

Unclassified

ENV/JM/MONO(2014)14

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

15-Jul-2014

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

REPORT OF THE JACVAM INITIATIVE INTERNATIONAL PRE-VALIDATION STUDIES OF THE
IN VIVO RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC
CARCINOGENS

Series on Testing and Assessment

No. 195

This document is only available in PDF format.

JT03360433

Complete document available on OLIS in its original format

This document and any map included herein are without prejudice to the status of or sovereignty over any territory, to the delimitation of international frontiers and boundaries and to the name of any territory, city or area.



ENV/JM/MONO(2014)14
Unclassified

English - Or. English

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 195

**REPORT OF THE JACVAM INITIATIVE INTERNATIONAL PRE-VALIDATION STUDIES OF
THE IN VIVO RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF
GENOTOXIC CARCINOGENS**

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among **FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD**

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2014

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 34 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in eleven different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides; Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (<http://www.oecd.org/chemicalsafety/>).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

This publication is available electronically, at no charge.

Also published in the Series on Testing and Assessment link

**For this and many other Environment,
Health and Safety publications, consult the OECD's
World Wide Web site (www.oecd.org/chemicalsafety/)**

or contact:

**OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France**

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

© OECD 2014

Applications for permission to reproduce or translate all or part of this material should be made to: Head of Publications Service, RIGHTS@oecd.org, OECD, 2 rue André-Pascal, 75775 Paris Cedex 16, France

FOREWORD

This document presents the pre-validation report of the *in vivo* Mammalian Alkaline Comet Assay. It describes the 1st to 3rd phases (pre-validation steps) of the validation study, while the outcome of the main validation study (4th phase) is reported separately in document No. 196 in the Series on Testing and Assessment.

The project for developing a Test Guideline for the *in vivo* Mammalian Alkaline Comet Assay was proposed by Japan and included in the work plan of the Test Guidelines Programme in 2008. The pre-validation report and the validation report were submitted for peer review to a subgroup of the expert group on the comet assay in January 2013. This pre-validation report was endorsed by the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) at its 25th meeting in April 2013. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 7th July, 2014.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

TABLE OF CONTENTS

| | |
|---|----|
| PREFACE | 8 |
| ORGANIZATION FOR THE COMET ASSAY INTERNATIONAL VALIDATION STUDY | 10 |
| EXECUTIVE SUMMARY | 11 |
| 1. First Phase pre-validation Study | 13 |
| 2. Second Phase pre-validation Study | 18 |
| 3. Third Phase Pre-validation Study | 29 |
| 4. Recruitment of labs for the definitive (4th phase) validation study | 37 |
| 5. References | 37 |
| PHASE 1-3, APPENDIX 1: TESTING PROCEDURE OF IN VIVO ALKALINE COMET ASSAY FOR THE INTERNATIONAL VALIDATION STUDIES (DRAFT, VER. 4) | 38 |
| PHASE 1-3, APPENDIX 2: INTERNATIONAL VALIDATION OF THE IN VIVO RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (VERSION 12)..... | 47 |
| Phase 1-3, Appendix 3: INTERNATIONAL VALIDATION OF THE IN VIVO RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (Supplementary Protocol for 2nd Pre-validation Study)..... | 56 |
| PHASE 1-3, APPENDIX 4: INTERNATIONAL VALIDATION OF THE IN VIVO RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (VERSION 13)..... | 58 |
| Phase 1-3, Appendix 5: INTERNATIONAL VALIDATION OF THE <i>IN VIVO</i> RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS, Study Plan for 3 rd Phase Validation Study | 66 |
| PHASE 1-3, APPENDIX 6: INTERNATIONAL VALIDATION OF THE IN VIVO RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (VERSION 14)..... | 68 |
| APPENDIX 7: FINAL REPORT ON THE RECRUITMENT OF LABORATORIES FOR PARTICIPATION IN THE 4TH PHASE INTERNATIONAL <i>IN VIVO</i> COMET ASSAY VALIDATION STUDY (DRAFT)..... | 77 |
| ANNEX 1: LIST OF LABORATORY CODE AND LABORATORY NAME (REPRESENTATIVE) | 85 |
| ANNEX 2: INVITATION TO PARTICIPATE IN AN INTERNATIONAL..... | 86 |

PREFACE

The *in vivo* rodent alkaline Comet assay is used worldwide for detecting DNA damage as evidenced by strand breaks. The assay can be applied to the investigation of genotoxic potential of test chemicals, and is currently identified as a second *in vivo* genotoxicity assay in the ICH-S2(R1) guidance along with the more usual *in vivo* micronucleus test in bone marrow or peripheral blood. The Comet assay protocol has been discussed in the meetings of the International Workshop on Genotoxicity Testing (IWGT) and the International Comet Assay Workshop (ICAW), and consensus articles have been published.

