VMT meeting

- Two dose level-assay instead of three dose levels (L, M, H) for the phase IV was proposed by Dr. Tice and discussed among VMT members. The consensus was three dose levels should be used in the phase IV validation study because it is necessary to show the dose-response relationship clearly. **Comet atlas including definition of hedgehog should be prepared as soon as possible, possibly by the kick-off workshop in Italy. Action by Dr. Nakajima and else.**

February 5

VMT meeting

Discussion and consensus (Chair: Dr. Uno)

1. Schedule and outline for Phase IV-a

Sending chemicals: the end of April?

Starting tests: May?

ICCVAM needs discuss the study plan and chemical list before starting the phase IV validation study.

Two dose level-assay instead of three dose levels (L, M, H) for the phase IV was proposed by Dr. Tice and discussed among VMT members. The consensus was three dose levels should be used in the phase IV validation study because it is necessary to show the dose-response relationship clearly.

Comet atlas including definition of hedgehog should be prepared as soon as possible, possibly by the kick-off workshop in Italy. Action by Dr. Nakajima.

February 6

VMT meeting

Discussion and consensus (Chair: Dr. Uno)

1. Chemical selection

Dr. Tice explained the strategy of selection of chemicals for validation study. He will look at the National Toxicology Program (NTP) database, etc. Additionally, the following issues were discussed.

- Phase IV, The top dose-level should be MTD or limit dose (2000 mg/kg).
- Dose and solvent should be selected by the laboratory
- To provide the information about the chemical toxicity, etc.
- First set of chemicals (phase IV-a): Dose-levels of each chemical will be informed to the laboratory?
- Up and down method to set top dose by the laboratories? NO. It takes time and not correct.
- Ask each laboratory to provide a protocol for dose-selection, and VMT review them and then prepare a standard protocol.
- Real-world: should be done by the laboratories by themselves.
- To follow the OECD guideline?
- Indication of solvent selection and how to select top dose-level for the first stage of the phase IV (i.e., phase IV-a)

Draft

- Repeat the same chemicals that used for the phase III + something easy chemical
- ICCVAM Genetic Toxicity Working Group meeting with PP(???)

Dr. Takeshi Morita (NIHS) explained the candidate chemical list which includes about 50 chemicals selected. However, the list does not contain halogenated hydrocarbons, pesticides, and heavy metals enough. Dr. Tice will fill the excel sheet using additional database that he can access, e.g., 270 pesticides EPA database. Dr. Raffaella Corvi (ECVAM) will provide database for chemical selection for toxicogonomics. Same chemicals should be used for the in *vitro* validation study. **The draft chemical list (as excel file) should be sent to Drs. Tice and Corvi (by Dr. Kojima???).**

2. Plan of the phase IV-a

Single chemical with all information including top-dose, vehicle, etc. will be provided to the laboratories.

3. Next VMT meeting

The schedule or venues of the next meeting were surveyed by questionnaire to the VMT and the all participated laboratories by Dr. Kojima. In the running for the next meeting, it will be tapped during the 10th ICEM 2009 - INTERNATIONAL CONFERENCE ON ENVIROMENTAL MUTAGENS, 8th International comet assay workshop or 7th World Congress on alternatives and animal use in the life sciences in August-September.

Closing remark

The Chair thanked participants for a very productive meeting and hearty JaCVAM's hospitality.

Annex1

Draft Agenda

The 5th meeting for

the International Comet assay validation study

Date: February 4, 2009, 9:00 a.m.-February 6, 3:00 p.m.

