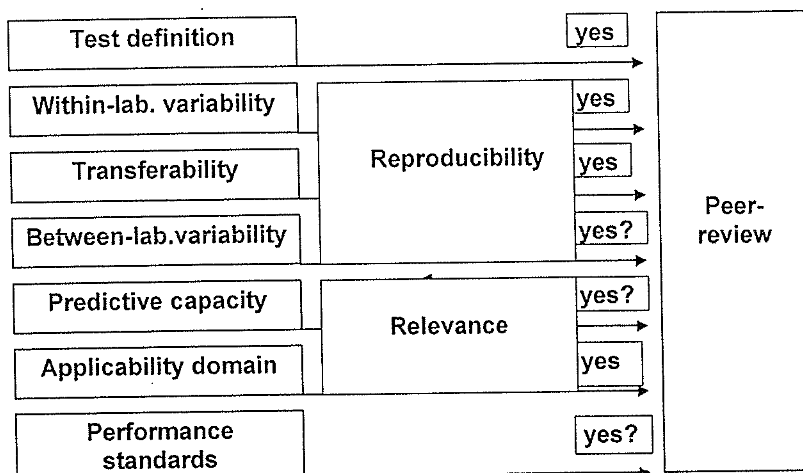
- This is version 1.0
- Before start this study, we will send the last version of this data sheet to each facility
- If any questions or comment of this data sheet (ver. 1.0), please e-mail at omori@pbh.med.kyoto-u.ac.jp

Comet assay validation: modular approach*



*from Thomas Hartung, ECVAM, 2003

INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (VERSION 10)

Issued by: the Validation Management Team (VMT)

Date: November 2, 2006 revised

Notes:

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

A. PURPOSE OF THIS DOCUMUNT

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. The purpose of this phase of the validation is to optimize the test method protocol. 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

Appropriate national and/or international regulations on animal welfare must be followed.

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. Test Substance (also the positive control in future phases of the validation study)

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 preferentially 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. At the discretion of the VMT, mice may be used.

1.2.2. Sex

In order to allow for a direct comparison with the rat hepatocyte UDS assay, males will be used. At the discretion of the VMT, females may be used.

1.2.3. Strain

Rat: Crl:CD (SD)

At the discretion of the VMT, other strains may be used.

1.2.4. Source

Charles River, Inc.

1.2.5. Justification of the strain selection

These strains are commonly used for safety assessment studies.

1.2.6. Age

At the time of purchase: 6-8 weeks of age

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

1.2.7. Body weight

The weight variation of animals should not exceed 20% of the mean weight at the time of dosing.

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

5 animals of the same sex (male).

1.2.9. 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".

1.2.9.1. Diet

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

1.2.9.2. Water

Animals will be given free access to tap water *via* an automatic water system or a water supply bottle.

1.2.10. 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 animals approved by the Study Director and/or the Animal Facility Veterinarian will be used.

1.2.11. Animal identification and group assignment

Animals will be identified uniquely and assigned to groups 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. The VMT will review SOPs used at the 2nd JaCVAM validation meeting planning at Tokyo in December.

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

Regular melting agarose (source and lot may be designated by the VMT) 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 (source and lot may be designated by the VMT) 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 or Trizma base 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-X 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 or Trizma base 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 and/or homogenization 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 (which will be provided by Invitrogen-Molcular 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	Low dose*	5
EMS	High dose**	5

* Low dose level will be 50% of the high dose.

**For EMS, the high dose level will be 200 mg/kg. It was previously reported that the daily dose of 100 mg/kg of EMS to Crl:CD(SD) male rats for 5 days resulted in about 12% decrease in body weight without clinical signs (H. Takagi et al., J. Toxicol. Sci., 25(1), 25-31, 2000), and thus the 200 mg/kg seems to be well tolerated for the multiple (two times) treatment to male rats.

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, an unacceptable level of animal toxicity or excessive cytotoxicity in the target tissue. Selection of doses will be based on the toxicity of the test substance but will not exceed 2000 mg/kg.