The assay, however, has not been validated formally with a standardized study protocol. In addition, since reports on the predictive capability of the *in vivo* rodent Comet assay for carcinogenicity are limited, the investigation of predictive capability in multiple laboratories using one validated study protocol would be more useful to understand the overall performance of the assay. The Japanese Environmental Mutagen Society/the Mammalian Mutagenicity Study Group (JEMS/MMS) decided to organise an (international) collaborative study of the *in vivo* Comet assay in 2003, and conducted a preliminary collaborative study on the Comet assay procedure, notably a comparison of assay results between whole cells and isolated nuclei. At the same time, other groups of scientists expressed a wish to establish an OECD guideline for the Comet assay.

A co-ordinated validation effort for the *in vivo* Comet assay was therefore required, and so the Japanese Center for the Validation of Alternative Methods (JaCVAM) organized an international validation study commencing in April, 2006. This was done in cooperation with the U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Centre for the Validation of Alternative Methods (ECVAM) and JEMS/MMS.

The purpose of the four phase validation studies was to evaluate the ability of the *in vivo* Comet assay to identify genotoxic chemicals as a potential predictor of rodent carcinogenicity, to demonstrate acceptable intra- and inter-laboratory reproducibility, and to confirm its applicability domain. At the same time it was hoped to consider the value of the *in vivo* Comet assay as an alternative follow-up assay to more the commonly used *in vivo* rodent Unscheduled DNA Synthesis (UDS) assay. The ultimate goal of this validation effort is to establish an OECD guideline for the *in vivo* rodent alkaline Comet assay. This report describes the pre-validation studies required to prepare for the main validation study.

Purpose of each phase study:

- 1st phase pre-validation study: Optimization of protocol using positive control (Ethyl methanesulfonate: EMS) in 5 leading laboratories
- 2nd phase pre-validation study: Optimization of protocol and reproducibility using three coded test chemicals and EMS, the positive control, which were assayed in five leading laboratories in accordance with the Comet assay protocol-version 12
- 3rd phase pre-validation study: Optimization of protocol and reproducibility using three coded test chemicals and EMS, the positive control, which were assayed in four leading laboratories in accordance with the Comet assay protocol-version 13
- 4th phase-1st step validation study: Reproducibility (Within and between laboratory-variability): Four coded test chemicals and EMS, the positive control, were assayed in 13 laboratories in accordance with the Comet assay protocol-version 14.1
- 4th phase-2nd step validation study: Reliability (predictive capacity with carcinogenicity): 40 coded chemicals were assayed in 14 laboratories in accordance with the Comet assay protocol-version 14.2

In this pre-validation report, the VMT has summarized the results of the 1st to 3rd phase validation studies.

Hajime Kojima, Ph.D.,

Secretary General,

Japanese Center for the Validation of Alternative Methods (JaCVAM)

National Institute of Health Sciences (NIHS)

ORGANIZATION FOR THE COMET ASSAY INTERNATIONAL VALIDATION STUDY

Validation Management Team (VMT)

M. Hayashi (Chair, BSRC)
R. Corvi (ECVAM)
M. Honma (NIHS)
L. Schechtman (Consultant)
R. Tice (NTP/ICCVAM)
Y. Uno (MTPC, JEMS/MMS)
Secretariat
H. Kojima (NIHS/JaCVAM)

Consultation Team (CT)

N. Asano (JEMS/MMS)
P. Escobar (Boehringer-Ingelheim)
D. Lovell (Univ. of Surrey)
T. Morita (NIHS)
M. Nakajima (BSRC)
Y. Ohno (NIHS/JaCVAM)
T. Omori (Doshisha Univ.)

Participant Laboratory

14 Laboratories, Lead laboratory*

1. AstraZeneca (UK) : Catherine Priestley
2. Bayer Schering Pharma (Germany) : Uta Wirnitzer
3. BioReliance* (USA) : Kamala Pant, Buba Krsmanovic
4. Covance (UK) : Carol Beevers, Lucinda Williams
5. Food and Drug Safety Center* (JPN) : Kohji Yamakage
6. Health Canada (Canada) : James P. McNamee
7. Huntingdon Life Sciences* (UK) : Brian Burlinson
8. Integrated Laboratory System (USA) : Cheryl A. Hobbs
9. Johnson & Johnson (Belgium) : Marlies De Boeck
10. Merck* (USA) : Andrew R. Kraynak, Richard D. Storer
11. Mitsubishi Chemical Medience (JPN) : Hironao Takasawa
12. Novartis Pharma (Switzerland) : Ulla Plappert-Helbig
13. Sumitomo Chemical (JPN) : Sachiko Kitamoto
14. The Institute of Environmental Toxicology (JPN) : Kunio Wada