Venue: Sango, 3F,
Kansai Airport Washington Hotel,
Osaka, Japan

February 4 (9:00-12:00)

Welcome address (9:00-9:30)

Chair: Makoto Hayashi

House keeping

Hajime Kojima

In *vitro* Comet-international validation study

Data analysis of phase I study and next study plan (9:30-12:00)

Chair: Masamitsu Honma

1. Introduction : Masamitsu Honma
2. Data analysis and statistical analysis of phase I study: Masaya Suzuki
3. Short presentation and discussion: leading laboratories
4. Progress of phase II study and next study plan: Masamitsu Honma

Lunch, 12:00-13:30

February 4 (13:30-18:00)

In *vivo* Comet-international validation study

Workshop - Image Analysis with Comet IV (13:30-18:00 including coffee break)

Chair: Makoto Hayashi

- Comet and Hedgehog, which is which? : Madoka Nakajima
- Discussion and consensus of judgment for questionable images

February 5

In *vivo* data analysis and next study plan (9:00-18:00)

Chair: Yoshifumi Uno

1. Introduction - progress overview: Yoshifumi Uno (9:00-9:20)

2. Data analysis of the 3rd phase validation study (9:20-12:00)
- Statistical analysis results: Takashi Omori
- Check on the draft data acceptance criteria for EMS data
- Check on the data variation between laboratories for 3 coded chemicals
- Necessity of protocol amendment based on the 3rd phase validation study data

Lunch, 12:00-13:30

3. Data analysis of the laboratory selection study (13:30-15:00)
- EMS and negative control: Yoshifumi Uno
- Two coded chemicals (if possible): Takashi Omori

Coffee break, 15:00-15:30

4. Outline of the 4th phase (definitive) validation study (15:30-17:30)
- Explanation of protocol version 14 (3-times administration): Yoshifumi Uno
- Outline of study plan and schedule: Yoshifumi Uno

5. Other business (17:30-18:00)
(Workshop: Image Analysis with Comet IV: continuation of the yesterday's session if further discussion is needed)

February 6 (9:00-15:00)

The closed meeting for the Validation Management Team

1. Discussion and consensus (9:00-12:00)
Chair: Makoto Hayashi, Yoshifumi Uno
- Chemical selection and distribution
- Time schedule proposal

Lunch, 12:00-13:30

2. Other business (13:30-15:00)
Chair: Makoto Hayashi

Adjourn

Meeting documents

Meeting Document #1	International Validation Study of in *Vitro* Alkaline Comet Assay, *Responses provided by* Masamitsu Honma
Meeting Document #2	Data analysis and statically analysis of phase I study, *Responses provided by Masaya Suzuk*
Meeting Document #3	*Image analysis using Comet Assay IV, Responses provided by Madoka Nakajima*
Meeting Document #4	International Validation of the *in Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens (Ver.14)
Meeting Document #5	In *Vivo* Comet Assay: Update on the on-going validation coordinated by JaCVAM, *Responses provided by Uno Yoshifumi*
Meeting Document #6	In *Vivo* Comet Assay: 3rd phase validation study, *Responses provided by Uno Yoshifumi*
Meeting Document #7	In *Vivo* Comet Assay: 4th phase validation study, *Responses provided by Uno Yoshifumi*
Meeting Document #8	Re2nd phase Figs and tables %tail DNA, *provided by Takashi Omori*
Meeting Document #9	3rd phase Figs and tables %tail DNA, *provided by Takashi Omori*
Meeting Document #10	In *Vivo* Comet Assay: Examination to select labs for 4th phase validation study, *Responses provided by Uno Yoshifumi*
Meeting Document #11	Memo for figs and tables on the phase 3 study

Annex 2
List of Participants

MMS/JaCVAM Joint Seminar

—The Pros & Cons of Comet Assay—

Date: August 13, 2006 From 2:00 pm to 5:00 pm
Venue: Hokkaido-koseinenkin-kaikan 3F Horai-room (Sapporo)

The host organization: *JEMS.MMS and JaCVAM*
The supporter: *Safety Research Institute for Chemical Compounds (Shinei, Kiyota-ku, Sapporo, Phone:011-885-5031)*
Japan Food Research Laboratories, Chitose (3 Bunkyo 2-chome, Chitose-shi, Hokkaido)
Supported by *the Health and Labour Sciences Research Grants (H18-Chemistry-General-003)*

Program

Chairpersons: Dr. M. Hayashi (NIHS)
Dr. L. Schechtman (ICCVAM)

Welcome address
Mr. Tsuguo Ikka (Safety Res. Inst. Chem. Compounds)