To minimize the numbers of animals used and to maximize the ability to integrate the Comet assay and the *in vivo* rodent micronucleus test into a single test, the focus of this validation effort is on a multiple treatment/single sample time protocol. In this 1st stage of validation, EMS will be administered two times with 21- and/or 24-hour interval and one sampling time at 3 hours (2-4 hours) after the final administration. However, some studies may be conducted that compare this protocol with a single treatment/multiple sample time protocol (i.e., with sample times of 2 to 4 and 23 to 25 hours after a single treatment). When testing coded substances, EMS (the positive control) will be administered 3 hours prior to tissue sampling, unless otherwise directed.

1.4.2. Administration to animals

The test substance will be usually administered orally by gavage. The dosage volume will usually be 0.1 mL per 10 g body weight in rats (mice can go up to 0.2 mL per 10 g body weight) 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 during the quarantine and acclimation periods (e.g. on the day of receipt, at the end of the acclimation period) and just prior to administration (the weight at this time will be used to determine the volume of each substance administered). In addition, except for situations where tissues are collected at 3 hours after a single treatment, individual body weight will also be measured before tissue removal. 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, consistent with Section C "Animal Welfare". The stomach and portions of the liver will be removed. An exsanguinations step may be performed prior to tissue collection. Exsanguination from the abdominal aorta may reduce the level of blood contaminating the tissue samples. 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 exsanguinations is not used), and stored on ice until processed.

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 cut with a pair of fine scissors 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 with a Cell Strainers, pore size of 40 μm, and the remaining suspension will be placed on ice), and the supernatant will be used to prepare comet slides.

Stomach: The forestomach will be removed and discarded. The glandular section will be cut open and washed free from food using cold mincing buffer. The 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 back of a scalpel blade or a Teflon scrapper. This layer will be discarded and the gastric mucosa rinsed with the cold mincing buffer. The glandular portion of the stomach will be moved to a clean Petri dish and with cold mincing buffer. The stomach epithelia will be carefully

scraped 4-5 times (or more, if necessary) with the back of a scalpel blade or Teflon scrapper to release the cells. After scraping, the sample may be minced mildly with a pair of fine scissors to release enough 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 Strainers, pore size of 40 μm, 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 for a minimum of 1 hour 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 (a re-circulating type may be preferable) 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, 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 +/- 2°C. To allow for a comparison of the effect of temperature on the sensitivity of the test, studies will be conducted comparing unwinding and electrophoresis at <10°C with 20 to 25°C. 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 5-15 (tentative criteria) % DNA in the tail.

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, 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 (i.e., 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 between 200X to 400X. For each sample (animal/tissue), fifty comets cells per slide will be analyzed, with 2 slides scored per sample. 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. 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, tail length in microns measured from the estimated edge of the head region closest to the anode, and, if possible for a particular image analysis system, Olive tail moment [= a measure of tail length (a distance between a center of head mass and a center of tail mass; microns) X a measure of DNA in tail (% tail DNA/100): Olive et al., 1990]. At the same time, there may be interest in collecting categorical DNA migration data, based on the four classification system introduced by Collins et al. (1993) on 500 cells per slide.

1.4.11. Neutral diffusion assay

To evaluate the extent of cytotoxicity associated with the treatment, one comet slide per sample will be used to evaluate the frequency of cells with low molecular weight DNA indicative of apoptosis or necrosis. After incubation in the lysis solution for 1 hour (± 5 minutes), the comet slide will be rinsed with purified water to remove residual detergent and salts. All slides will be dehydrated by immersion into absolute ethanol (≥99.6%) for at least 5 minutes, allowed to air dry, and then stored until scored. Once scored, slides should be retained and stored under low humidity conditions (i.e., in a desiccator) for potential rescoring. Coded slides will be stained according to laboratory specific SOPs with SYBR Gold according to manufacturer's specifications. The frequency of cells with

diffuse DNA (see Appendix 1) among 100 comets will be visually scored using a fluorescence microscope at magnification between 200X to 400X.

