No.	Candidate chemical	CAS	IARC group	CPDB	EU GHS[$]	Genotoxicity Data[$$] In vivo Comet	Ames	in vitro CA	in vivo MN[$$]	Liver UDS	LD_{50}[$$$] rat, po (mg/kg)	Chemical profiles (class, mode of action, etc)	Note
37	Saccharin Na	128-44-9	3	+	NL	+	-	-	-		mouse po 17g	Heterocyclic cmpd, -ve in TG test and adduct, carcinogenicity to male rats only (bladder)	
● **Non-genotoxic non-carcinogens (9)**													
38	Ampicillin trihydrate	7177-48-2	3	-	NL		-	-	-		10g	Heterocyclic cmpd, beta-lactam antibiotics	
39	*o*-Anthranilic acid	118-92-3	3	-	NL		-	+	-		5410	Aromatic cmpd	
40	*t*-Butylhydroquinone	1948-33-0	NL	-	NL		-	+	-		700	Aromatic cmpd	
41	Ethionamide	536-33-4	3	+	NL		-	+			1320	Amide, carcinogenicity -ve in rats; possible thyroid tumours in mice (Kirkland et al., 2008), +ve only in female mice in CPDB, -ve in both species in NCI-CG-TR-46, liver toxicity	
42	Isobutyraldehyde	78-84-2	NL	-	NL		-	+	-		>2000	Aliphatic cmpd	
43	D-Mannitol	69-65-8	NL	-	NL		-	-	-		13500	Aliphatic cmpd	Non-selection due to use in phase 3 and phase 4 step 1
44	D,L-Menthol	15356-70-4	NL	-	NL	-	-	+	-		2900	Heterocyclic cmpd	
45	Sodium chloride	7647-14-5	NL	-	NL		-	-	-		3000	Inorganic metal cmpd, stomach toxicity	
46	Trisodium EDTA (monohydrate)	150-38-9 (10378-22-0)	NL	-	NL	-	-	-			2150	Aliphatic cmpd, chelating agent	
● **Positive Control (Genotoxic carcinogen)**													
47	Ethyl methanesulfonate (EMS)	62-50-0	2B	NL	NL	+	+	+	+		ip 350 mouse po 470	Sulfonate, DNA alkylation	Used in all studies

No.	Candidate chemical	CAS	IARC group	CPDB	EU GHS[$]	Genotoxicity Data[$$] In vivo Comet	Ames	in vitro CA	in vivo MN[$$]	Liver UDS	LD_{50}[$$$] rat, po (mg/kg)	Chemical profiles (class, mode of action, etc)	Note

*: Genotoxic was defeined as Ames +ve and/or standard in vivo assay +ve.
**: Erythrocytes (bone marrow or peripheral blood) MN assay with mouse or rat

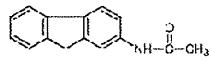
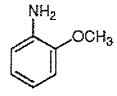
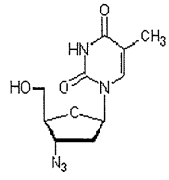
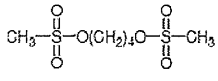
$: EU CLP Regulations (L353/1, 31.12.2008) for carcinogenicity and mutagenicity
$$: Data from Sasaki et al (2000), Sekihashi et al (2002), COM (2006), Kirkland et al (2008), IARC monographs, Morita et al (1997), Madle et al (1994), Kirkland and Speit (2008), and other sources
$$$: Data from RTECS (Registry of Toxic Effects of Chemical Substances)

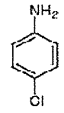
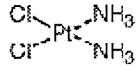
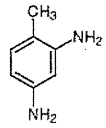
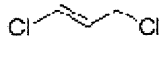
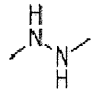
Abbreviations: CPDB, carcinogenic potency data base; CA, chromosomal aberraton; MN, micronucleus; UDS, unscheduled DNA synthesis; TG, rodent transgenic mutation model
M, mice; R, rats; ip, intraperitoneal injection; iv, intravenous injection; po, per os; NL, not listed; E, equivocal; Inc, inconclusive; W, Woko; T, Tokyo Kasei; S, Sigma-Aldrich;
GHS, grobally harmonized system of classification and labelling of chemicals; Carc, carcinogenicity; Muta, mutagenicity;
+, positive; -, negative

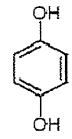
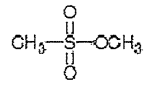
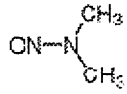
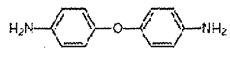
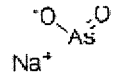
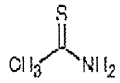
References: IARC, http://monographs.iarc.fr/ENG/Classification/index.php
CPDB, http://potency.berkeley.edu/
COM, MUT/06/03, Draft, 2006.
Kirkland et al, Mutat Res, 653, 99-108, 2008.
Kirland and Speit, Mutat Res, 654, 114-132, 2008.
Maddle et al, Mutat Res, 312, 263-285, 1994.
Morita et al, Mutat Res, 389, 1-122, 1997.
NCI-CG-TR-46, National Cancer Institute, Carcinogenesis, Technical Report Series, No. 46, 1978.
http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr046.pdf
Sasaki et al, Cri Rev in Toxicol, 30, 629-799, 2000.
Sekihashi et al, Mutat Mes, 517, 53-74, 2002.
RIVM, Muller and Bos, The occurrence of Carcinogenic, Mutagenic and Reprotoxic (CMR) substances in consumer preparations, RIVM report 32001001/2004.
http://rivm.openrepository.com/rivm/bitstream/10029/8883/1/320010001.pdf

