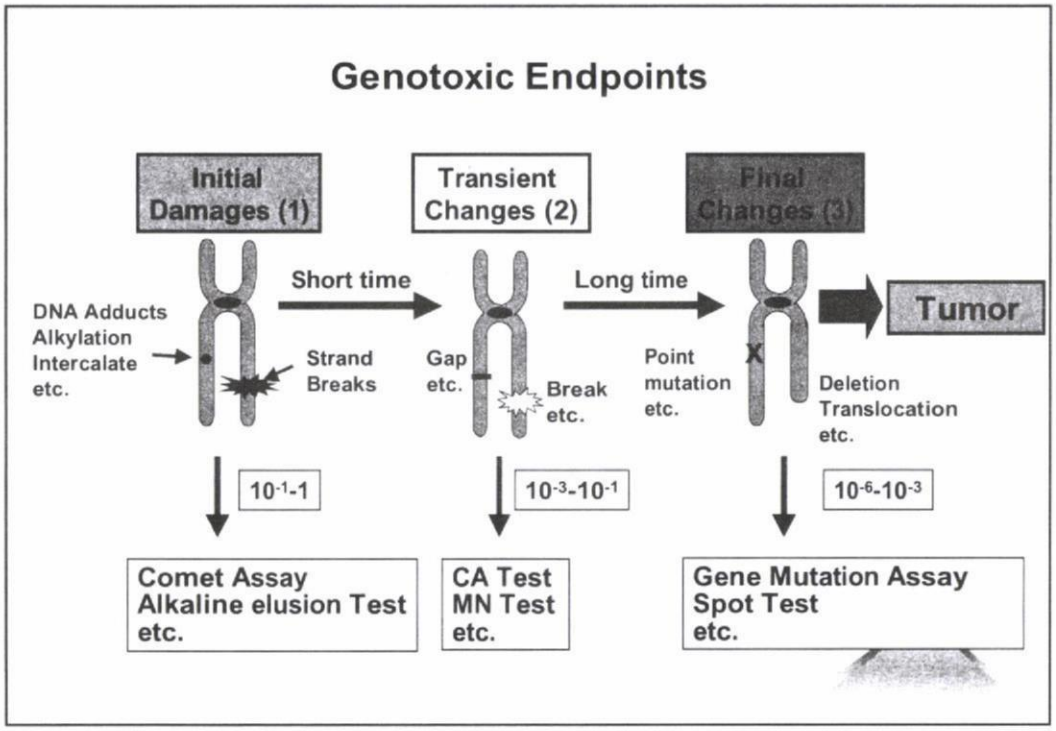


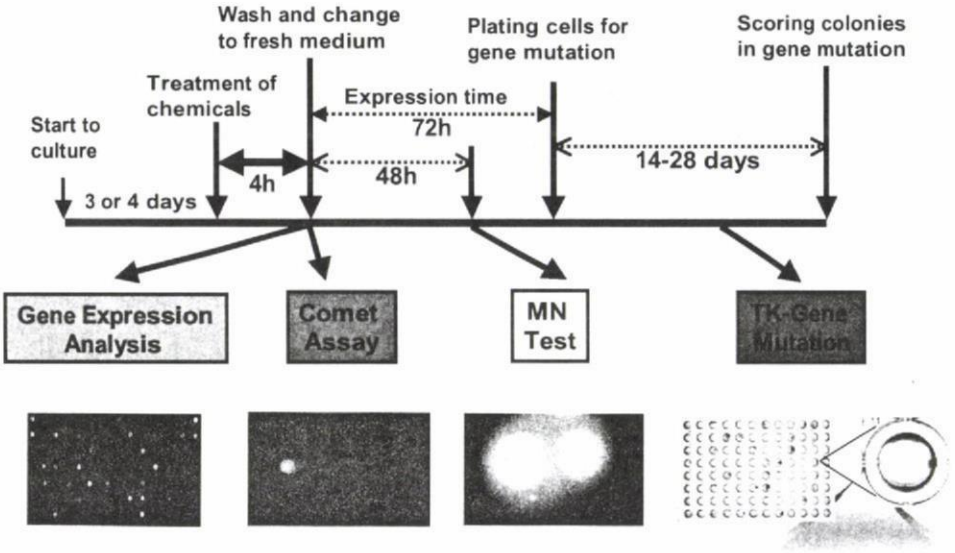
Genotoxicity Test Guideline in Japan

?

	エームス試験	染色体異常試験	MLA	In vivo 小核試験
新規化学物質および製造中間体(安衛法、1996)	●	●		
一般化学物質(化審法、1996)	●	●		○
動物医薬品(1988)	●	●		○
医薬品(薬事法, ICH、1999)	●	◎	◎	●

●:必須、◎どちらか一方を選択、○:必要に応じて実施

Test Battery and Treatment



Comparison of Test Results

	Chemicals	Ames	Comet	CA	MN	MLA	TK
1	Acrylamide						
2	N-Aminoethyl ethanolamine						
3	Bleomycin sulfate						
4	Camptothecin						
5	Catechin						
6	Colchicine						
7	Cytocine arabinoside						
8	5-Fluorouracil						
9	Glycidamide						
10	Griseofulvin						
11	Hexamethyl phosphoramide						
12	Hydroxyurea						
13	Methotorexate						
14	MNNG						
15	Monocrotaline						
16	4NQO						
17	Quercetin						
18	Vinblastine sulfate						