1.4.12. Histopathology

When a positive Comet assay response is obtained for a tissue, a histopathological assessment will be conducted to evaluate for the presence of examined for the tissue according to the SOP in each testing facility. In this 1st stage of validation, we do not need histopathologial examination.

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. However, other measures of DNA migration (tail length, Olive tail moment, and also categorical data, if available) will be analyzed also. 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. Generally, data should be evaluated for normality and, if normally distributed, for equality of variances. The statistical test should be two-tailed if both a decrease or an increase in DNA migration can be measured. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate transformation of the data (e.g., logit, probit). Each laboratory should make their own conclusion about the *in vivo* genotoxicity of a test substance using their standard approach.

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.

3.1.3. Study report

The study report from each testing facility will at least include the following information:

3.1.3.1. Test substance and positive/negative controls

Identification; CAS number; supplier; lot number; physical nature and purity; physiochemical property relevant to the conduct of the study, if known; justification for choice of vehicle; and solubility and stability of the substances in the solvent/vehicle, if known.

3.1.3.2. Test animals

Species/strain used; number, age and sex of animals; source, housing conditions, quarantine and acclimation procedure, and animal identification and group assignment procedure; individual weight of the animals on the day of receipt, at the end of the acclimation period, and before administration (at the time of grouping), including body weight range, mean and standard deviation for each group; and choice of tissue(s) and justification.

3.1.3.3. Regents to prepare regent solutions

Identification; supplier; lot number; and time limit for usage if known.

3.1.3.4. Test conditions

Data from range-finding study, if conducted; rationale for dose level selection; details of test substance preparation; details of the administration of the test substance; rationale for route of administration; methods for verifying that the test substance reached the general circulation or target tissue, if applicable; details of food and water quality; detailed description of treatment and sampling schedules; method of measurement of toxicity, including histopathology; detailed methods of single cell preparation; method of slide preparation, including agarose concentration, lysis conditions, alkali conditions and pH, alkali unwinding time and temperature, electrophoresis conditions (pH, V/cm, mA, and temperature at the start of unwinding and the start and the end of electrophoresis) and staining procedure; criteria for scoring comets and number of comets analyzed per slide, per tissue and per animal; evaluation criteria; criteria for considering studies as positive, negative or equivocal.

3.1.3.5. Results

Signs of toxicity, including histopathology in the appropriate tissue(s) if applicable; individual and mean values for DNA migration and % cells with low molecular weight DNA in individual tissue, animal, and group; concurrent positive and negative control data; dose-response relationship, where possible; and statistical evaluation.

3.1.3.6. Discussion of the results and/or conclusion, as appropriate.

4. ARCHIVES AND REVIEW

The study report and all raw data (including slide samples and image data) from this study will be retained according to the SOP in each testing facility. All raw data will be submitted to the management team for review if required.

5. REFERENCES

Burlinson B, et al., 4th International Workgroup on Genotoxicity Testing: result of the in vivo comet assay workgroup (in preparation).

Collins AR, et al., Direct enzymatic detection of endogenous oxidative base damage in human lymphocyte DNA. Carcinogenesis, 14, 1733-1735, 1993.

Hartmann A, et al., Recommendation for conducting the *in vivo* alkaline Comet assay. Mutagenesis, 18(1), 45-51, 2003.

Lovell DP, G Thomas G, R Dubow., Issues related to the experimental design and subsequent statistical analysis of in vivo and in vitro comet studies. Teratog Carcinog Mutagen. 19(2), 109-119, 1999.