Table 3 Summary of in vivo Genotoxicity Data on Selected Test Chemicals for International Validation Study for Comet Assay for Phase 4 Step 2 Studeis

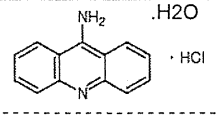
20120308

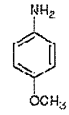
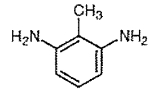
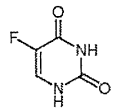
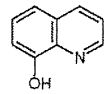
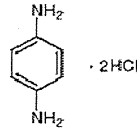
No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
	Genotoxic* carcinogens (19)								
1	2-Acetylaminofluorene [53-96-3]		UDS	+	Rat	po	5, 50		**1**, 2
			MN	+	Rat	po	125-500 x 2d	Positive also in mouce	3
	<IARC, Not listed; CPDB, +ve>		Comet	+	Mouse	po	600	Positive in C, L, K, Lu	**4**, 5
			Liver TG	+	BigBlue mouse	Diet	72 x 28d		**10**, 65
			In vitro Ames/CA	+/+					**12, 73**
2	Acrylonitrile [107-13-1]		UDS	-	Rat	po	75, 60 x 5d		**1**, 6
			MN	-	Mouse	ip	6-45	Negative in both BM and PB	7
	<IARC, 2B; CPDB, +ve>		MN	-	Mouse	po	4-32	Negative in BM	7
			MN	-	Mouse	iv	10-40	Negative in both BM and PB	7
			MN	-	Rat	po	10-40	Negative in BM	7
			MN	+	Rat	iv	24.5-98	Positive in PB	7
			MN	+	Rat	iv	31-125 x 2d	Positive in BM, but negative in PB	3
			Comet	+	Rat	ip	30	Positive in S, Co, K, Bl, Lu	8
			Liver TG	Not done	MutaMouse	drink. water	for 28d	Negative in BM, Br, Lu, Sp lymp, testis	**10**
			In vitro Ames/CA	+/+					**7, 73**
3	*o*-Anisidine [90-04-0] (o-Anisidine HCl [134-29-2])		UDS	-	Rat	po	50-1104		**1**, 9
			MN	-	Mouse	ip	400-800		7
	<IARC, 2B; CPDB, +ve>		Comet	+	Rat	po	1000	Positive in K, Bl, Lu	8
			Liver TG	-	BigBlue Mouse	po	750 x 3d	Positive in Bl	**10**, 11
			In vitro Ames/CA	+/+					**7, 73**
4	Azidothymidine [30516-87-1]		MN	+	Mouse	po	500-2000 x 3d, 200-2000 x 3d		28
			MN	+	Mouse	ip	17 x 10 d/2 wks		29
	<IARC, 2B; CPDB, +ve>		MN	+	Rat	po	500 x 7d	Negative with single iv dose	**62**
			In vitro Ames/CA	-/+					**12**
5	Benzene [71-43-2]		MN	+	Rat	po	500-2000	Positive also in mouse	3
			Comet	+	Rat	po	2000	Positive in S, C, L, K, Ub, Lu, Br	**4**, 8
	<IARC, 1; CPDB, +ve>		Liver TG	-	BigBlue mouse	inh	1350 ppm x 84d		**10**
			In vitro Ames/CA	-/+					**7, 73**
6	Busulfan (Myleran) [55-98-1]		MN	+	Mouse	ip	10-40		7
			Comet	+	Mouse	ip	40	Positive in C	**4**, 17
	<IARC, 1; CPDB, +ve>		In vitro Ames/CA	+/+					**7, 73**
7	Cadmium chloride [10108-64-2]		MN	+	Rat	po	15, 15 x 60d	as Cd dose?	30

No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
			MN	+	Mouse	ip	1.9-7.6		31
	<IARC, 1; CPDB, +ve>		MN	-	Mouse	Drink. water	300 ppm for 7 days		**24**, 25
			Comet	-	Mouse	ip	1		**4**
			In vitro Ames/CA	-/+					**7, 73**
8	*p*-Chloroaniline [106-47-8]		MN	+	Mouse	po	300 x 3d	Negative in a test at 180 mg/kg (ref. 15)	**12**, **13**, 14, **15**
			Comet	+	Mouse	po	200	Positive in S, C, L, Ub, Lu, Br	**4**, 16
	<IARC, 2B; CPDB, +ve>		In vitro Ames/CA	+/+					7, **12, 73**
9	Cisplatin [15663-27-1]		MN	+	Mouse	ip	0.03-10		7
			Comet	+	Mouse	ip	10	Positive in C, Lu, Br	**4**, 17
	<IARC, 2A; CPDB, Not listed>		Liver TG	+	LacZ mouse	ip	6		**10**, 66
			In vitro Ames/CA	+/+					7, **12, 73**
10	2,4-Diaminotoluene [95-80-7]		UDS	+	Rat	po	150 (+ve, Ref. 2); 300 (E or weak, Ref. 18)		**1**, 2, 18
			MN	-	Mouse	ip	30-240		7
	<IARC, 2B; CPDB, +ve>		MN	+	Rat (PVG)	po	150-300	Negative in F344 rats at 50-150 mg/kg	7, 18
			Comet	+	Rat	po	130	Positive in S, C, K, Br	8
			Liver Comet	-	Rat	po	25-100 x 29d	MN negative in BM	63
			Liver TG	+	BigBlue Mouse	po	66 x 12d		**10**, 19
			In vitro Ames/CA	+/+					7, **12, 73**
11	1,2-Dibromoethane [106-93-4]		UDS	+w	Rat	po	10-100		**1**, 20
			UDS	+	Rat	ip	100		**1**, 20
	<IARC, 2A; CPDB, +ve>		MN	-	Mouse	ip	25-150; 80-100 x 3d		7
			Comet	+	Mouse	ip	100	Positive in S, C, L, K, Ub, Lu	**4**, 21
			Liver TG	-	MutaMouse	ip	60, 16 x 5d		**10**, 67
			In vitro Ames/CA	+/+					**7, 73**
12	1,3-Dichloropropene [542-75-6]		UDS	-	Rat	po	125		**1**, 22
			MN	-	Mouse	ip	18.9-150		7
	<IARC, 2B; CPDB, +ve>		MN	-	Rat	po	125	Negative in BM, L, Spleen	76
			MN	+	Mouse (female)	po	187, 234	Negative in males at 140 and 180 mg/kg	77
			Comet	+	Mouse	ip	150	Positive in S, C, L, K, Ub, Lu, Br, BM	**4**, 21
			In vitro Ames/CA	+/-					**7, 73**
13	1,2-Dimethylhydrazine 2HCl [306-37-6] (1,2-Dimethylhydrazine [540-73-8])	 2HCl	UDS	+	Rat	po	20	as free base	**1**, 2
			MN	+	Rat	po	200 x 2d; 25-100 x 2d		3, 23
			MN	+	Mouse	ip	2.5-10 x 2d; 2.5-10 x 4d		7