Negative Weak positive

Summary (I)
-Comet-

- **The *in vitro* Comet assay can detect Ames positive chemicals at high probability.**
- **The *in vitro* Comet assay can not detect indirect DNA acting mutagens including spindle poisons and metabolic antagonists.**
- **The *in vitro* Comet assay may be alternative for the Ames assay.**

International Pre-Validation Study of the In Vitro Alkaline Comet Assay

In order to establish a robust in vitro Comet assay protocol 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. Form the studies, we pursuit the possibility of the in vitro Comet assay as alternative for other in vitro or in vivo genotoxicity tests.

Organization

Validation Management Team (VMT)
M. Hayashi (Chair, JaCVAM/NIHS)
R. Corvi (ECVAM)
M. Honma (NIHS)
Y. Uno (MTPC, JEMS/MMS)
L. Schechtman (Consultant)
R. Tice (NIEHS)

Secretariat
H. Kojima (JaCVAM/NIHS)

Leading laboratory
M. Nakajima (An-Pyo Ctr., JP)
K. Yamakage (FDSC, JP)
P. Escober (formerly Bio-Reliance, USA)
B. Burlinson (HLS, UK)
A. Kraynak (Merk, USA)

Consultation Team
N. Asano (Nitto Denko, JEMS/MMS)
D. Lovell (Univ. of Surrey)
T. Morita (NIHS)
N. Nakashima (PMDA)
Y. Ohno (JaCVAM/NIHS)
T. Omori (Kyoto Univ.)
YF. Sasaki (Hachinohe Nat.Coll.Tech.)
M. Suzuki (An-Pyo Ctr.)
S. Hoffman (ECVAM)
G. Speit (Unv. of Ulm)
A. Collins (Unv. of Oslo)
S. Park (KFDA)
Y. Seo (Kyung Hee Unv.)

Local Committee in JPN
Mainly from JEMS/MMS members

Specific Issues of the In Vitro Comet Assay Protocol

1. Cells, Cell lines
   - Suspension cells vs. adherent cells
2. Duration of treatment with chemicals
   - Short vs. long
3. Dose selection
   - Cytotoxic parameter, level of cytotoxicity
4. Metabolic activation
   - S9 condition
5. Relevance to other genotoxic responses
   - Comparison of sensitivity and reactivity with other genotoxicity tests
6. Statistical analysis

Basic Protocol for Alkaline Comet Assay

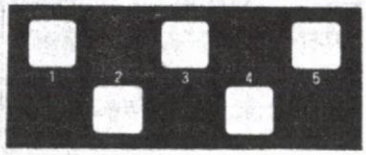
* In vitro alkaline Comet assay protocol is identical after cell preparation.

		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.

Specially Coated Slides Are Available for Alkaline Comet Assay

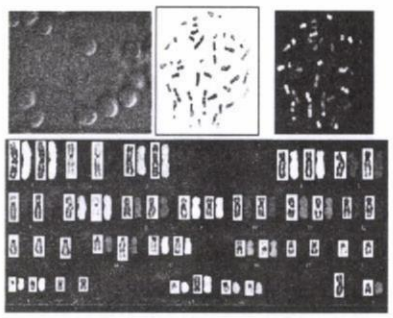
A New Mas-coat Slide Designed for Comet Assay Produced by Matsunami Company



*Sample available!

