In vitro Micronucleus Assay Results

TK6 cells after 4h Exposure to Mitomycin C, without Metabolic Activation (-S9)

Group No.	MMC Treatment (μg/mL)	Analyzed Cell No.	Number of Micronucleated cell (% Incidence)
1	Saline	1000	8 (0.8)
2	0.125	1000	59 (5.9)
3	0.25	1000	121 (12.1)
4	0.5	1000	223 (22.3)
5	1	TOX	- (-)
6	2	TOX	- (-)
7	4	TOX	- (-)

TOX: Not scored due to cytotoxicity

Micronucleus assay was performed on Day 2.

3. 2-Aminoanthracene (2AA)

Cytotoxity Assays Results

TK6 cells after 4h Exposure to 2-Aminoanthracene, with Metabolic Activation (+S9)

Group No.	2AA Treatment (μg/mL)	Daily Cell Growth[A] Measured by COULTER COUNTER			
		Day 1	Day 2	Day 1 × Day 2	Relative Suspension Growth (%)
1	DMSO	2.74	3.89	10.64	100.0
2	0.0625	2.90	4.01	11.62	109.3
3	0.125	2.71	3.96	10.73	100.8
4	0.25	2.85	4.05	11.53	108.4
5	0.5	2.69	4.05	10.90	102.4
6	1	2.70	3.59	9.69	91.0
7	2	1.49	3.07	4.58	43.0

[A] Cell conc. on the day / Cell conc. on the day before

Day 1: After 24h from end of treatment

Day 2: After 48h from end of treatment

Group No.	2AA Treatment (μg/mL)	% Viabilty[B] Measured by Dye Exclusion Assay		
		Day 0	Day 1	Day 2
1	DMSO	95.8	97.5	97.3
2	0.0625	100.0	97.6	98.0
3	0.125	100.0	99.1	100.0
4	0.25	88.9	98.8	99.0
5	0.5	97.6	98.8	98.9
6	1	97.4	97.4	98.8
7	2	96.0	88.9	92.3

[B] Number of unstained cells / number of total cells) x 100

Day 0: Just after treatment

Day 1: After 24h from end of treatment

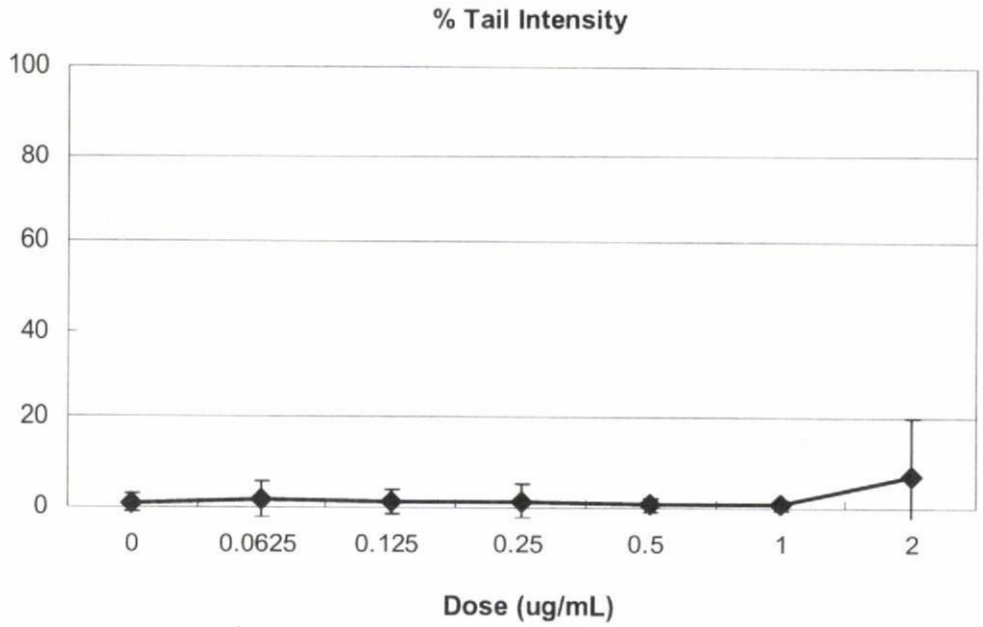
Day 2: After 48h from end of treatment

Comet Assay Results

TK6 cells after 4h Exposure to 2-Aminoanthracene, with Metabolic Activation (+S9)