Opening remarks
Dr. Norihide Asano (President of MMS)

1. **The Pros & Cons of Comet Assay**
   Dr. Ray R. Tice (NICEATM)

2. **Utility of Alkaline DNA Strand Breakage Assays in Genotoxicity Testing**
   —Comparison of the Alkaline Elution and Comet Assays
   Dr. Rick Storer (Merck Research Laboratories)

3. **Case study—1**
   Dr. Phil Clay (Syngenta)

4. **Case study—2**
   Dr. Patricia Escobar (BioReliance, invitrogen)

Coffee break

Chairpersons: Dr. H. Kojima (JaCVAM)
Dr. R. Tice (NICEATM)

5. **Outcomes of MMS Collaborative trial**
   Dr. Madoka Nakajima (Anpyo-Center)

6. ***In vitro* Comet assay—A possible candidate as a member of the standard test battery**
   Dr. Masamitsu Honma (NIHS) & Dr. K. Yamakage (FDSC)

7. **Limitation of the comet assay**
   Dr. Brian Burlinson (Huntingdon Life Sciences)

Closing remarks
Dr. Yasuo Ohno (JaCVAM/NIHS)

JaCVAM initiative International validation on *in vivo* and *in vitro* comet assay

Japanese Center for the Validation of Alternative Methods (JaCVAM)

1 The purpose

1.1 To validate the *in vivo* comet assay as an alternative follow-up assay to the more commonly used *in vivo* liver UDS assay. Moreover, we would like to evaluate the use of the *in vivo* comet assay for the assessment of DNA damage by chemicals in multiple tissues and to investigate the correlation with carcinogenicity data in those tissues.

1) The intra- and inter-laboratory reproducibility of this assay will also be evaluated.
2) To clarify some technical aspects and to recommend the standard technical procedure of this assay, including whole cell *vs* isolated nuclei issue.
3) To discuss and recommend the method to assess cytotoxicity: histopathological method *vs* any other methods.

1.2 To validate the *in vitro* comet assay as a method of detecting potential DNA damaging effects of test chemicals and also as an alternative to the *in vivo* comet assay.

2 Organization

2.1 Management Team

M. Hayashi (JaCVAM/NIHS)
Y. Uno (MMS*/Mitsubishi Phama Co.)
T. Hurtung or any other representative (ECVAM)
L. Schechtman (ICCVAM/FDA)
R. Tice (NICEATM)

Secretariat
H. Kojima (JaCVAM/NIHS)

*Mammalian Mutagenicity Study Group, which is a sub-organization to the Japanese Environmental Mutagen Society

2.2 Consultation team

N. Asano (MMS/Nitto Denko Co.)
B. Burlinson (Huntingdon, UK)
M. Honma (NIHS)
D. Lovell (Statistician, University of Surrey)
T. Morita (NIHS)
N. Nakashima (OECD)
Y. Ohno (JaCVAM/NIHS)
T. Omori (Statistician, Kyoto University)
YF Sasaki (Hachinohe National College of Technology)
B. Young (Bio-Reliance, USA)

2.3 Local Committee
N. Asano (MMS/Nitto Denko Co.)
M. Hayashi (JaCVAM/NIHS)
M. Honma (NIHS)
H. Kojima (JaCVAM/NIHS)
T. Morita (NIHS)
M. Nakajima (MMS/Anpyo-Center)
T. Omori (Statistician, Kyoto University)
Y.F. Sasaki (Hachinohe National College of Technology)
Y. Uno (MMS/Mitsubishi Phama Co.)
K. Yamakage (MMS/FDSC)

2.4 SD Team for pre-validation
K. Yamakage (FDSC)
M. Nakajima (Anpyo-Center)
Patricia Escobar (Invitrogen)
B. Burlinson (Huntingdon)
P. Clay (Syngenta)

2.5 SD Team for main validation
FDSC (Dr. K. Yamakage)
Anpyo-Center (Mr. M. Nakajima)
Invitrogen (Dr. Patricia Escobar)
Huntingdon (Dr. B. Burlinson)
Syngenta (Dr. P. Clay)
Merck (Dr. R.D. Storer)

To be added up to approximately 10 qualified laboratories in total.