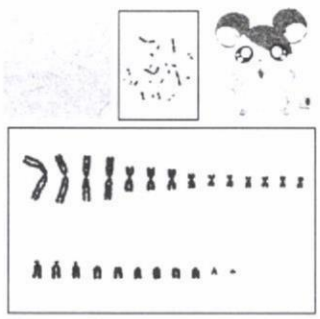
Cell lines

TK6

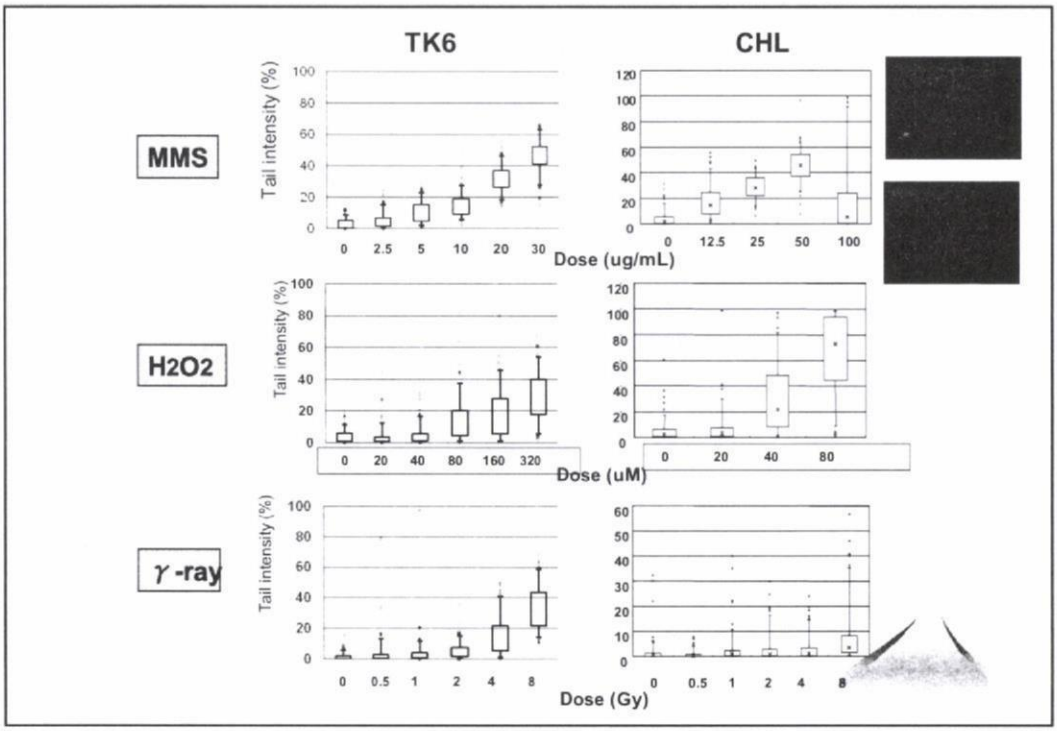


- Lymphoblast cell (Suspension)
- PDL: 11-13h
- MN, TK-mutation
- p53-wild type

CHL



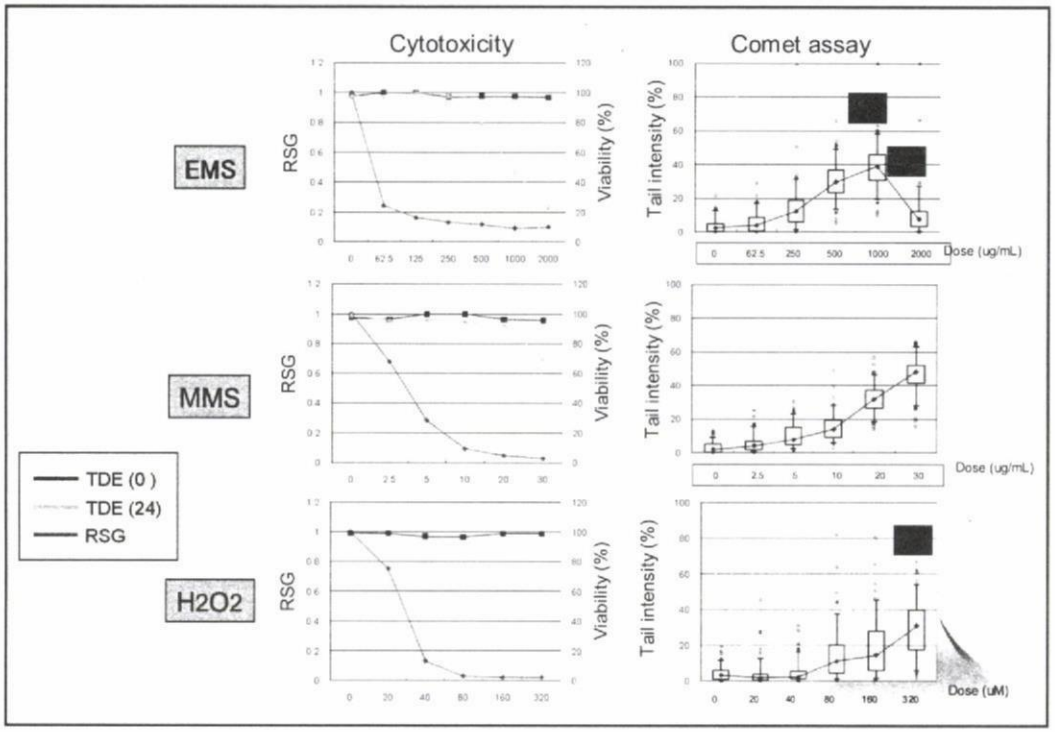
- Fibroblast cell (Adherent)
- PDL: 15-17h
- Chrom Ab., MN
- p53-mutant type