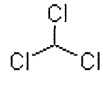
No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
	<IARC, 2A; CPDB, +ve>		Comet	+	Rat	po	100	Positive in S, C, L, K, Ub, Lu, Br, BM	**4**, 8
			Liver Comet	+	Rat	po	12.5-50, 1.25-5 x 29d	MN negative in BM & PB by 29d	63
			In vitro Ames/CA	+/+					7
14	Hydroquinone [123-31-9]		MN	+	Mouse	ip	30-100		**12**, 32
	<IARC, 3; CPDB, +ve>		MN	+	Mouse	po	80	Weaker than ip	**33**, 34
			In vitro Ames/CA	-/+					**12, 73**
15	Methyl methanesulfonate [66-27-3]		UDS	+	Rat	po	20-100		**1**, 2
			MN	+	Rat	po	36-144 x 2d		3
	<IARC, 2A; CPDB, +ve>		Comet	+	Rat	ip	80	Positive in S, C, L, K, Ub, Lu, Br, BM	**4**, 8
			Liver TG	+	Mouse	ip	100		**10**
			In vitro Ames/CA	+/+					7, **12**
16	*N*-Nitrosodimethylamine [62-75-9]		UDS	+	Rat	po	10		**1**, 2
			MN	+	Mouse	po	25		**24**, 25
	<IARC,2A; CPDB, +ve>		Comet	+	Mouse	ip	6.25-50	Positive in S, C, L, K, Ub, Lu, Br, BM	**4**, 17
			Liver Comet	+	Rat	po	0.5-4 x 15d	MN negative in PB	63
			Liver TG	+	Mouse, Rat	po	Various doses and duration		**10**
			In vitro Ames/CA	+/+					7, **12, 73**
17	4,4'-Oxydianiline [101-80-4]		UDS	-	Rat	po	40-725		**1**, 26
			MN	+	Mouse	ip	37.5-150 x 3d		27
	<IARC, 2B; CPDB, +ve>		Comet	+	Mouse	po	500	Positive in S, L, K, Ub, Lu, Br	**4**, 16
			In vitro Ames/CA	+/+					**73**
18	Sodium arsenite [7784-46-5]		MN	+	Mouse	ip	5-10	Positive in water, but negative in corn oil as vehichle	**12**, 35
	<IARC, 1; CPDB, -ve>		In vitro Ames/CA	-/+					7, **12**
19	Thioacetamide [62-55-5]		MN	+	Mouse	po	50 -200		**24**, 57
			MN	+	Mouse	po	375-1500		68
	<IARC, 2B; CPDB, +ve>		Comet	+	Mouse	ip	200	Positive in S, C, Ub	**4**
			In vitro Ames/CA	-/-					7

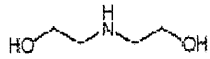
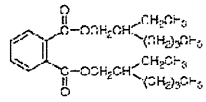
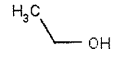
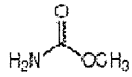
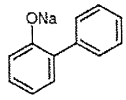
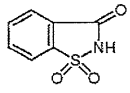
Genotoxic non-carcinogens (6)

20	9-Aminoacridine hydrochloride monohydrate [52417-22-8]		No in vivo data	

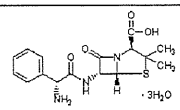
No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
	(9-Aminoacridine [90-45-9], 9-Aminoacridine HCl [134-50-9]) <IARC, Not listed; CPDB, Not listed>		In vitro Ames/CA	+/+					**73**
21	*p*-Anisidine [104-94-9] (*p*-Anisidine HCl [20265-97-8])		No in vivo data						
	<IARC, 3; CPDB, -ve>		In vitro Ames/CA	+/+					**73**
22	2,6-Diaminotoluene [823-40-5] (2,6-Diaminotoluene 2HCl [15481-70-6])		UDS	-	Rat	po	150; 150, 300		**1**, 2, 18
			UDS	+	Rat	po	1000, 1000 x 2d		**1**, 36
			MN	+	Mouse	ip	15.6-62.5 x 3d	as 2HCl salt	27
	<IARC, Not listed; CPDB, -ve>		MN	+w	Rat	po	300, 600	=< x2 from negative control	18
			MN	-	gpt Delta Rat	Diet	500 ppm (13 wks)		37
			Comet	-	Rat	po	250		**4**, 8
			Comet	+w	Rat	po	125-500 x 3d, 15-60 29d	MN negative in BM by 3 & 29d, positive in PB by 29d	63
			Liver TG	-	BigBlue Mouse	Diet	120 x 30&90d		**10**, 64
			In vitro Ames/CA	+/+					**73**
23	5-Fluorouracil [51-21-8]		MN	+	Rat (male)	ip	20-80		3
			MN	+	Rat	po	20, 40	4 wks old, Positive in PB, but negative in liver	38
	<IARC, 3; CPDB, +ve>		Comet	-	Mouse	ip	100		**4**, 39
			In vitro Ames/CA	-/+					**73/74**
24	8-Hydroxyquinoline [148-24-3]		UDS	-	Rat	po	100-500; 600, 600 x 2d		**1**, 36, 40
			MN	-	Mouse	ip	10.8-43 x 3d		27
	<IARC, 3; CPDB,-ve>		Comet	-	Mouse	po	600		**4**
			In vitro Ames/CA	+/+					**73**
25	*p*-Phenylenediamine 2HCl [624-18-0] (*p*-Phenylenediamine [106-50-3])		MN	-	Mouse	ip	20-100		41
			MN	-	Rat	po	300 x2d		**78**
			Comet	-	Rat	po	75		**4**, 8
	<IARC, 3; CPDB, -ve>		In vitro Ames/CA	+/+					**73**

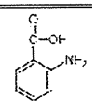
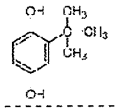
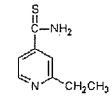
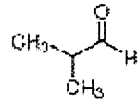
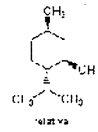
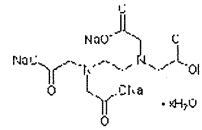
Non-genotoxic carcinogens (7)

26	Chloroform [67-66-3]		UDS	-	Rat	po	40, 400		**1**, 2
			MN	-	Mouse	ip	238-952 x 2d		**42, 43**

No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
			MN	+	Rat	po	480	in kidney cells, addition of folic acid (iv route) for increasing proliferation	**42**, 44
	<IARC, 2B; CPDB, +ve>		Comet	-	Mouse	po	400		**4**
			Liver TG	-	BigBlue mouse	Inh	154 x 10-180d		**10**
			In vitro Ames/CA	-/-					7, **73**
27	Diethanolamine [111-42-2]		MN	-	Mouse	skin	80-1250 x 90d	MNNCE in blood	**12**, 58, 69
	<IARC, 2B; CPDB, Not listed>		In vitro Ames/CA	-/-					**73**
28	Di(2-ethylhexyl)phthalate [117-81-7]		UDS	-	Rat	po	500		**1**, 6
			MN	-	Mouse	ip	500-2000 x 2d		7
	<IARC, 2B; CPDB, +ve>		Comet	-	Mouse	po	2000		**4**
			TG	-	BigBlue mouse	Diet	360-720 x 120d		**10**, 45
			In vitro Ames/CA	-/-					**12, 73**
29	Ethanol [64-17-5]		MN	-	Mouse	Drink. Water	10 and 20% for 3 or 7 wks		**46**, 71
	<IARC, 1; CPDB, +ve>		In vitro Ames/CA	-/-					**75**
30	Methyl carbamate [598-55-0]		MN	-	Mouse	ip	500-2000, 2000-3000		**12**, 47
	<IARC, 3; CPDB, +ve>		In vitro Ames/CA	-/-					**73**
	o-Phenylphenol sodium salt [132-27-4] (*o*-Phenylphenol [90-43-7])		CA	-	Rat	Diet	0-2.0%		**48, 49**, 50
			CA	-	Mouse	po	250-4000; 50-800 x 5d		**48**
			MN	-	Rat	Diet	0-12500 ppm		52
	<IARC, 2B; CPDB, +ve; for sodium salt>		MN	+	Rat	Diet	80-12500 ppm	in urinaly bladder, as free base	**48**, 53
	<IARC, 3; CPDB, +ve; for free base>		Comet	+	Rat	po	2000	Positive in S, C, L, K, Ub, Lu, Br	**4**, 8
			Comet	-	Rat	po	2000		**49**, 54
			Comet	+	Mouse	po	100-2000	Positive in C, S, Ub, Lu, L, K	55
			In vitro Ames/CA	-/+					**42**
32	Saccharin [81-07-2]		CA	-	Mouse	?	4000	as Na salt	**12, 56,** 70
	(Saccharin sodium [128-44-9])		Comet	+	Mouse	po	100-2000	Positive in S, C as Na salt	**49**, 55
			Liver TG	-	BigBlue rat	Diet	Dose not specified (X 10d)	as Na salt	**10**
	<IARC, 3; CPDB, +ve for sodium salt, -ve for free		In vitro Ames/CA	-/-					**12**

Non-genotoxic, non-carcinogens (8)

33	Ampicillin trihydrate [7177-48-2] (Ampicillin [69-53-4])		MN	-	Rat	po	3000, 5000		**12**, 59
	<IARC, 3; CPDB, -ve>		In vitro Ames/CA	-/-					**12, 73**