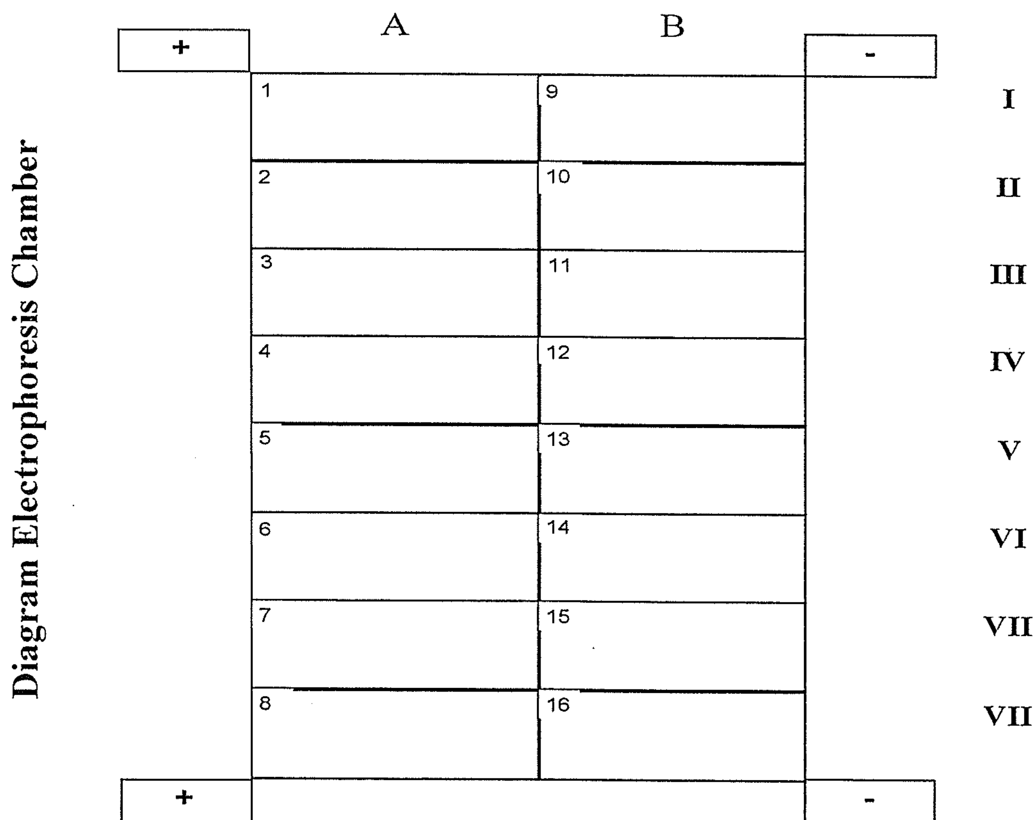
Olive PL, et al., Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the "comet" assay. Radiat. Res., 122, 86-94, 1990.
Tice RR et al., Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen., 35, 206-221, 2000.

Wiklund SJ, E Agurell., Aspects of design and statistical analysis in the Comet assay. Mutagenesis 18(2):167-175, 2003.

Attachment 1:

SLIDES UNWINDING & ELECTROPHORESIS RECORDING SHEET

Electrophoresis Run #				**Initials & Date**
Approximate alkaline electrophoresis buffer volume in chamber				
Unwinding				
Time	Total	Start	End	
Buffer Temperature				
Electrophoresis				
Running time	Total	Start	End	
Volts				
Milliamperes				
Buffer Temperature				
Thermometer No.				
Electrophoresis chamber No.				
Power supply No.				