3 Time schedule

3.1 April 13, 2006 Yoga, Japan
Local Organizing Committee meeting,

3.2 August 14-15, 2006 Sapporo, Hokkaido
Management Team and Kick-off meeting
(Management Team members, Expert and Observer team, and representatives from laboratories for pre-validation)

3.3 September-November, 2006 *In vivo* pre-validation

3.4 December, 2006 Data cleaning and analysis

3.5 February-March, 2007 Management team meeting (telephone conference?) for the evaluation of the pre-validation study and planning for the main validation and also preparation of the pre-validation *in vitro* study

3.6 March, 2007 Preparation of the report for the MHLW budget

3.7 April-May, 2007 *In vivo* main validation/*in vitro* pre-validation

3.8 August, 2008 Management team meeting for the *in vitro* pre-validation

study and also for the main validation study

3.9 February-March, 2008 Management team meeting for the assessment of the *in vivo* main-validation study and the evaluation of *in vitro* pre-validation and planning the *in vitro* main validation study

3.10 Summer, 2008 Drafting of the *in vivo* comet assay test guideline and propose to OECD

3.11 February-March, 2009 Management team meeting for the assessment of the *in vitro* main-validation

3.12 Summer, 2009 Drafting of the *in vitro* comet assay test guideline and propose to OECD

4 Success criteria

To be discussed at the kickoff meeting in summer, 2006.

5 Funding

Grant form MHLW and MMS

6 Pre-validation study

The protocol used will be proposed for review at the Kick-off meeting

Negative (solvent) control; positive control (to be selected at the kick-off meeting); two dose levels of a positive control and coded (?) chemical.

1) Test animal species

Mouse

2) Study design

Compound	Dose (mg/kg)	Number of animals
Corn oil (negative control)	0	4
EMS (positive control)	200	4
EMS (positive control)	400	4
Unknown	?	4
Unknown	?	4

Twice repeat treatment at each laboratory.

3) Route for administration

Oral gavage

4) Tissues to be investigated: Liver and stomach.

5) Preparation of whole cells or isolated nuclei

Each laboratory will use the mincing method to obtain whole cells and the homogenization method to obtain isolated nuclei.

7 Main validation study will be discussed at the Management Team based on the outcomes of the pre-validation study.

8 Others

Collaborate with the COMICS
Etc.

JaCVAM 1st International Workshop

Evaluation of Comet Assay -Present State and Future

Date：March 11 (Tue), 2008, 13：30～17：00

Venue：NIHS hall, 3 rd floor at 11th building, NIHS

Co-chair Hajime Kojima (JaCVAM, NIHS)
Raffaella Corvi (ECVAM)

1. Overview — Brian Burlinson (Huntingdon, UK)
2. *In vivo* validation study — Yoshifumi Uno (Mitsubishi Tanabe Pharma)

Coffee Break

3. Practical issues to be discussed at protocol in this validation study — Andy Kraynak (Merk, USA)
4. *In vitro* validation study — Masamitsu Honma (NIHS)

Closing — Makoto Hayashi (NIHS)

JaCVAM 第1回ワークショップ

日時：平成20年3月11日（火）１０：００～１２：００
場所：国立医薬品食品衛生研究所　11号館3階講堂
名称：昨今の試験法ガイドラインを巡る国際動向

演題および演者
開会挨拶　　　　西島正弘(国立医薬品食品衛生研究所)
座長　　　　　　林　真（国立医薬品食品衛生研究所)
１．欧州の動向とECVAMの活動
　　　　　　　　Raffaella　Corvi　（ECVAM)
２．日本の動向とJaCVAMの活動
　　　　　　　　小島　肇（国立医薬品食品衛生研究所)
３．OECDテストガイドラインの概要、手続き、規制における活用、最近の動向
　　　　　　　　戸田英作（環境省環境保健部化学物質審査室)
閉会挨拶　　　　大野泰雄(国立医薬品食品衛生研究所)

INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (VERSION 14)

Issued by: the Validation Management Team (VMT)

Date: February 6, 2009 revised

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 hours).