No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
34	*o*-Anthranilic acid [118-92-3]		MN	-	Mouse	ip	75-300, 150-600		**12**, 60
	<IARC, 3; CPDB, -ve>		In vitro Ames/CA	-/+					**12, 73**
35	*t*-Butylhydroquinone [1948-33-0]		MN	-	Mouse	ip	9-400 x 3d		**12**, 58
	<IARC, Not listed; CPDB, -ve>		In vitro Ames/CA	-/+					**12, 73**
36	Ethionamide [536-33-4]		No in vivo data						
	<IARC, 3; CPDB, +ve; NCI, -ve>		In vitro Ames/CA	-/+					**12, 73**
37	Isobutyraldehyde [78-84-2]		MN	-	Mouse	ip	39-1250 x 3d, 156-625 x 3d		**12**, 58
	<IARC, Not listed; CPDB, -ve>		MN	-	Rat	ip	313-1250 x 3d		**12**, 58
			In vitro Ames/CA	-/+					**12, 73**
38	D,L-Menthol [15356-70-4]		MN	-	Mouse	ip	250-1000 x 3d		27
			Comet	-	Mouse	po	2000		**4**
	<IARC, Not listed; CPDB, -ve>		In vitro Ames/CA	-/+					**12, 73**
39	Sodium chloride [7647-14-5]	NaCl	UDS	-	Rat	po	1000	Negative in stomach	61
			MN	-	Mouse	ip	2000		56
	<IARC, Not listed; CPDB, -ve>		In vitro Ames/CA	-/-					**73**
40	Trisodium EDTA monohydrate [10378-22-0]		MN	-	Mouse	po	500-2000	as disodium salt	**72**
	(EDTA [60-00-4], Trisodium EDTA trihaydarate [150-38-9],		MN	-	Mouse	ip	186	as disodium salt	**72**
	Disodium EDTA dihydrate [6381-92-6])		MN	+	Mouse	ip	5-20	as disodium salt	**72**
			Comet	-	Mouse	po	6月22日		**4**
	<IARC, Not listed; CPDB, -ve>		In vitro Ames/CA	-/-					**12, 73**

*: Genotoxic compounds are definede as chemicals which are positive in the Ames test or standered in vivo genotoxicity test.

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No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.

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Table 4 バリデーション研究における被験物質選択のための一般原則

	検討項目	目的	概説
1	当該バリデーション研究の目的	最小被験物質数の導出	施設内・施設間再現性の他に、当該バリデーション研究の目的に応じた必要とされる最小限の被験物質数を導出する。
2	バリデーション研究対象試験の毒性分野や種類	候補物質の収集	試験系の類似性(in vitro, in vivo, 動物種など)を考慮し、当該毒性分野あるいは関連分野にて検討され、その知見を有する物質について、以下の絞込みに対応できるよう十分な数の候補物質を収集する。
3	化学物質クラス	科学的絞込み	対象試験の種類にもよるが、特定の化学物質クラスに偏らないように広範囲なクラスから選択し、結果に普遍性をもたせる。
4	化学物質構造	科学的絞込み	必要に応じ、類似の化学構造を有する物質を増減させる。
5	作用機序・作用様式	科学的絞込み	対象試験における毒性発現の種々の作用機序あるいは作用様式をカバーする。
6	物性	科学的絞込み	対象試験に応じ、性状(気体、液体、固体)、蒸気圧(揮発性物質によるin vitro試験系への影響)、溶解性(試験系や溶媒/媒体の選択に影響)、色(測定系への影響)などを考慮し、試験実施に適切な物質を選択する。
7	既存情報の充実性	科学的絞込み	関連する毒性、代謝、安定性などに関する情報が豊富なものを選択する。
8	(急性)毒性値	実務的絞込み	被験物質必要量の算出に利用。特にin vivo試験では、低毒性物質では必要量が多くなるため、メーカーやロットの同一性の確保が困難となりやすい。
9	入手可能性	実務的絞込み	当該物質が市販されているか、適切な純度のものはあるか、同一ロットによる必要量があるか、など入手の可否を検討する。
10	取扱容易性	実務的絞込み	当該物質の包装形態、容量、保管要件(室温、冷蔵など)、各国規制(輸出入/輸送制限など)等を検討し、ブラインド化の容易性や国内外への搬送可能性を検討する。
11	価格	実務的絞込み	当該バリデーション研究の予算内で対応可能なものを選択する。

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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小島 肇夫	序章、動物実験代替法と動物実験の住み分け 第1章第2節　日本における各種承認申請に必要な安全性試験と代替法の受理の現状 第1章第3節　REACH.GHSなどの各種規制との違い 第2章　皮膚腐食性試験の実験手法 第4章　眼刺激性試験代替法の実験手法	小島 肇夫	最新　動物実験代替法の技法ノウハウ	技術情報協会	東京	2011	3-9 19-23 24-29 33-43 71-87

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Satoshi Kano, Hiroaki Todo, Katsunori Furui, Kenichi Sugie, Yoshihiro Tokudome, Fumie Hashimoto, Hajime Kojima, Kenji Sugibayashi	Comparison of Several Reconstructed Cultured Human Skin Models by Microscopic Observation : Their Usefulness as an Alternative Membrane for Skin in Drug Permeation Experiments	AATEX	16(2)	51-8	2011
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小島 肇夫	技術講座　安全性評価試験(1)―安全性評価の考え方―	ｺｽﾒﾃｯｸｼﾞｬﾊﾟﾝ	Vol.1(6)	10-3	2011
小島 肇夫	技術講座　安全性評価試験(2)―安全性評価試験法―	ｺｽﾒﾃｯｸｼﾞｬﾊﾟﾝ	Vol.1(7)	18-22	2011
小島 肇夫	技術講座　安全性評価試験(3)―バリデーション―	ｺｽﾒﾃｯｸｼﾞｬﾊﾟﾝ	Vol.2(1)	73-7	2012
小島 肇夫	技術講座　安全性評価試験(4)―バリデーションセンター―	ｺｽﾒﾃｯｸｼﾞｬﾊﾟﾝ	Vol.2(2)	65-9	2012
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Validation Study of the *In Vitro* Skin Irritation Test with the LabCyte EPI-MODEL24