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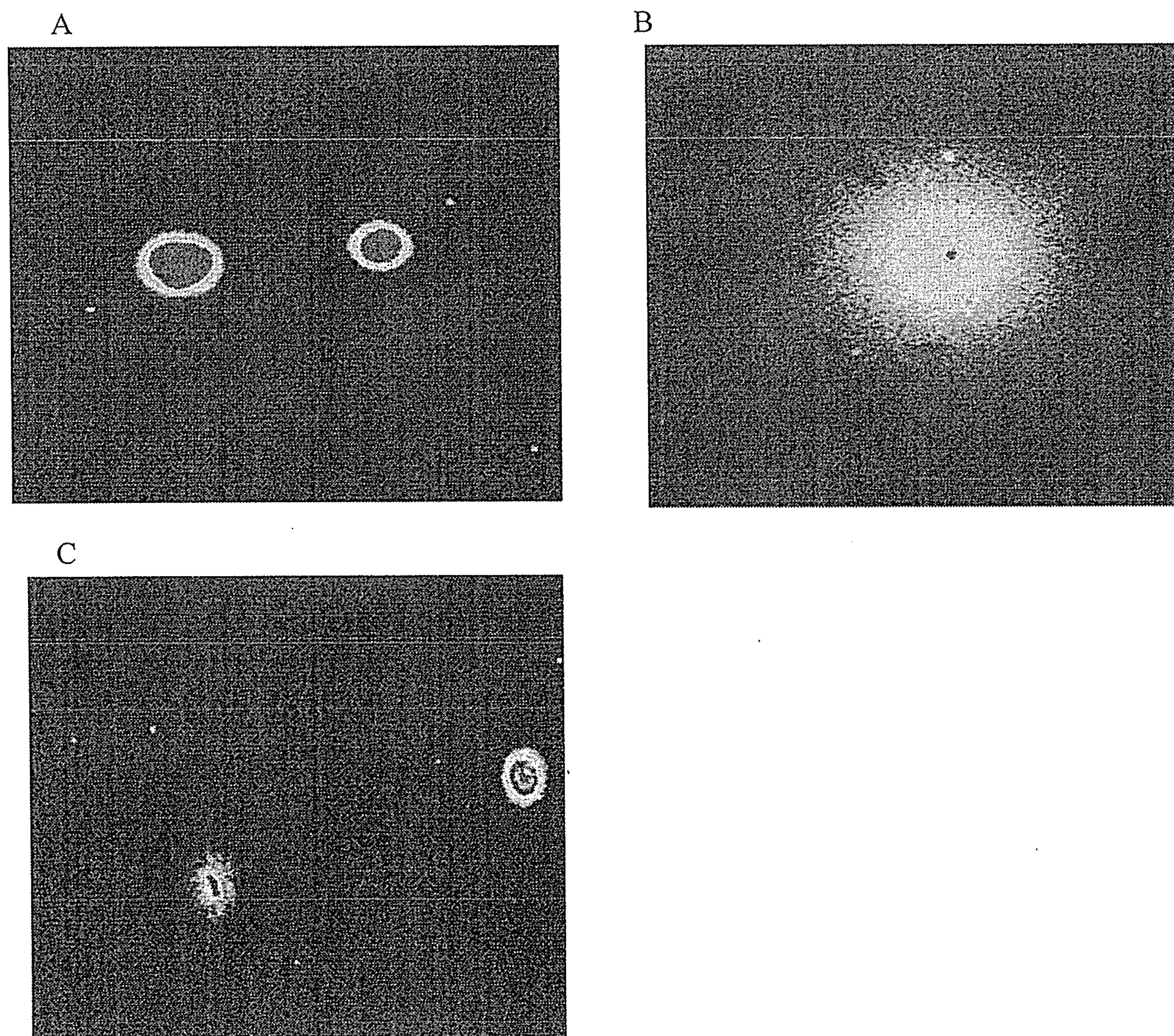
Position of slide in

Appendix 1:

Photographs of cells with diffuse DNA indicative of low molecular weight DNA. Comet slides were removed from lysis after one to 24 hours, rinsed to remove detergents, stained with SYBRGreen, and examined at 250X magnification. Control cells with high molecular weight DNA are in picture A. The remaining 3 pictures (B, C) show a progression of cells with low molecular weight DNA, depending on when the slides were removed from lysis (B at 1 hr, C at 24 hrs). The cells with low molecular weight DNA were from an experiment in which maintaining cells on low serum for 73 hours induced either apoptosis or necrosis, depending on the cell line. Under the experimental conditions used, it was not possible to distinguish between apoptosis and necrosis based on the appearance of the cells with diffuse DNA.