Group No.	2AA Treatment (μg/mL)	Analyzed Cell No.	Data[A] from Comet Assay IV		
			Tail Moment	Tail Length	% Tail Intensity
1	DMSO	100	0.23	33.86	1.12
2	0.0625	100	0.54	36.04	1.88
3	0.125	100	0.31	33.58	1.40
4	0.25	100	0.46	36.38	1.59
5	0.5	100	0.13	2.96	0.79
6	1	100	0.16	34.30	0.77
7	2	100	3.54	56.25	7.40

[A] Mean of analyzed cells



In vitro Micronucleus Assay Results

TK6 cells after 4h Exposure to 2-Aminoanthracene, with Metabolic Activation (+S9)

Group No.	2AA Treatment (μg/mL)	Analyzed Cell No.	Number of Micronucleated cell (% Incidence)
1	DMSO	1000	19 (1.9)
2	0.0625	1000	10 (1.0)
3	0.125	1000	14 (1.4)
4	0.25	1000	4 (0.4)
5	0.5	1000	9 (0.9)
6	1	1000	8 (0.8)
7	2	1000	18 (1.8)

Micronucleus assay was performed on Day 2.

Cytotoxity Assays Results

TK6 cells after 4h Exposure to 2-Aminoanthracene, with Metabolic Activation (+S9)

Group No.	2AA Treatment (μg/mL)	Daily Cell Growth[A] Measured by COULTER COUNTER			
		Day 1	Day 2	Day 1 × Day 2	Relative Suspension Growth (%)
1	DMSO	3.55	3.15	11.17	100.0
2	0.5	3.15	3.68	11.57	103.6
3	1	2.87	3.52	10.09	90.4
4	2	1.90	3.11	5.91	52.9
5	3	1.38	2.96	4.10	36.7
6	4	0.93	1.51	1.41	12.6

[A] Cell conc. on the day / Cell conc. on the day before

Day 1: After 24h from end of treatment

Day 2: After 48h from end of treatment

Group No.	2AA Treatment (μg/mL)	% Viabilty[B] Measured by Dye Exclusion Assay		
		Day 0	Day 1	Day 2
1	DMSO	97.4	96.8	99.3
2	0.5	100.0	98.5	99.3
3	1	100.0	97.3	97.5
4	2	87.5	88.7	92.4
5	3	95.2	71.0	90.4
6	4	91.4	27.3	34.2