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Summary — A validation study on an *in vitro* skin irritation assay study was performed with the reconstructed human epidermis (RhE) LabCyte EPI-MODEL24, developed by Japan Tissue Engineering Co. Ltd (Gamagori, Japan). The protocol that was followed in the current study was an optimised version of the EpiSkin protocol (LabCyte assay). According to the United Nations Globally Harmonised System (UN GHS) of classification for assessing the skin irritation potential of a chemical, 12 irritants and 13 non-irritants were validated by a minimum of six laboratories from the Japanese Society for Alternatives to Animal Experiments (JSAAE) skin irritation validation study management team (VMT). The 25 chemicals were listed in the European Centre for the Validation of Alternative Methods (ECVAM) performance standards. The reconstructed tissues were exposed to the chemicals for 15 minutes and incubated for 42 hours in fresh culture medium. Subsequently, the level of interleukin-1 alpha (IL-1α) present in the conditioned medium was measured, and tissue viability was assessed by using the MTT assay. The results of the MTT assay obtained with the LabCyte EPI-MODEL24 (LabCyte MTT assay) demonstrated high within-laboratory and between-laboratory reproducibility, as well as high accuracy for use as a stand-alone assay to distinguish skin irritants from non-irritants. In addition, the IL-1α release measurements in the LabCyte assay were clearly unnecessary for the success of this model in the classification of chemicals for skin irritation potential.

Key words: in vitro, *interleukin-1 alpha (IL-1α), MTT, reconstructed human epidermis, skin irritation, validation.*

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Introduction

Since 1946, the Draize rabbit test for skin irritation has been widely used to evaluate the skin irritation potential of xenobiotics (1, 2). However, the relevance to humans of the data provided by the test is limited by species differences, so a significant number of alternative testing methods have been developed to date, including the use of *in vitro* tissue constructs based on human keratinocytes (3, 4). These constructs closely resemble human epidermis with respect to biochemical profile (e.g. lipid composition), tissue architecture (e.g. cell layering and formation of a stratum corneum), and the presence of a functional skin barrier.

Three commercially available test methods based on reconstructed human epidermis (RhE) have been validated by the European Centre for the Validation of Alternative Methods (ECVAM; 5–7) as being suitable for determining the potential hazardous (i.e. skin irritant) properties of xenobiotics. These methods are also in compliance with the new United Nations Globally Harmonised System (UN GHS) rules for the classification and labelling of substances, implemented in the EU through regulations on the Classification, Labelling and Packaging of Substances and Mixtures. In December 2008, the EU adopted a new classification system based on the UN GHS system for Classification and Labelling (8), but which continues to use two categories to distinguish non-irritant (No Category) chemicals from irritant (Category 2) chemicals. According to the new UN GHS rules for the classification and labelling of skin irritation, the cut-off *in vivo* score to distinguish between No Category and Category

2 chemicals has changed from a value of 2.0 to 2.3. Consequently, chemicals with an *in vivo* score of between 2.0 and 2.3 had been considered irritants under the existing EU classification system, but are now classified as non-irritants under the new UN GHS system, which does not use the optional GHS Category 3.

The three *in vitro* test methods validated by ECVAM are based on identical tissue engineering technology, and, essentially, the same test protocol was followed through several validation studies (9–17), as is evident from their associated Standard Operating Procedures (SOPs). For these reasons, they are suitable for the development of a general test method procedure, which will include minimal performance criteria for similar and modified methods (6, 7, 18, 19). The EU system for the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH; 20), the EU Cosmetics Directive (21), and other legislative requirements worldwide, are a clear indication of the need for an internationally-harmonised and consistent test procedure for *in vitro* skin irritation testing based on RhE, similar to test guidelines promoted by the Organisation for the Economic Co-operation and Development (OECD; 22).

A RhE model, the LabCyte EPI-MODEL24, was developed by Japan Tissue Engineering Co. Ltd. (J-TEC; Gamagori, Aichi, Japan), and this was the skin model used in an *in vitro* skin irritation assay that has undergone protocol optimisation based on the EpiSkin protocol (23). According to this protocol, tissues are exposed to the test chemicals for 15 minutes, and are then incubated for 42 hours in fresh culture medium without the test chemicals. After this period, the amount of interleukin-1 alpha (IL-1α) released into the conditioned medium is measured, as is tissue viability (via the MTT assay). This system is referred to as the 'LabCyte assay' throughout this paper.

A multi-laboratory assessment of the LabCyte assay was performed under the direction of the Japanese Society for Alternatives to Animal Experiments (JSAAE) and the Japanese Centre for the Validation of Alternative Methods (JaCVAM), and was based on the ECVAM performance standards for *in vitro* skin irritation test methods based on reconstructed human epidermis (18, 19). The present paper reports the results of a three-phase validation study, which was performed by a minimum of six laboratories. The test substances were chosen in accordance with the ECVAM performance standards, and also from the revised list described in the new ECVAM Scientific Advisory Committee (ESAC) statement from 2009 (19). The objective of this investigation was to conduct a series of validation studies to assess the reliability (within-laboratory and between-laboratory reproducibility) and relevance (predictive capacity) of this assay, by using a challenge set of 25 coded test chemicals (12 irritants and 13 non-irritants) for which high-quality *in vivo* data were available. The validation study was undertaken in accordance with the principles and criteria documented in the *OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* (24), and according to the modular approach to validation described by Hartung *et al.* (25).