資料 15

Draft Minutes

International Validation Kick off Meeting On Comet Assay

Date: August 14-15, 2006
(August 14, 9:30 to 17:30, August 15, 9:00 to 12:00)
Venue: Sapporo Koseinenkin kaikan,
Sapporo, Japan

Participants:
M. Hayashi (NIHS), T. Hartung (ECVAM), L. Schectman (FDA), R. Tice (NICEATM), Y. Uno (Mitsubishi Pharma Co.), H. Kojima(JaCVAM, NIHS), N. Asano (Nitto Denko Co. : MMS president), B. Burlinson (Huntingdon), M. Honma (NIHS), D. Lovel (Univ. of Surrey), T. Morita (NIHS), N. Nakashima (OECD), P. Clay (Syngenta, CTL), P. Escobar (Invitrogen), R. Storer (Merck), M. Nakajima (Anpyo-Center), K. Yamakage (FDSC), Y. Nakamura(LSG Co.: observer),

1 Welcome address
Dr. Kojima opened the kick off meeting on the comet assay by welcoming everyone. He then asked everyone to introduce themselves. Dr. Ohno, who has grant for this validation study, also welcomed everyone. After the greeting, the following agenda items were discussed.

2. Overview of the pre-validation study
Dr. Hayashi presented a power point "**International validation study of *in vivo* & *in vitro* Comet assay**" that provided the rationale for the international validation of the *in vivo* and *in vitro* comet assays and described how such a study might be conducted..

3. Validation process
Dr. Tice presented an overview of the test method validation process used by the U.S. ICCVAM (see power point titled "**Pros and Cons of the Comet Assay for Human Risk Assessment**" and"***Comet assay validation: modular approach***".

4. Process for OECD guideline
Dr. Nakashima presented an overview of the OECD, the mutual acceptance of data (MAD) treaty, test guidelines and Good Laboratory Practice guidelines (see power point "***OECD Activities on Chemical Safety***").

5. Other information
Dr. Hartung presented an overview of the COMICs project being initiated by ECVAM in the EU to validate the *in vitro* version of the comet assay, as well as related information on ECVAM activities (see poewer point "***Comet assay and cell array for fast and efficient genotoxicity testing***").

6. Selection of validation management team chair
The members were unanimous in their approval of Dr. Hayashi as the chairperson of the validation management team.

7. Object of this validation
Dr. Hayashi discussed the proposed purpose of this meeting (see Word file "***JaCVAM initiative International validation on in vivo and in vitro comet assay***"). The purpose

was to:
1) clarify some technical aspects of the comet assay and to recommend a standardardized procedure, and
2) discuss how the intra- and inter-laboratory reproducibility of this assay will be evaluated.

8. Structure of validation team and responsibilities
Dr. Hayashi discussed the proposed organization and responsibilities of all members (see Word file "***JaCVAM initiative International validation on in vivo and in vitro comet assay***"). He stated that Merck will not participate in the pre-validation study as the lead laboratory.

9. Protocol issues
Dr. Hayashi discussed several protocol issues (see Word file "***TESTING PROCEDURE OF IN VIVO ALKALINE COMET ASSAY FOR THE INTERNATIONAL VALIDATION STUDIES(DRAFT, VER. 3)***"). These included:

9-1 GLP
This study will be conducted in the spirit of Good Laboratory Practice

9-2 Positive control
Ethyl methanesulfonate (EMS) will be used as a positive control. All laboratories will use the same batch of EMS; Invitrogen will use both EMS and methyl methansesulfonate (MMS).

9-3 Negative control (solvent/vehicle)
In the absence of instruction from the management team, an appropriate solvent/vehicle will be chosen for each test substance by each testing facility from the following ones: physiological saline, 0.5% (w/v) sodium carboxymethylcellulose aqua solution, corn oil.

9-4 Test animals and size of study,
The following conditions were determined; Rat: Crl: CD (SD), male, at the time of dosing: 7-9 weeks of age, 5 animals/group raised according to national regulations.