[B] Number of unstained cells / number of total cells) x 100

Day 0: Just after treatment

Day 1: After 24h from end of treatment

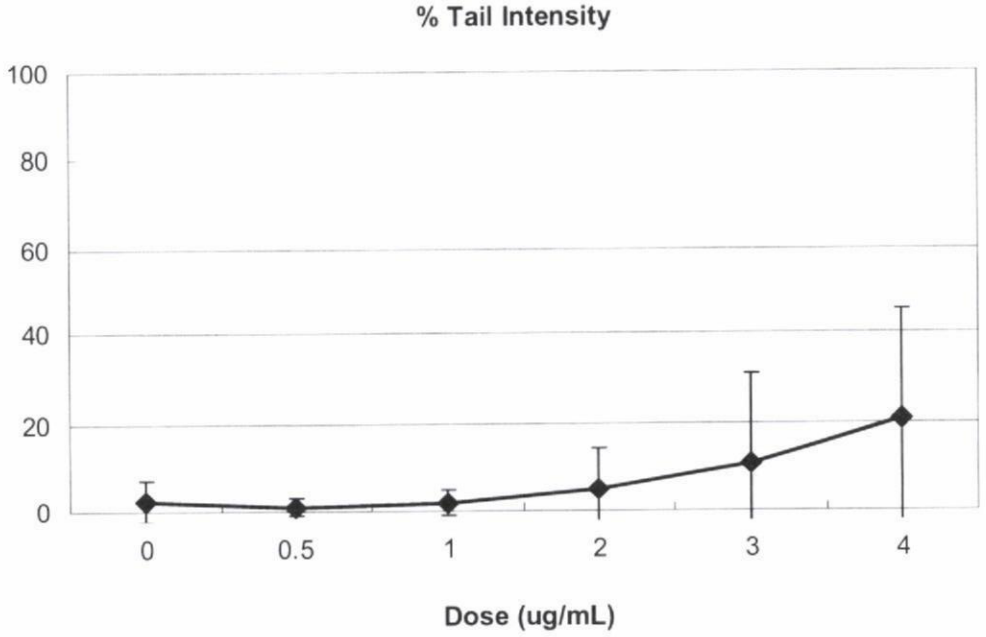
Day 2: After 48h from end of treatment

Comet Assay Results

TK6 cells after 4h Exposure to 2-Aminoanthracene, with Metabolic Activation (+S9)

Group No.	2AA Treatment (µg/mL)	Analyzed Cell No.	Data[A] from Comet Assay IV		
			Tail Moment	Tail Length	% Tail Intensity
1	DMSO	100	0.51	30.16	2.40
2	0.5	100	0.19	28.44	1.13
3	1	100	0.34	31.09	1.99
4	2	100	1.58	39.05	4.88
5	3	100	5.47	48.33	10.81
6	4	100	11.21	72.57	21.07

[A] Mean of analyzed cells



In vitro Micronucleus Assay Results

TK6 cells after 4h Exposure to 2-Aminoanthracene, with Metabolic Activation (+S9)

Group No.	2AA Treatment (μg/mL)	Analyzed Cell No.	Number of Micronucleated cell (% Incidence)
1	DMSO	1000	7 (0.7)
2	0.5	1000	6 (0.6)
3	1	1000	15 (1.5)
4	2	1000	23 (2.3)
5	3	1000	18 (1.8)
6	4	1000	30 (3.0)

Micronucleus assay was performed on Day 2.

4. Cycloheximide (CHX)

Cytotoxity Assays Results

TK6 cells after 4h Exposure to Cycloheximide, without Metabolic Activation (-S9)

Group No.	CHX Treatment (μg/mL)	Daily Cell Growth[A] Measured by COULTER COUNTER			
		Day 1	Day 2	Day 1 × Day 2	Relative Suspension Growth (%)
1	Ethanol	4.34	3.49	15.14	100.0
2	125	0.91	1.15	1.04	6.9
3	250	0.85	1.10	0.94	6.2
4	500	0.80	1.32	1.05	7.0
5	1000	0.90	1.10	0.99	6.5
6	2000	0.85	1.22	1.05	6.9
7	4000	0.71	0.95	0.67	4.5

[A] Cell conc. on the day / Cell conc. on the day before

Day 1: After 24h from end of treatment

Day 2: After 48h from end of treatment

Group No.	CHX Treatment (μg/mL)	% Viabilty[B] Measured by Dye Exclusion Assay		
		Day 0	Day 1	Day 2
1	Ethanol	100.0	100.0	99.0
2	125	100.0	81.5	84.6
3	250	92.3	84.4	72.2
4	500	86.4	92.9	57.1
5	1000	95.0	96.2	58.8
6	2000	95.0	58.8	23.5
7	4000	88.5	76.5	66.7