Materials and Methods

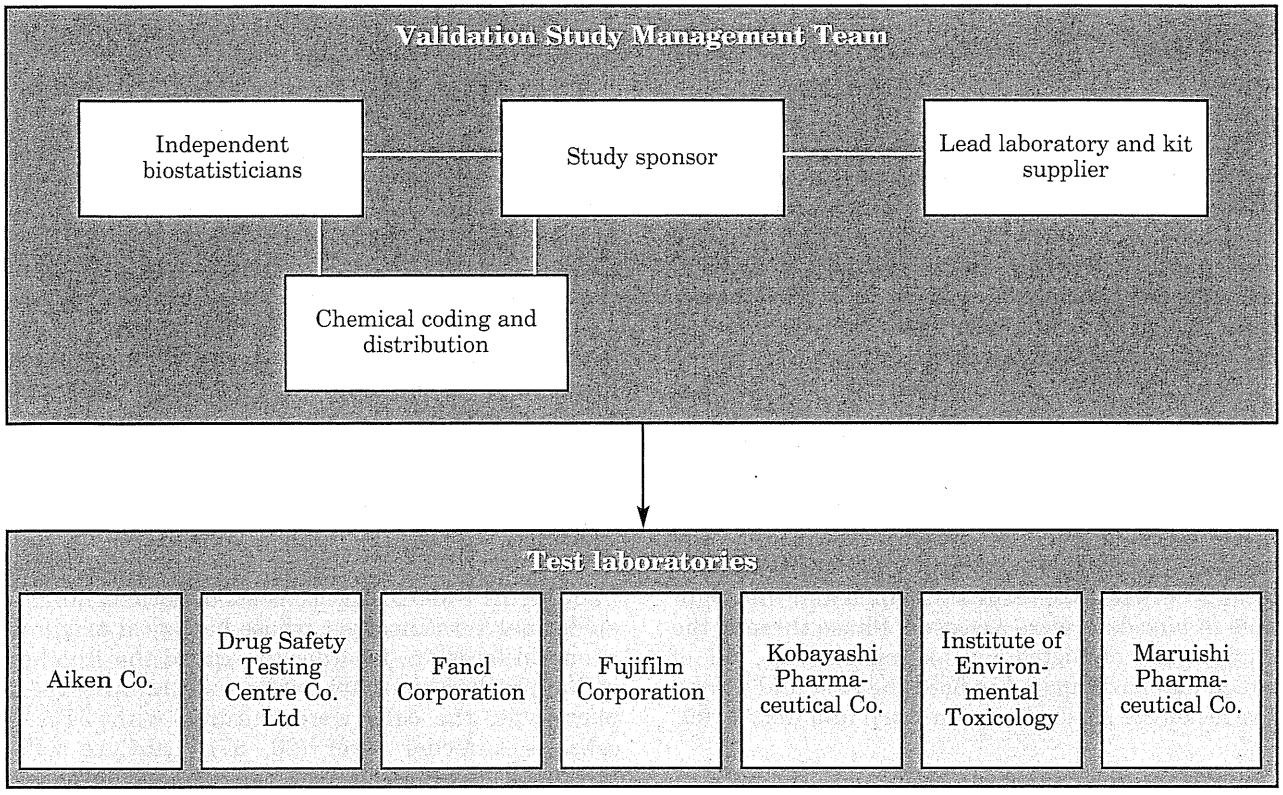
Validation study management structure

The skin irritation validation management team (VMT) for the LabCyte assay was organised by the JSAAE. The management structure and members of the study are shown in Figure 1 and Table 1. The VMT played a central role in overseeing the conduct of the validation study, and was responsible for: selecting test chemicals, producing goal statements, planning the project (including the

Table 1: Details of the JSAAE skin irritation validation study management team

No.	Role	Name	Affiliation
1	Chair, chemical coding and distribution	Hajime Kojima	JaCVAM and NIHS
2	Protocol check	Kenji Idehara	Daicel Corporation
3	Protocol check	Isao Yoshimura	Tokyo University of Science
4	Lead laboratory and kit supplier	Masakazu Kato	J-TEC
5	Independent biostatistician and study sponsor	Takashi Omori	Kyoto University (present post: Doshisya University, Japan)
6	Independent biostatistician	Etsuyoshi Miyaoka	Tokyo University of Science
7	Independent biostatistician	Kenya Ishiyama	Tokyo University of Science

Figure 1: Structure of the JSAAE skin irritation validation study management team



study protocol and amendments), the outcome of quality control (QC) audits, data management procedures, timeline and study progression, study interpretation, conclusions, and the publication of reports. In addition, the VMT was responsible for making the final decision on which laboratories were to participate in the validation study. The chemical selection group, JaCVAM, was in charge of defining the test chemical selection criteria and selecting the test chemicals, liaising with suppliers, performing final checks on the chemicals provided, coding the test substances, and distributing them to the different laboratories. The independent biostatistics group was responsible for the collection, screening and analysis of the data, and for preparing spreadsheets.