9-5 Preparation of reagent solution
The solutions will be prepared, stored appropriately, and used within a time that is apporiate for each solution. The conc. of agarose gel is 1.0% (w/v) for the bottom layer and 0.5 % (w/v) low-melting agarose gel. The DNA stain will be Cyber Gold.

9-6 Administration to animals and sampling
The test substance will be usually administered twice to animals orally by gavage, 21 hours apart. Animals will be anesthetized with ether or a proper anesthetic at 3 hours after the second dosing. The the liver and the stomach will be the main tissues collected.

9-7 Experimental design
Unknown chemicals will be tested 2 or 3 dose, and EMS at 250 mg/kg. The Management team recommends benzo[a]pyrene(as mild mutagen), and 2,6-diaminotoluene (as weak mutagen).

9-8 Isolated nuclei vs whole cell
Single cells will be obtained using mincing or scrapping.

9-9 Slide preparation, electrophoresis, staining
The DNA will be left to be unwind for 20 minutes. After alkali unwinding, the slides will be electrophoresed at 0.7 to 1 V/cm, and at an amperage of 0.25-0.30 A. The goal is to achieve 5-10 % migration.
The electrophoresis solution should be maintained a constant temperature +/- 2 C. Both roomtemperature and between 2 and 10 C is being considered. To confirm the

liquid temperature changes, the liquid temperature of at least three points will be measured and recorded at the start of alkali unwinding, the start of the electrophoresis, and the end of electrophoresis.

9-10 Comet visualization and analysis

Fifty comets per slide will be analyzed, with at least two slides scored per sample.

9-11 Endpoint and analysis (including IA *vs* categorization)

Dr. Yamakage presented the endpoint that he used (see power point "***Comparative Data on Image Analyzer for Comet Assay***"). As the endpoint, the percentage of DNA in tail will be calculated using an image analyzer system.

9-12 Cytotoxicity (histopathology *vs* others)

Dr. Tice recommended the Neutral diffusion assay. Only when a positive result in comet analysis is obtained in a tissue, histopathology will be examined for the tissue according to the SOP in each testing facility.

10 Data collection

Dr. Hayashi, on behalf of Dr. Omori, presented the data collection template (see power point "***About data sheet ver.1.0 for our validation study***").

11 Time schedule proposal

Dr. Hayashi discussed the proposed time schedule proposal (see Word file "***JaCVAM initiative International validation on in vivo and in vitro comet assay***"). Dr. Hartung commented that this assay is incomplete to start a validation study. Prior to start the pre-validation study, he insisted on optimizing a protocol in this assay. Therefore, each laboratory will pre-test for pre-validation using above protocol (chemical: EMS only) at September-November, 2006. In the beginning of December, next meeting will be held in Tokyo, Japan. JaCVAM will prepare the venue, schedule and budget for the next meeting.

12 *In vitro* comet assay

Dr. Honma presented a proposal of an *in vitro* comet assay validation study (see power point "***The in vitro Comet assay-Study Plan-***"). This validation starts next year.

Abbreviation

Anpyo-center	Biosafety Research Center, Foods, Drugs and Pesticides
ECVAM	European Center for the validation of alternative methods
FDA	Food and Drug Administration
FDSC	Food and Drug Safety Center
Huntingdon	Huntingdon Life Sciences
Invitrogen	BioReliance,invitrogen bioservices
JaCVAM	Japanese Center for the validation of alternative methods
MMS	Mammalian Mutagenicity Study
Merck	Merck Research Laboratories
NICEATM	Interagency Center for the Evaluation of Alternative Toxicological Methods
NIHS	National Institute of Health Sciences
OECD	Organisation for Economic Co-operation and Development

資料16

Comet Pre-Validation Study- Check Sheet: Protocol / SOP

Date issued: Dec. 10, 2006

* Please fill in each column if you have any deviations, modifications or comments on the standard protocol