EXECUTIVE SUMMARY

The *in vivo* rodent alkaline Comet assay is used internationally to investigate the *in vivo* genotoxic potential of test chemicals. This assay, however, has not previously been formally validated. The Japanese JaCVAM, with cooperation of the U.S. NICEATM and ICCVAM, the European ECVAM, and the Japanese JEMS/MMS, organized an international validation study to evaluate the reliability of the assay using liver and glandular stomach as target organs following 2 or 3 daily treatments and to assess its ability to identify genotoxic carcinogens, thereby serving as a potential predictor of rodent carcinogenicity. The ultimate goal of this exercise is to establish an OECD guideline. The validation effort involved 4 phases. The study protocol was optimized in the 1st to 3rd phases (pre-validation steps). In the 4th phase-1st step, assay reproducibility was confirmed among laboratories using four coded test chemicals and the positive control ethyl methanesulfonate. In the 4th phase-2nd step, the predictive capability for discriminating between genotoxic carcinogens, genotoxic non-carcinogens, non-genotoxic carcinogens and non-genotoxic non-carcinogens was investigated using 40 selected coded chemicals with known genotoxic and carcinogenic activity. The validation study involved 14 laboratories in Japan, North America, and Europe. Classification of results as positive or negative was based on a pre-established statistical approach that considered dose response and increased migration at individual dose levels and histopathology results were also considered for final judgement. In the pre-validation steps reported here, we describe how the protocol was optimized, acceptability criteria established, positive control responses defined and some initial observations on intra- and inter-laboratory reproducibility made. The outcome of the main validation study (4th phase) is reported separately in document [ENV/JM/MONO(2014)10].

Title: Progress report of the JaCVAM initiative international validation study of the *in vivo* rodent alkaline Comet assay for the detection of genotoxic carcinogens: pre-validation studies in the 1st to the 3rd phases

Issued: Yoshifumi Uno, D.V.M., Ph.D. and a Validation Management Team (VMT) member

Notes: this document is prepared to summarize the *in vivo* Comet assay pe-validation process and results in the 1st to the 3rd phases. The methods are mentioned minimally in this document, because the details are described in the study protocol(s) and the study plan(s). An article for submission to a scientific journal will be provided separately based on this document, the study protocols and the study plans (and the other documents if necessary and available).

1. First Phase pre-validation Study

1-1. Purpose

The purposes of the 1st phase pre-validation study were to optimize the study protocol, and to investigate whether or not comparable data could be obtained in five leading laboratories when a consensus protocol was used. The original protocol (protocol-version (v.) 1) was kindly provided by Dr. Nakajima, a member of Biosafety Research Center (BSRC, formerly Anpyo-center), and the Japanese local committee members of this validation effort reviewed and revised it as v.4 for the validation purpose. The v.4 protocol was reviewed at the international kick-off meeting of the project held in August, 2006, and the 1st phase pre-validation study started with the v.4 protocol (Appendix 1) and the consensus* in the meeting

* The following are consensus items:

- 1) GLP: This study will be conducted in the spirit of Good Laboratory Practice.
- 2) Positive control: Ethyl methanesulfonate (EMS) will be used as a positive control. All laboratories will use the same batch of EMS.
- 3) Negative control (solvent/vehicle): In the absence of instruction from the VMT, 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, and corn oil.
- 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.
- 5) Preparation of reagent solution: The solutions will be prepared, stored appropriately, and used within a time that is appropriate for each solution. The concentration of agarose gel is 1.0% (w/v) for the bottom layer and 0.5% (w/v) low-melting point agarose gel. The DNA stain will be SYBR Gold.
- 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 another suitable anesthetic at 3 hours after the second dosing. The liver and the glandular stomach will be the main tissues collected.
- 7) Experimental design: The VMT proposed that unknown chemicals having weakly genotoxic activity *in vivo* such as benzo[*a*]pyrene and 2,6-diaminotoluene would be tested at 2 or 3 different dose levels, and EMS at 250 mg/kg. The participants, however, felt that such huge experimental design would be unfeasible and a simpler experimental design would be preferable as the first step of the pre-validation studies. Finally the VMT decided that EMS only would be examined at two dose levels, namely 100 and 200 mg/kg.
- 8) Isolated nuclei vs. whole cell: Single whole cells will be obtained using mincing or scraping.
- 9) Slide preparation, electrophoresis, staining: The DNA will be left to unwind for 20 minutes. After alkali unwinding, the slides will be electrophoresed at 0.7 to 1 V/cm, and at amperage of 0.25-0.30 A. At this stage the goal is to achieve 5-10% migration for both liver and stomach, although this may be refined in the later phases. The electrophoresis solution should be maintained at a constant temperature $\pm 2^{\circ}\text{C}$. Both room temperature and between 2 and 10°C is being considered. To check for any important 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.
- 10) Comet visualization and analysis: Fifty comets per slide will be analyzed, with at least two slides scored per sample.
- 11) Endpoint and analysis (including discussion about which image analysis [IA] or categorization is a better approach for analysis): As the endpoint, the percentage of DNA in tail will be calculated using an image analyzer system.