The following seven laboratories (and study directors) participated in the first and second phases of the validation study for the evaluation of the LabCyte assay:

— Laboratory A: Aiken Co. Ltd (Yoko Ando and Yui Asako)

— Laboratory B: Kobayashi Pharmaceutical Co. Ltd (Yoshihiro Yamaguchi and Maki Nakamura)

— Laboratory C: The Institute of Environmental Toxicology (Tadashi Kosaka and Koichi Hayashi)

— Laboratory D: Fancl Corp. (Tamie Suzuki and Runa Izumi)

— Laboratory E: Fujifilm Corporation (Atsuko Yuasa and Shinichi Akimoto)

— Laboratory F: Maruishi Pharmaceutical Co. Ltd (Yukihiko Watanabe and Osamu Mitani)

— Laboratory G: Drug Safety Testing Center Co. Ltd (Shinsuke Shinoda and Saori Hagiwara)

Six of the laboratories also participated in the third phase. Only Laboratory E did not participate in all three phases of the study. J-TEC, the lead laboratory in the VMT, did not participate in the practical application of the protocol in the validation study.

Study design

Before this validation study on the operation of the LabCyte EPI-MODEL24 was carried out, a one-day training course was held by J-TEC, in April 2008. All of the technicians from each laboratory participated in this training course.

The validation study was conducted in three stages, as follows. In the first phase of the study, the proposed test protocol was confirmed and its transferability was assessed by testing three coded chemicals (ethanol, glycerol and naphthalene acetic acid) and a positive control (5% w/v sodium lauryl sulphate [SLS]) in seven laboratories, between June and July 2008.

During the second phase, the VMT confirmed within-laboratory and between-laboratory reproducibility, as well as the correlation between the results obtained and the identities of the 20 coded chemicals, 19 of which are described in the original ECVAM performance standards (18). These tests were conducted by seven laboratories, between September 2008 and January 2009.

Since the statement regarding the revised ECVAM performance standards (7, 19) became available after the second phase of the study had finished, the VMT decided to conduct a third phase. During this last phase, six additional chemicals were tested for within-laboratory and between-laboratory reproducibility, and the correlations between the results obtained and the available *in vivo* data were assessed. Phase three of the study was conducted by six laboratories, all of which had participated in both the first and second phases of the study, between April and May 2009.

Test chemicals

Throughout all phases of the study, the negative control consisted of distilled water and the positive control was 5% w/v SLS (Wako Pure Chemical Industries Ltd, Osaka, Japan).

In the first phase, the VMT selected and JaCVAM distributed the three coded chemicals (ethanol, glycerol and naphthalene acetic acid; Wako Pure Chemical Industries Ltd) to each of the laboratories taking part in the study (see Table 2).

In the second phase, the VMT selected 19 of 20 chemicals for testing, according to the reference list provided in the original ECVAM performance standards (18). Their chemical names, CAS numbers, GHS labels and *in vivo* scores are listed in Table 2. One of the chemicals in the original ECVAM performance standards reference list, tri-isobutyl phosphate, was not available for purchase in Japan. Ultimately, the VMT approved the use of a 5% w/v SLS solution — the same chemical that JaCVAM had proposed for use as the positive control — to replace tri-isobutyl phosphate as chemical No. 13. To avoid any potential bias in the final selection, the VMT did not inform the laboratory representatives about these discussions.

In the third phase of the study, the VMT selected six new chemicals to be tested, according to the reference list in the revised ECVAM performance standards (19). Their chemical names, CAS numbers, GHS labels and *in vivo* scores are listed in Table 2. JaCVAM suggested the final list of chemicals, which was then approved by the VMT. As before, in order to avoid any bias in the final selection, the VMT did not inform the laboratory representatives of these discussions.

As shown in Table 2, the chemicals were purchased from Wako Pure Chemical Industries Ltd, Kanto Chemical Co. Inc. (Tokyo, Japan), Sigma-Aldrich Corporation (St Louis, MO, USA), Sigma-Fluka (St Louis, MO, USA), Alfa Aesar (Haverhill, MA, USA) and Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). JaCVAM contracted an independent laboratory to code and distribute the chemicals, and a chemical manager (or safety officer) carried out these tasks. The Material Safety Data Sheet for each chemical was also distributed with the chemicals.

Reconstructed human cultured epidermal model

LabCyte EPI-MODEL24 consists of normal human epidermal keratinocytes, whose biological origin is neonatal foreskin. In order to expand the number of human keratinocytes while maintaining their phenotype, the cells were cultured with 3T3-J2 cells as a feeder layer (26, 27). LabCyte EPI-MODEL24 involves growing keratinocytes on an inert substrate — a tissue culture filter with a surface of 0.3cm^2 — at the air–liquid interface for 13 days in an optimised medium. The composition of the optimised medium for the culture of LabCyte EPI-MODEL24 is based on Dulbecco's modified Eagle's medium and Ham's F12 medium (in a 3:1 ratio), with epidermal growth factor, insulin, hydrocortisone, other proprietary stimulators of epidermal differentiation, antibiotics, and 5% v/v fetal bovine serum (FBS). Although the FBS was purchased from several different suppliers, after reviewing safety data and performance, FBS from Japan Bio Serum (Fukuyama, Japan) was used.

Ultimately, this tissue model results in a multilayer structure consisting of a fully-differentiated epithelium that has features of the normal human epidermis, including a stratum corneum. For dispatch, the LabCyte EPI-MODEL24 samples are embedded in an agarose gel containing appropriate nutrients, and shipped in 24-well plates at around 18°C (13).

J-TEC audited the batch release criteria for LabCyte EPI-MODEL24, in order to ensure compliance with the principles of Good Laboratory Practice (GLP), and to guarantee that only certified tissues were used for the prediction and classification of irritants (28). For this purpose, in order to demonstrate the barrier function of the reconstructed epithelium layer, the optical density (at 570nm and at 650nm as reference absorbance)