Subject	Item checked	Testing facility				
		Merck	BioReliance	HLS	FDSC	An-pyo
General	Individual protocol	Yes	Yes	Yes	Yes	Yes
	GLP facility	Yes	Yes	Yes	Yes	Yes
	QC check	data reviewed by 2nd biologist	No	No	No	No
	Animal welfare	Yes	Yes	Yes	Yes	Yes
Test substance	Receive process	not sure what this is?	Yes	Yes	No	VMT
	Identification	Ethyl methanesulfonate	Ear tags	Tail Marking	?	Yes
	CAS No.	62-50-0	62-50-0	62-50-0	62-50-0	62-50-0
	Supplier	Sigma	Sigma	Sigma	VMT	Sigma-Aldrich
	Lot	125K1797	125k1797	125K1797	Sigma, 125K1797	074K1260
	Physical nature	Liquid	clear colorless liquid	Clear Liquid	Liquid, transparent	?
	Purity	≥98%	NA	n/a	not available	?
	Preparation procedure	Dissolved in 0.9% saline just before start of each dosing session	Each concentraton (20 mg/ml and 10 mg/mL) was weigh separately and then diluted with saline and vortex for severla minutes to obtained clear colorless solution.	weiged and diluted with saline.	Dilute with Physiological saline, 10 mg/mL for 100 mg/kg and 20 mg/mL for 200 mg/kg	
	Solvent/Vehicle	Physiological saline lot#001K023	Physiological Saline (Braun- Lot No. 76H005)	Physiological Saline (Aqupharm, lot 05K10BC)	Physiological saline (Otsuka, lot. 3K86S)	Saline
Test animal	Species	Rat	Rat	Rat	Rat	Rat
	Sex	Male (Also used female)	Male	Male	Male	Male
	Strain	Sprague-Dawley (CD)	Sprague-Dawle (Hsd:SD)	Sprague Dawley	Sprague-Dawley(SD) [Crl:CD(SD)、SPF]	Crl:CD(SD)

Source	Charles River, USA	Harlan, Frederick MD, Barrier 208A	Charles River, UK.	Charles River Laboratories Japan (Crlj), Atsugi breeding center	Charles River Japan Laboratory, Inc.
Age	7 weeks	6-7 weeks	6-8 weeks	6w (purchased)	7weeks
Body weight	Range: 178-238g ; Average:217g	218.70 - 237.50 grams (randomization)		243.0-270.5g (end of acclimation)	211 to 231g
Number/group	5	5	5	5	5animals/group
Maintenance				-	?
Room temp.	Environmentally controlled allowable range: 19-25 C	72 ± 3°F (22- 25YC)	19 - 25 'C	Allowable temperature 21.0-25.0°C	23.8 to 24.6℃
Room humidity	Environmentally controlled allowable range: 40-60%	50 ± 20% relative humidity	40 - 70 %	Allowable relative humidity 40.0-75.0%	51 to 61%
Lighting	12 hr light cycle	12 hour light/dark cycle.	12 hour light/dark cycle	12 hours (Lighting is provided from 7:00 a.m. to 7:00 p.m.)	12 hours (light on:7 a.m., light off:7 p.m.)
Ventilation frequency	12-15 room changes per hour	At least 10 changes of fresh HEPA-filtered air every hour	About 15 air changes per hour	About 15 air changes per hour	8 times/h or more
Diet/Water	PMI Certified Rodent diet #5002 Pellets ad libitum water ad libitum	tap water and a certified laboratory rodent chow	Mains Water R + M No.1	CE-2 pellet feed (CLEA Japan), ad libitum / Tap water (supplied by Hadano-shi), Ad libitum (automatic supply with nozzle)	MF(Oriental Yeast) / Automatic water system
Animal No./cage	1	5	3	1	3 animals/cage
Quarantine	1 week	8 days (SOP is minimum 5)	5	7 days	5days
Acclimation	1 week	Quarantine and acclimation is the same.	Quarantine and acclimation is the same.	7 days	Yes