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 (see note 1).

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 (see note 2).

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

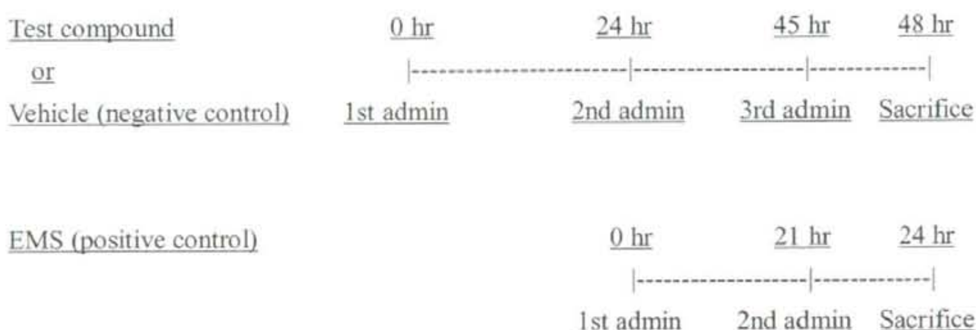
Compound	Dose (mg/kg)	Number of animals (see note 1)
Vehicle (negative control)	0	5
EMS (positive control)	200	5
Test compound	Low (1/4 of high)	5
Test compound	Medium (1/2 of high)	5
Test compound	High*	5

*High dose selection (see note 3): 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 three times orally by gavage, 24 and 21 hours apart, i.e. the second administration is 24 hours after the first administration, and the third administration is 21 hours after the second administration (at 3 hours before animal sacrifice). EMS will be administered twice orally by gavage at 24 hours and 3 hours before animal sacrifice. The administration regimes are summarized in a figure below, which enable us to detect comet and micronucleus at the same time (see note 4). 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~~ third administration of a test substance and at 3 hours after second treatment of EMS ~~treatment~~, consistent with Section C "Animal Welfare and 3Rs". The stomach and portions of the liver will be removed (see note 5). 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. For 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

Single cell preparation should be done within one hour after animal sacrifice. 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 (see note 6). ~~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

Slide preparation should be done within one hour after single cell 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 (see note 7)~~(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 during 20 minutes~~(Notes: the voltage may be defined more strictly, e.g. 0.7 exactly, based on the 3rd phase validation study results)~~, with a constant voltage at approximately ~~0.~~300 mA (see note 8). 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 . 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 1-8% 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 (see note 9)~~(Notes: to be re-evaluated after statistical analysis)~~. Approximately 10 areas/slide should be observed at 5 cells or less/field (see note 10)~~(may require dilution of cell suspension~~

~~during the single cell preparation process)~~, taking care to avoid any selection bias, overlap counting of cells, and edge areas of slides. 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 if the image analysis system can not properly score them (see note 11)~~This instruction means that the automatic image analysis results will be basically accepted as they are. However the following cases will be excluded from the comet data analysis: the automatic recognition is judged incorrect (e.g. the center of nucleus is not recognized correctly); and the staining of nucleus and/or migration is judged wrong. Add pictures in an appendix, and VMT will prepare a color atlas to instruct how to judge comet and hedgehog soon — 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, tail length in microns measured from the estimated edge of the head region closest to the anode (see note 12), 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]. (see note 13)~~Notes: at Atagawa meeting held on March 13-14, 2008, there were some discussions about necessity of tail length and Olive tail moment. Again, there was brief discussions about this point at Osaka meeting on February 4, 2009. As a tentative consensus, these parameters are no longer necessary to analyze statistically in this validation effort, because %DNA in tail seems a sufficient endpoint for validation. But data on tail length and tail moment will be collected to prepare for the future analysis)~~

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~~ both sides test (P<0.05) and Trend test (P<0.05) ~~is~~ will be ~~also~~ applied to "Effect" ~~for data analysis~~ to judge positive or negative as assay results. For the positive control group, the Student's t-test (P<0.05) will be applied to the "Effect".

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: