identify substances that do not require classification for eye irritation (UN GHS No Category), as well as a step within a Top-Down approach to identify mild ,moderate or severe ocular irritants and substances that do not require classification for eye irritation (UN GHS No Category) from other toxicity classes, specially for limited types of substances.

Acknowledgments

The Validation Executive Committee was organized under the Japanese Society for Alternative to Animal Experiments (JSAAE), and it performed the present study. In performing the present study, the JSAAE, through its Validation Committee, provided both laboratory personnel and financial support. We are very grateful for the MHLW funding through JaCVAM activity

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OECD TEST GUIDELINES PROGRAMME

Standard Project Submission Form

If you require further information please contact the OECD Secretariat
Return completed forms to Laurence Musset and Camilla Francis

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PROJECT TITLE

New TG: Short Time Exposure (STE) Test for Identifying Ocular Irritants

SUBMITTED BY (Country / European Commission / Secretariat)

Japan

DATE OF SUBMISSION TO THE SECRETARIAT

February –th, 2011

DETAILS OF LEAD COUNTRY/CONSORTIUM

Country /Organisation:	Japan
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PROJECT OUTCOMES

☒ New Test Guideline	☐ Guidance document
☐ Revised Test Guideline	☐ Detailed Review Paper
☐ Deletion of an existing Test Guideline	☐ Other, please specify below

PROPOSED WORK PLAN and RESOURCE NEEDS:

1. Draft workplan for development of the proposal, including any need to establish Ad Hoc Expert Group and mode of meetings (face-to-face, teleconference; electronic discussion group). Indicate key milestones, including first and subsequent drafts of documents and timing of meetings.

In 2001, Kao Corporation developed the method of the Short Time Exposure (STE) test for Identifying ocular Irritants (attachment No.1, ref. 1 listed in attachment No.3).

During 2006-2009, inter-laboratory studies of the STE test were conducted collaboratively between two or three laboratories for evaluation of transferability and between-laboratory reproducibility of chemicals (ref. 2, 3).

From 2008 to 2009, domestic 1st validation study was conducted by the STE test validation executive committee organized by Validation Committee of the Japanese Society for Alternative to Animal Experiments (JSAAE). This validation study was completed by November 2009 successfully.

Then 2010, domestic 2nd (additional) validation study with more chemicals was conducted by the STE test validation management team (VMT) organized by JaCVAM. This 2nd validation study was completed by December 2010 successfully.

The work plan towards the adoption draft TG by WNT is as follows.

- By September 2011, an international Ad Hoc peer review panel will be established by NICEATM (National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods)/ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods and JaCVAM (Japanese Centre for the Validation of Alternative Methods). The panel will review the background review documents and draft TG on the STE test.
- By October 2011, a public face-to-face meeting of the panel would be held, and a peer review report prepared
- In December 2011, a revised draft TG and peer review report will be circulated to WNT for comments.
- In April 2012, the final draft TG and peer review report will be submitted to WNT for adoption.

2. Will additional information, including generation or collection of data, be required? If yes, please describe the anticipated process and timelines.

No. The collection of data, including the data of formal validation studies, was completed.

3. Indicate the estimated overall resource need (time/money) for member country / consortium and Secretariat

Resources for the preparation of documents and the peer review will be provided by Japan and NICEATM-ICCVAM. Other, member countries will be invited to also review and comment on the STE test.

4. Is this proposal intended to replace an existing Test Guideline or lead to the deletion of an existing Test Guideline?

- ◆ No. The STE test is intended to be used in combination with other *in vitro* methods, which can identify ocular corrosive and severe irritants (e.g., Bovine Corneal Opacity and Permeability (BCOP) Test Method for Identifying Ocular Corrosives and Severe Irritants (TG 437), or the Isolated Chicken Eye (ICE) for Identifying Ocular Corrosives and Severe Irritants (TG 438).

ESSENTIAL INFORMATION

In this section, please provide the information required by the Working Group of National Coordinators of the Test Guidelines Programme to assess the suitability of the project for the workplan of the Test Guidelines Programme

1. What is the existing or expected regulatory need/data requirement that will be met by the proposed outcome of the project? Please provide details below or as an attachment.

At this time, existing *in vitro* methods approved as test guidelines (e.g., BCOP [TG 437] and ICE [TG438]) can only identify ocular corrosive/severe irritants that correspond to GHS Category 1. There exists no test guideline assessable for mild or moderate ocular irritants that correspond to GHS Category 2.

The STE test is a cytotoxicity based method using SIRC cells (rabbit corneal cell line) that has been proposed for the identification of mild or moderate ocular irritants (attachment No.1, ref.1) and substances not labeled as irritants.

Therefore, using the STE test in combination with other *in vitro* methods that can identify ocular corrosive/severe irritants (e.g., BCOP, ICE) may allow the identification of ocular irritants over the entire potency range for chemicals.

or as attachment No.__