[B] Number of unstained cells / number of total cells) x 100

Day 0: Just after treatment

Day 1: After 24h from end of treatment

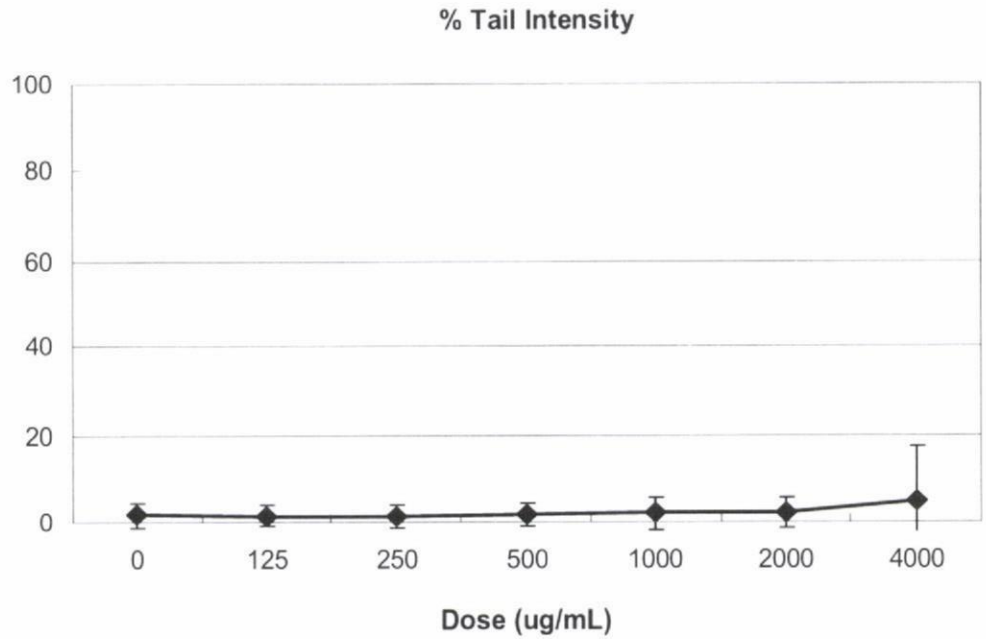
Day 2: After 48h from end of treatment

Comet Assay Results

TK6 cells after 4h Exposure to Cycloheximide, without Metabolic Activation (-S9)

Group No.	CHX Treatment (μg/mL)	Analyzed Cell No.	Data[A] from Comet Assay IV		
			Tail Moment	Tail Length	% Tail Intensity
1	Ethanol	100	0.39	30.90	1.71
2	125	100	0.28	30.37	1.50
3	250	100	0.25	28.73	1.44
4	500	100	0.35	31.28	1.80
5	1000	100	0.40	33.32	2.17
6	2000	100	0.43	31.82	2.12
7	4000	100	2.08	37.12	4.99

[A] Mean of analyzed cells



In vitro Micronucleus Assay Results

TK6 cells after 4h Exposure to Cycloheximide, without Metabolic Activation (-S9)

Group No.	CHX Treatment (μg/mL)	Analyzed Cell No.	Number of Micronucleated cell (% Incidence)
1	Ethanol	1000	11 (1.1)
2	125	1000	12 (1.2)
3	250	1000	17 (1.7)
4	500	1000	21 (2.1)
5	1000	1000	27 (2.7)
6	2000	TOX	- (-)
7	4000	TOX	- (-)

TOX: Not scored due to cytotoxicity

Micronucleus assay was performed on Day 2.

5. Triton X-100 (TRX)

Cytotoxity Assays Results

TK6 cells after 4h Exposure to Triton X-100, without Metabolic Activation (-S9)

Group No.	TRX Treatment (μg/mL)	Daily Cell Growth[A] Measured by COULTER COUNTER			
		Day 1	Day 2	Day 1 × Day 2	Relative Suspension Growth (%)
1	Saline	3.71	3.59	13.31	100.0
2	6.25	3.54	3.92	13.87	104.2
3	12.5	3.35	3.53	11.80	88.7
4	25	3.22	3.69	11.90	89.4
5	50	3.24	3.36	10.89	81.8
6	100	2.84	3.70	10.51	78.9
7	200	1.11	1.04	1.15	8.6

[A] Cell conc. on the day / Cell conc. on the day before

Day 1: After 24h from end of treatment

Day 2: After 48h from end of treatment

Group No.	TRX Treatment (μg/mL)	% Viabilty[B] Measured by Dye Exclusion Assay		
		Day 0	Day 1	Day 2
1	Saline	100.0	100.0	99.4
2	6.25	94.4	96.8	99.3
3	12.5	96.9	99.1	98.3
4	25	97.3	97.6	97.8
5	50	100.0	99.1	99.3
6	100	100.0	96.6	99.2
7	200	25.0	0.0	0.0

[B] Number of unstained cells / number of total cells) x 100

Day 0: Just after treatment

Day 1: After 24h from end of treatment

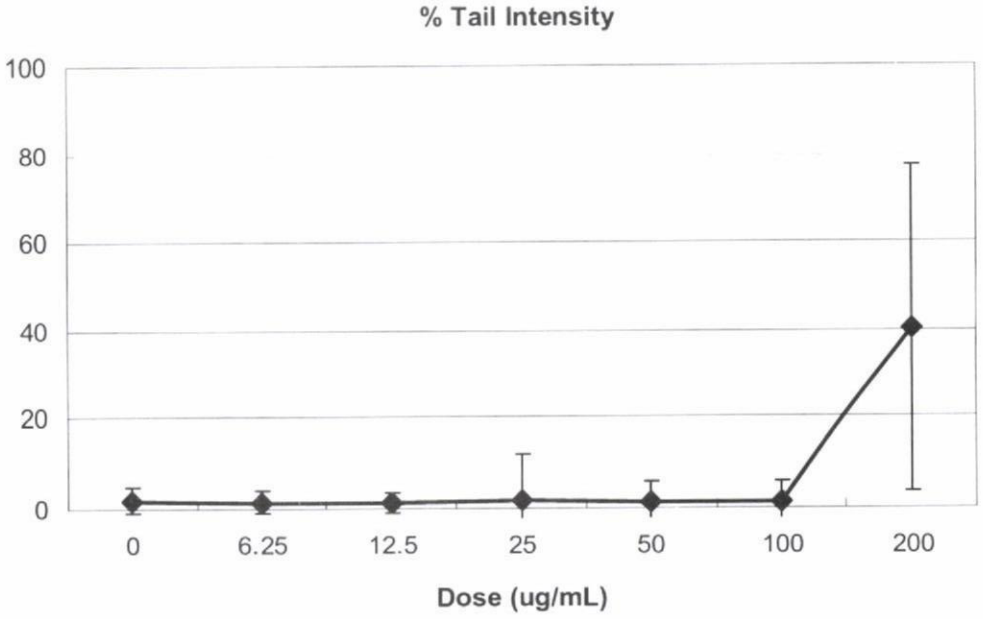
Day 2: After 48h from end of treatment

Comet Assay Results

TK6 cells after 4h Exposure to Triton X-100, without Metabolic Activation (-S9)

Group No.	TRX Treatment (μg/mL)	Analyzed Cell No.	Data[A] from Comet Assay IV		
			Tail Moment	Tail Length	% Tail Intensity
1	Saline	100	0.40	35.04	1.94
2	6.25	100	0.27	33.23	1.50
3	12.5	100	0.28	32.25	1.44
4	25	100	0.39	32.49	2.01
5	50	100	0.28	28.75	1.44
6	100	100	0.31	28.10	1.47
7	200	33	27.77	102.48	40.53

[A] Mean of analyzed cells



200 μg/mL TRX

Hedgehog (small head)

In vitro Micronucleus Assay Results

TK6 cells after 4h Exposure to Triton X-100, without Metabolic Activation (-S9)

Group No.	TRX Treatment (μg/mL)	Analyzed Cell No.	Number of Micronucleated cell (% Incidence)
1	Saline	1000	4 (0.4)
2	6.25	1000	8 (0.8)
3	12.5	1000	4 (0.4)
4	25	1000	7 (0.7)
5	50	1000	4 (0.4)
6	100	1000	8 (0.8)
7	200	TOX	- (-)

TOX: Not scored due to cytotoxicity

Micronucleus assay was performed on Day 2.

INTERNATIONAL PRE-VALIDATION STUDY OF THE IN VITRO ALKALINE COMET ASSAY

Issued by the VMT (Masamitsu Honma)
Version 4.3, January 6, 2008 revised

A. PURPOSE OF THIS DOCUMENT

This document is provided to conduct an international pre-validation study of the in vitro alkaline Comet assay. In order to establish a robust in vitro Comet assay procedure and to make consensuses for evaluation and interpretation of the Comet results (including cytotoxicity), five leading laboratories conduct the in vitro Comet assay for 5 genotoxic or non-genotoxic chemicals. The management members review and validate the Comet results with the consultation of experts. The outcome of pre-validation study will lead to the main validation study, in which we will validate the capacity and limitation of the in vitro Comet assay from large experiments and pursuit the possibility of the in vitro Comet assay as alternative for other in vitro or in vivo genotoxicity tests.

B. ORGANIZATION

1. Validation management team (VMT)
   M. Hayashi (JaCVAM/NIHS), R. Corvi (ECVAM), M. Honma (NIHS), Y. Uno (MTP/MMS), L. Schechtman (Consultant), R. Tice (NIEHS/ICCVAM/NICEATM), H. Kojima (JaCVAM/NIHS; Secretariat)
2. Consultation team
   N. Asano (MMS/Nitto Denko), D. Lovell (Univ. of Surrey), T. Morita (NIHS), N. Nakashima (PMDA), Y. Ohno (JaCVAM/NIHS), T. Omori (Kyoto Univ.), YF Sasaki (Hachinohe Natl. Coll. of Tech.), M. Suzuki (An-Pyo Ctr.), S. Hoffmann (ECVAM), G. Speit (Unv. of Ulm), A. Collins (Unv. of Oslo), S. Park (KFDA), Y. Seo (Kyung Hee Univ.)
3. Leading laboratory
   M. Nakajima (An-Pyo Ctr., JP)
   K. Yamakage (FDSC, JP)
   P. Escober (Bio-Reliance, USA)
   B. Burlinson (HLS, UK)
   A. Kraynak (Merk, USA)

C. ASSUREANCE OF DATA QUALITY

The study will be conducted by the leading laboratories which have a facility of Good Laboratory Practice compliant (GLP).

D. TESTING PROCEDURE

1. BASIC PROCEDURES OF ALKALINE COMET ASSAY

We understand that the basic procedures for the Comet assay including cell lysis, un-winding, electrophoresis, neutralization, DNA staining, visualization, and Comet analysis are identical between in vivo and in vitro experiments. Those procedures were already established in the international validation study of the in vivo rodent alkaline Comet assay before. In this pre-validation study, the laboratory should conduct the Comet assay according to the established procedure. The summary of basic procedure is shown in Table 1.

Table 1

		In Vivo Comet Standard Procedure
Agarose gel and sample preparation	**Bottom gel**	1.0-1.5%-low-gelling temperature-agarose in PBS (if used)
	Sample gel (A)	0.5%-low-gelling temperature-agarose in PBS
	Solution of suspended cells (B)	Cells in HBSS with 20 mM EDTA and 10% DMSO*
	Mixture/ Final conc. of agarose	(A):(B)= 9:1/ 0.45%
Lysis and electroporation	**Lysis solution**	2.5M NaCl, 100mM Na2EDTA, 10mM Tris-base, 10% DMSO, 1% Triton-X (pH 10) *
	Lysys condition	Overnight, 4C
	Rinse solution/ Condition	Distiled water/ Dipping
	Electrophoresis solution	0.3M NaOH, 1mM EDTA (pH >13), <10C
	Electrophoresis condition	Unwinding 20min + Electrophoresis 0.7-1 V/cm (300mA), <10C
Staining	**Neutralization/ Dehydrization**	0.4M-Tris-base (pH 7.5) at least 5 min/ Absolute ethanol at least 5 min
	Staining dye/ Time	SYBR Gold/ 10 min
Scoring and statistics	**Comet analysis**	Comet IV, Tail length, Tail moment, % tail DNA

* DMSO and/or Triton X should be added just before use.

2. SPECIFIC ISSUES FOR THE IN VITRO ALKALINE COMET ASSAY; MATERALS AND METHODS

2-1. Cells, cell lines

The TK6 human lymphoblast cell line must be commonly used in the pre-validation study. Other cells including human peripheral lymphocytes, L5178Y, V79, CHO, CHL/IU, or HepG2 can be used as a second choice if the laboratory prefers.

2-2. Media, cell culture condition, and cell stocks

Appropriate culture media, and incubation conditions (culture vessel, CO_2 and concentration, temperature should be used in maintaining culture. For TK6 cells, culture medium consists of RPMI1640 medium (GIBCO by Invitrogen Corporation; Cat. No.11875) supplemented with 200 ug/ml sodium pyruvate, 100 U/ml penicillin-100 ug/ml streptomycin (GIBCO by Invitrogen Corporation; Cat. No.15140), and 10 % (v/v) heat-inactivated fetal bovine serum (FBS).

The TK6 cells are always maintained in the culture medium at 37C in an atmosphere of 5% CO_2 and 100% humidity. Cell density is measured by a hemocytometer or an automatic cell counter and the cells are routinely diluted to ~2 X 10^5 cells/ml each day to prevent overgrowth (>1.5 X 10^6/ml).

The laboratory will thaw the delivered the cells and expansively culture in the medium and maintain approximately 1 week. Logarithmic growth is normally maintained with population doubling times of 11-15 h. The cell stocks should be made at approximately 1 X 10^6 cells/ml, 1ml/tube in culture medium containing 10% dimethylsulfoxide (DMSO).

Note) Each laboratory purchase TK6 cell line and FBS from ATCC by itself. ATCC can provide same lots of the TK6 cell (CRL-8015, lot#: 3296817) and FBS (30-2020, lot#: 6504229). TK6 can well grow with FBS and horse serum (HS), and the both conditions are available for any genotoxicity studies. However, the population doubling time with FBS is a little faster than that with HS.

2-3. Metabolic activation

Cells should be exposed to the test chemicals both in the presence and absence of the metabolic activation system (S9-mix). The S9 is prepared from the livers of rats treated with Aroclor 1254 or a combination of phenobarbitone and beta-naphthoflavon. The composition of S9-mix and the

condition of the treatment should be same in the MLA (Honma et al., 1999). The standard S9-mix is prepared by combining 4ml S9 and 2ml each 180 mg/ml glucose-6-phosphate, 25mg/ml NADP and 150mM KCl. The concentration of S9-mix was 5% during treatment and the final concentration of S9 was 2%. The preparation of S9-mix is shown in Table 2.

Table 2

Components	Composition	Concentration in S9-mix	Concentration in culture
S9	4 ml	40 vol%	2 vol%
G-6-P	2ml of 180 mg/ml sol.	118 mM	5.90 mM
NADP	2 ml of 25 mg/ml sol.	6.4 mM	0.32 mM
KCl	2 ml of 150mM sol.	30 mM	1.50 mM

*Treatment in culture: 5% S9-mix

Note) VMT purchases a same lot of S9 from BIOPREDIC International in France (lot# FRA270001), and the company delivers it to each leading laboratory. The amount of S9 is not enough for all experiments in the collaborative study. Laboratories should use this S9 at least for 2-Aminoanthracene, which requires metabolic activation. For other testing chemicals, they can use their own S9. Although VMT does not specify the company and lot for the cofactors, high quality products must be recommended.

2-4. Duplicate cultures

The Comet assay for each chemical in the absence and the presence S9 should be conducted in duplicate, because the duplicate results will be appropriately evaluated statistically.

2-5. Duration of the treatment

The culture cells are treated with the chemical for 4 hours, and then the Comet assay should be immediately conducted. The 4 hours treatment is the optimal condition for in vitro Comet assay for most of cells.

2-6. Test chemicals

The following five chemicals are chosen for the pre-validation study. The first 3 chemicals exhibit positive responses in other genotoxicity test. The last 2 chemicals are non-genotoxic chemicals. The laboratory should examine the Comet assay for the five chemicals in the absence of S9-mix as well as in