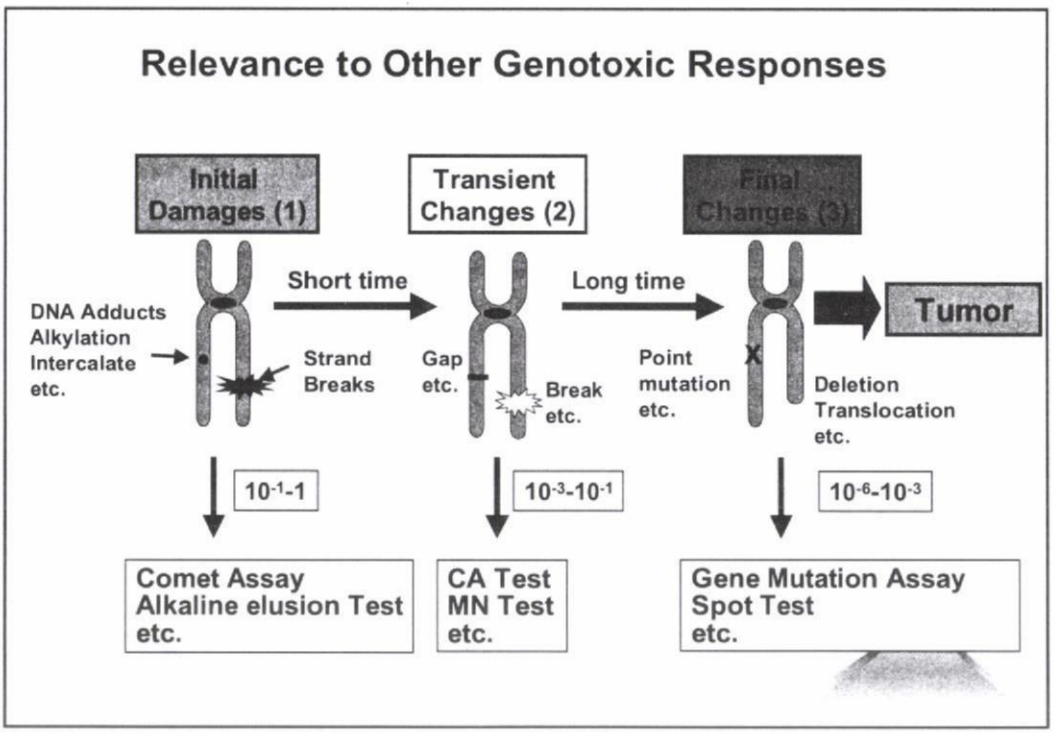


Cytotoxicity, Dose Selection

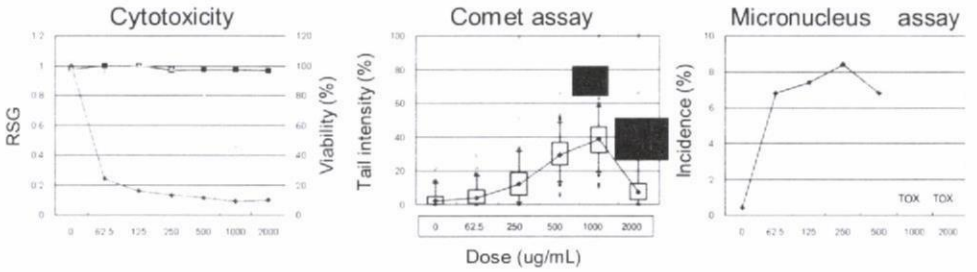
Possible cytotoxic parameters for Comet assay

- Trypan blue dye exclusion test (TDE)
- ATP assay
- Neutral diffusion assay
- Relative survival (colony formation)
- Relative cell (suspension) growth (RSG) for 24 h after the treatment
- Trypan blue dye exclusion at 24 h later after the treatment
- Others (Dual dye viability staining , mitotic index, etc.)

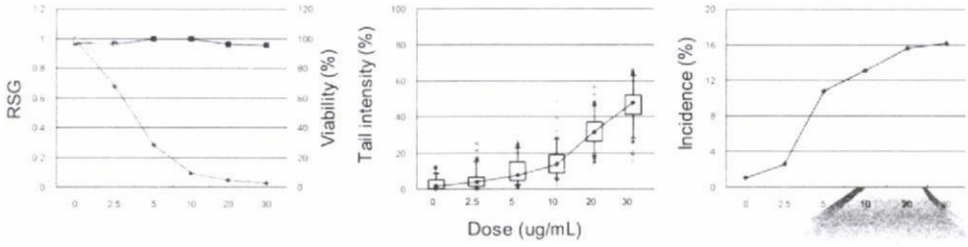

Cytotoxicity
Comet assay
EMS
MMS
H2O2
RSG
Viability (%)
Tail intensity (%)
Dose (ug/mL)
Dose (uM)
TDE (0)
TDE (24)
RSG


Relevance to Other Genotoxic Responses
Initial Damages (1)
Transient Changes (2)
Final Changes (3)
DNA Adducts
Alkylation
Intercalate
etc.
Short time
Strand Breaks
Gap etc.
Break etc.
Long time
Point mutation etc.
Tumor
Deletion
Translocation
etc.
10-1-1
10-3-10-1
10-6-10-3
Comet Assay
Alkaline elusion Test
etc.
CA Test
MN Test
etc.
Gene Mutation Assay
Spot Test
etc.

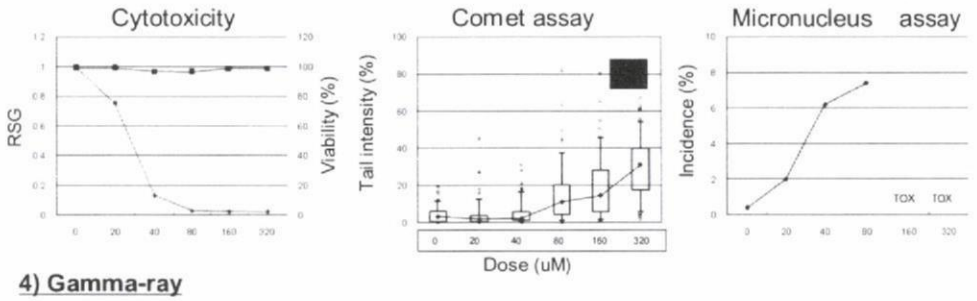
1) Ethyl methanesulfonate (EMS)



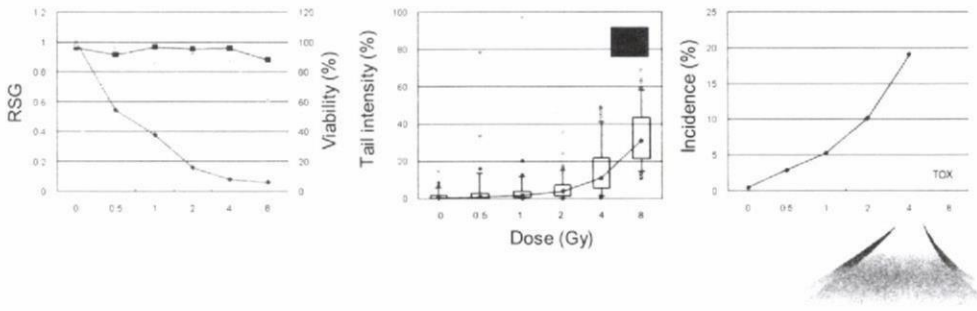
2) Methylmethane sulfonate (MMS)



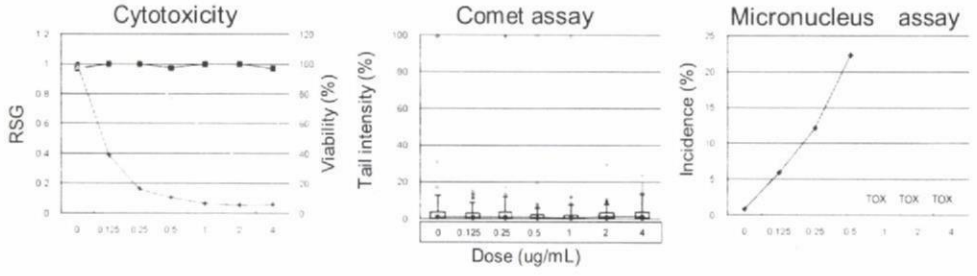
3) Hydrogen peroxide (H_2O_2)



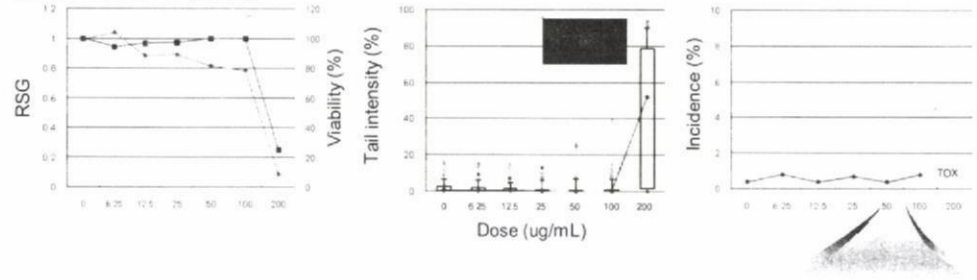
4) Gamma-ray



5) Mitomycin C (MMC)



6) Triton X-100 (TRX)



Summary (II)

- Both TK6 (Suspension cells) and CHL (adherent cells) are available for the Comet assay. However, TK6 cells is easy for the sample preparation.
- Trypan blue dye exclusion (TDE) assay is not appropriate for estimating cytotoxicity in the Comet assay. Relative cell growth must be better as a cytotoxic parameter.
- The *in vitro* Comet assay is less sensitive than other *in vitro* micronuclei assays.

Pre-Validation Study

Test Chemicals

1. Ethymethansulfonate (EMS): alkylating agent (DMSO)

   S9-mix +/-; 0, 62.5, 125, 250, 500, 1000, 2000 ug/ml

2. Mitomycine C (MMC): cross-linker (physiological saline)

   S9-mix +/-; 0, 0.125, 0.25, 0.5, 1, 2, 4 ug/ml

3. 2-Aminoanthracene (2AA): aromatic hydrocarbon (DMSO)

   S9-mix -; 0, 125, 250, 500, 1000, 2000, 4000 ug/ml

   S9-mix +; 0, 0.125, 0.25, 0.5, 1, 2, 4 ug/ml

4. Cycloheximide (CHX): inhibitor for protein synthesis (ethanol)

   S9-mix +/-; 0, 62.5, 125, 250, 500, 1000, 2000 ug/ml

5. Triton-X (TRX): detergent (physiological saline)

   S9-mix +/-; 0, 6.25, 12.5, 25, 50, 100, 200 ug/ml

*Because test chemicals are supplied from VNT, every laboratory examine same lot of chemicals.

Experimental Condition

1. Cell lines
- TK6 (Same lot of cells are supplied from ATCC)

2. S9
- Rat induced liver S9 :(Same lot of S9 are supplied from BIOPREDIC International)

3. Treatment time
- 4h

4. Cytotoxic parameter
1) Trypan blue dye exclusion test just after the treatment
2) Relative cell growth for 24 h after the treatment

4. Comet analysis
- According to the in vivo protocol

5. Measuring Comet
- SYBR gold staining
- Comet IV, Tail length, Tail moment, Tail intensity, 100 cells/dose/tube

6. Statistical analysis
- Not yet established

Action Plan

2007. 11	Start of pre-validation study
2008. 3	Data collecting and interim validation
2008. 6	Final validation and establishment of protocol
2008. 9	Announcement of main-validation study
2008. 12	Start of main-validation study

Thank you for your attention!

INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (VERSION 13)

Issued by: the Validation Management Team (VMT)

Date: March 14, 2008 revised

Notes: Will likely need to specify shelf life for some solutions as we reconcile lab-specific protocols

A. PURPOSE OF THIS DOCUMENT

This document is provided to clarify the conduct of an international validation study to evaluate the ability of the *in vivo* rodent alkaline Comet assay to identify genotoxic carcinogens, as a potential replacement for the *in vivo* rodent hepatocyte unscheduled DNA synthesis (UDS) assay. A study protocol will be developed by the testing facilities based on the information provided in this document.

B. ASSURANCE OF DATA QUALITY

The study will be conducted in facilities that are Good Laboratory Practice compliant. Consistency between raw data and a final report is the responsibility of each testing facility. The VMT may review the data for consistency, if deemed necessary.

C. ANIMAL WELFARE AND 3Rs

Appropriate national and/or international regulations on animal welfare must be followed. The 3R-principle for experimental animal use must be considered for determining the experimental design.

D. TESTING PROCEDURE

1. MATERIALS AND METHODS

1.1. Test substances and positive/negative controls

1.1.1. Test substance

With the exception of ethyl methanesulfonate (EMS), test substances will be supplied to each testing facility by the VMT. When coded substances are supplied, appropriate safety information will be provided in a sealed envelope to be opened only by an appropriate individual within the organization who is not involved in the study and/or in the case of

an emergency. If opened, appropriate documentation and justification will need to be provided to the VMT.

1.1.2. Test substance preparation

Each test substance will be dissolved or suspended with an appropriate solvent/vehicle just before administration (see section 1.1.4.).

1.1.3. Positive control

EMS (CAS No. 62-50-0); the source and lot number to be used will be provided by the VMT. EMS will be dissolved in physiological saline just before administration (within 2 hour).

1.1.4. Negative control (solvent/vehicle)

Solvents/vehicles for test substance preparation will be used as negative controls. An appropriate solvent/vehicle for a test substance may be indicated by the VMT. In the absence of instruction from the VMT, an appropriate solvent/vehicle will be chosen for each test substance by the testing facility in the following order: physiological saline, 0.5% w/v sodium carboxymethylcellulose aqua solution, corn oil. The source and lot of the corn oil will be specified by the VMT.

1.2. Test animals

1.2.1. Species

Although either rats or mice can be used in this assay, the validation study will use rats. The rat is the species most commonly used in toxicological studies and is the preferred species in the *in vivo* rodent hepatocyte UDS assay.

1.2.2. Sex

In order to allow for a direct comparison with the rat hepatocyte UDS assay, males will be used.

1.2.3. Strain

Rat: Crl:CD (SD)

1.2.4. Source

Charles River Laboratories, Inc.

1.2.5. Age

At the time of purchase: 6-8 weeks of age (body weight 150 g - 320 g)

At the time of dosing: 7-9 weeks of age

1.2.6. Body weight

The weight variation of animals should be +/- 20% of the mean weight at the time of dosing.

1.2.7. Number of animals in each dose group at each sampling time

Five males for the validation study. We will decide afterwards based upon power calculation.

1.2.8. Animal maintenance

Animals will be reared under appropriate housing and feeding conditions according to the standard operating procedures (SOP) in each testing facility, consistent with Section C "Animal Welfare".

1.2.8.1. Diet

Animals will be fed *ad libitum* with a commercially available pellet diet.

1.2.8.2. Water

Animals will be given free access to tap water *ad libitum* .

1.2.9. Animal quarantine and acclimation

Animals will be quarantined and acclimated for at least 5 days prior to the start of the study, according to SOPs in each testing facility. Only healthy animals approved by the Study Director and/or the Animal Facility Veterinarian will be used.

1.2.10. Animal identification and group assignment

Animals will be identified uniquely and assigned to groups by randomization on the basis of body weight according to the SOP in each testing facility.

1.3. Preparation of Comet assay solutions

The following solutions will be prepared, consistent with laboratory SOPs, unless otherwise specified.

1.3.1. 1.0-1.5% (w/v) standard agarose gel for the bottom layer (if used)

Regular melting agarose will be dissolved at 1.0-1.5% (w/v) in Dulbecco's phosphate buffer (Ca^{++}, Mg^{++} free and phenol free) by heating in a microwave.

1.3.2. 0.5 % (w/v) low-melting agarose (Lonza, NuSieve GTG Agarose) gel for the cell-containing layer and, if used, a top layer

Low-melting agarose will be dissolved at 0.5% (w/v) in Dulbecco's phosphate buffer (Ca^{++}, Mg^{++} free and phenol free) by heating in a microwave. During the study this solution will be kept at 37-45°C and discarded afterward.

1.3.3. Lysing solution

The lysing solution will consist of 100 mM EDTA (disodium), 2.5 M sodium chloride, and 10 mM tris hydroxymethyl aminomethane in purified water, with the pH adjusted to 10.0 with 1 M sodium hydroxide and/or hydrochloric acid. This solution may be refrigerated at <10°C until use. On the same day of use, 1 % (v/v) of triton-X100 and 10 % (v/v) DMSO will be added to this solution and the complete lysing solution will be refrigerated at <10°C for at least 30 minutes prior to use.

1.3.4. Alkaline solution for unwinding and electrophoresis

The alkaline solution consists of 300 mM sodium hydroxide and 1 mM EDTA (disodium) in purified water, pH >13. This solution will be refrigerated at <10°C until use. The pH of the solution will be measured just prior to use.

1.3.5. Neutralization solution

The neutralization solution consists of 0.4 M tris hydroxymethyl aminomethane in purified water, pH 7.5. This solution will be either refrigerated at <10°C or stored consistent with manufacturer's specifications until use.

1.3.6. Mincing buffer

The mincing buffer consists of 20 mM EDTA (disodium) and 10% DMSO in Hank's Balanced Salt Solution (HBSS) (Ca^{++}, Mg^{++} free, and phenol red free if available), pH 7.5 (DMSO will be added immediately before use). This solution will be refrigerated at <10°C until use.

1.3.7. Staining solution

The fluorescent DNA stain is SYBR Gold (Invitrogen-Molecular Probes), prepared and used according to the manufacturer's specifications.

1.4. Comet assay procedure

1.4.1. Experimental design

Compound	Dose (mg/kg)	Number of animals
Vehicle (negative control)	0	5
EMS (positive control)	300	5
Test compound	Low (1/4 of high)	5
Test compound	Medium (1/2 of high)	5
Test compound	High*	5

*High dose selection: in general, in the absence of VMT directions, the high dose level of

a test compound will be selected as the dose producing signs of toxicity such that a higher dose level, based on the same dosing regimen, would be expected to produce mortality, or an unacceptable level of animal distress. Selection of doses will be based on the toxicity of the test substance but will not exceed 2000 mg/kg.

1.4.2. Administration to animals

The test substance will be administered twice orally by gavage, 21 hours apart. EMS will be administered once orally by gavage. The dosage volume will be 0.1 mL per 10 g body weight in rats on the basis of the animal weight just before administration.

1.4.3. Measurement of body weight and examination of animal conditions

Individual body weights will be measured in accordance with local SOPs and just prior to administration (the weight at this time will be used to determine the volume of each substance administered). The clinical signs of the animals will be observed from just after dosing to just before tissue removal with an appropriate interval according to the SOP in each testing facility.

1.4.4. Tissue sampling

Animals will be humanely killed at 3 hours after second administration of a test substance and at 3 hours after EMS treatment, consistent with Section C "Animal Welfare and 3Rs". The stomach and portions of the liver will be removed. Tissues will be placed into ice-cold mincing buffer, rinsed sufficiently with the cold mincing buffer to remove residual blood (more rinses would likely be needed if exsanguination is not used), and stored on ice until processed. Fro histopathology, samples will be obtained from the same liver lobe, and from a minimal possible area of stomach.

1.4.5. Preparation of single cells

The liver and the stomach will be processed as follows:

Liver: A portion of the left lateral lobe of the liver will be removed and washed in the cold mincing buffer until as much blood as possible has been removed. The size of the portion will be at the discretion of the laboratory but will be standardized. The portion will be minced with a pair of fine scissors to release the cells. The cell suspension will be stored on ice for 15-30 seconds to allow large clumps to settle (or, the cell suspension will be strained through a Cell Strainer to remove lumps and the remaining suspension will be placed on ice), and the supernatant will be used to prepare comet slides.

Stomach: The stomach will be cut open and washed free from food using cold mincing buffer. The forestomach will be removed and discarded. The glandular stomach will be then placed into cold mincing buffer and incubated on ice for from 15 to 30 minutes.

After incubation, the surface epithelia will be gently scraped two times using the a scalpel blade or a Teflon scrapper. This layer will be discarded and the gastric mucosa rinsed with the cold mincing buffer. The stomach epithelia will be carefully scraped 4-5 times (or more, if necessary) with a scalpel blade or Teflon scrapper to release the cells. The cell suspension will be stored on ice for 15-30 seconds to allow large clumps to settle (or, the cell suspension will be strained with a Cell Strainer to remove clumps and the remaining suspension will be placed on ice), and samples of the supernatant used to prepare comet slides.

1.4.6. Slide preparation

Comet slides will be prepared using laboratory specific procedures. The volume of the cell suspension added to 0.50% low melting agarose to make the slides will not decrease the percentage of low melting agarose by more than 10% (i.e., not below 0.45%) .

1.4.7. Lyses

Once prepared, the slides will be immersed in chilled lysing solution overnight in a refrigerator under a light proof condition. After completion of lysing, the slides will be rinsed in purified water or neutralization solution to remove residual detergent and salts prior to the alkali unwinding step.

1.4.8. Unwinding and electrophoresis

Slides will be randomly placed onto a platform of submarine-type electrophoresis unit and the electrophoresis solution added. A balanced design will be used (i.e., in each electrophoresis run, there should be the same number of slides from each animal in the study; see Attachment 1, an example of use to keep track of each slides during each electrophoresis run. Each laboratory will need to provide its own electrophoresis box chart, as different boxes can accommodate different numbers of slides). The electrophoresis solution will be poured until the surfaces of the slides are completely covered with the solution. The slides will be left to be unwind for 20 minutes. Next, the slides will be electrophoresed at 0.7 to 1 V/cm (Note: voltage may be defined more strictly, e.g. 0.7 exactly: depending on the 3rd phase validation study results), with a constant voltage at approximately 0.30 A. The current at the start and end of the electrophoresis period should be recorded. The temperature of the electrophoresis solution through unwinding and electrophoresis should be maintained at a constant temperature <10°C (Note: the range of temperature may be defined more strictly, e.g. 4-8°C: depending on Andy's further experiments). The temperature of the electrophoresis solution at the start of unwinding, the start of electrophoresis, and the end of

electrophoresis should be recorded. The electrophoresis duration should result in an average DNA migration in the negative control group of 1-10% DNA in the tail for the liver, and 1-30% (preferably 1-20%) DNA in the tail for the stomach.

1.4.9. Neutralization and dehydration of slides

After completion of electrophoresis, the slides will be immersed in the neutralization buffer for at least 5 minutes. All slides will be dehydrated by immersion into absolute ethanol (≥99.6%) for at least 5 minutes if slides will not be scored soon, allowed to air dry, and then stored until scored at room temperature, protected from humidity > 60 %. Once scored, slides should be retained and stored under low humidity conditions (e.g., in a desiccator) for potential rescoring.

1.4.10. DNA staining, comet visualization and analysis

Coded slides will be blind scored according to laboratory specific SOPs. The slides will be stained with SYBR Gold according to manufacturer's specifications. The comets will be measured via a digital (e.g. CCD) camera linked to an image analyzer system using a fluorescence microscope at magnification of 200X. For each sample (animal/tissue), fifty comets cells per slide will be analyzed, with 2 slides scored per sample. At least 10 areas/slide will be observed at 5 cells or less/area (it will need to dilute cell suspension adequately in the single cell preparation process), taking care to avoid overlap counting of cells and outside areas of slides. To be re-evaluated after stat analysis Heavily damaged cells exhibiting a microscopic image (commonly referred to as hedgehogs) consisting of small or non-existent head and large, diffuse tails will be excluded from data collection. Add pictures in an appendix – indicate if scorable by software then should be scored. However, the frequency of such comets should be determined per sample, based on the visual scoring of 100 cells per sample. The comet endpoints collected will be % tail DNA. (Comment: in Atagawa mtg., there were some discussions about necessity to measure tail length and Olive tail moment. As a tentative consensus, these parameters are no longer necessary to measure in this validation effort, because %DNA in tail seems a sufficient endpoint for validation)

1.4.11. Histopathology

When a positive Comet assay response is obtained for a tissue, a sample histopathological assessment will be conducted to evaluate for the presence of examined for the tissue according to the SOP in each testing facility.

2. STATISTICS

Different approaches for data analysis have been proposed for comet data generated across a range of test substance dose levels (Lovell et al. 1999; Hartmann et al. 2003; Wiklund and Agurell 2003). The primary endpoint of interest for DNA migration is the % tail DNA. In addition, the distribution of migration patterns among cells within an animal will be considered. The percentage of "hedgehogs" and of cells with low molecular weight DNA will also be evaluated as a function of treatment. The unit of analysis for a specific tissue is the individual animal. Each laboratory may make their own conclusion about the *in vivo* genotoxicity of a test substance using their standard approach.

In data analysis process of this validation study, three conceptual key terms, i.e. "Endpoint", "Estimate", and "Effect" are defined and used. Briefly, "Endpoint" is defined as individual observed values for a parameter such as % DNA in tail. "Estimate" is defined as a mean or median calculated with values of a particular "Endpoint" in each animal. "Effect" is defined as difference (or ratio) of an average of "Estimate" between a negative control group and a treatment group. A general purpose in data analysis of validation studies is to investigate how large variation exists among data from testing facilities, and "Effect" is considered as a good yardstick (criterion) to understand the variation of Comet parameters among testing facilities. Thus "Effect" will be used in this validation study. Dunnett's one side test is also applied for data analysis.

3. DATA AND REPORTING

3.1.1. Treatment of results

Individual animal data and group summaries will be presented in a fixed tabular form that will be provided from the VMT.

3.1.2. Evaluation and interpretation of results

A positive response is defined as a statistically significant change in the % tail DNA in at least one dose group at a single sampling time in comparison with the negative control value. The positive control should produce a positive response, and if not, the study data will not be acceptable. Where a positive response is obtained in a test substance group, the investigator(s) will assess the possibility that a cytotoxic rather than a genotoxic effect is responsible based on the percentage of cells with low molecular weight DNA and histopathology. Positive results indicate that the test substance induce DNA damage in the target tissue(s) investigated. Negative results indicate that, under the test conditions used, the test substance does not induce DNA damage *in vivo* in the tissue(s) evaluated.