- 12) Cytotoxicity (histopathology vs. others): Dr. Tice recommended the Neutral diffusion assay, but finally it was decided that histopathology would be applied to evaluation of cytotoxicity based on a consensus in the IWGT meeting on Comet assay (Burlinson, et al, 2007). Only when a positive result in comet analysis is obtained in a tissue, histopathology of tissue samples taken at the same time as for comet analysis will be examined for that tissue according to the SOP in each testing facility.

In parallel with the progress of 1st phase experiments, the protocol was being revised based on comments from the validation project members. The resultant v.10 protocol was presented at the 2nd international meeting held in December, 2006, reviewed by the participants, and then fixed as v.12. The protocol-v.12 was applied to the 2nd phase pre-validation study.

1-2. Experimental period

September-November, 2006

1-3. Participant laboratories

Five laboratories* participated in the 1st phase pre-validation study, as the leading laboratories that have a lot of experience with the Comet assay.

* BioReliance (Lab.2), Biosafety Research Center (BSRC, Lab.5), Food and Drug Safety Center (FDSC, Lab.4), Huntingdon Life Sciences (HLS, Lab.3), Merck Research Laboratories (Merck, Lab.1)

1-4. Animal species

Rats were selected in this validation effort because they are routinely used in toxicology studies.

1-5. Test chemical, vehicle, dose level, and administration

Ethyl methanesulfonate (EMS) was selected, because it is a well-known genotoxic chemical. EMS was dissolved in physiological saline, and administered to male Crj:CD(SD) rats at the dose levels of 100 and 200 mg/kg on 2 occasions (21 or 24 hr interval*) with oral administration.

* The difference between laboratories was caused due to unclear description on the dosing regimen in the protocol. The 21 hr interval was used in all laboratories except for Merck.

1-6. Organs analyzed

Liver and stomach (glandular stomach) were selected for this pre-validation effort, because the former is the primary organ for the metabolism of absorbed chemicals, and the latter is a site of first contact of chemicals following orally administration. The analysis of these organs was recommended for screening of genotoxic chemicals in the previous discussion in ICAW (Hartmann, et al., 2003).

1-7. Data analysis

Initially the statisticians looked for a suitable method to investigate the validity of data within/between laboratories. Dr. Omori, the main statistician, proposed and tried some approaches for data analysis in the 1st phase pre-validation study, e.g. by using difference of means of %DNA in tail between the negative control group and the positive control group. Three key conceptual terms, "Endpoint", "Estimate" and "Effect" were actually defined (below) and used for the data analysis in and after the 2nd phase pre-validation study, but, for the convenience of explanation in this document, these terms are also used to show results of the 1st phase pre-validation study.

Briefly, "Endpoint" is defined as the individual observed values for a parameter such as % DNA in tail. "Estimate" is defined as a mean calculated from the Endpoint values in each animal. "Effect" is defined as the difference (hereafter designated as Effect (diff.)) or ratio (hereafter designated as Effect (ratio)) of an average of the Estimates between a negative control group and a treatment

group. A key step in validation studies is to investigate how large is the variation that exists among the data on the same chemical tested in several testing facilities, i.e. to determine the inter-laboratory reproducibility, and Effect is considered as the most appropriate criterion to evaluate this variation of Comet assay responses among the different testing facilities.

Dunnett's test was mainly used for the statistical analysis, because this test is commonly applied to data analysis of toxicity studies. In the 1st and the 2nd phase pre-validation studies, the one-sided test ($p < 0.05$) was used, because only increases in comet parameters (% tail DNA) were expected. In contrast, the two-sided test ($p < 0.05$) was used in the 3rd phase pre-validation study (and the 4th phase validation study), because, by virtue of the chemicals tested, both increases and decreases in Comet parameters could be detected.

The VMT has identified through the 1st to the 3rd phase pre-validation studies that Effect (diff.) is more appropriate for the comparison of variation/reproducibility than Effect (ratio), because Effect (ratio) depends on the magnitude of negative control values (i.e. lower negative control values produce easily higher Effect (ratio)) and may mislead the evaluation of responses induced following a test chemical administration. Therefore Effect (diff.) is mainly used to reveal the results of data analysis in this document.

1-8. Results

Figs. 1 and 2 show Effect (diff.) of %DNA in tail in liver and glandular stomach respectively following EMS treatment in the 5 laboratories. All laboratories showed statistically significant increases (Dunnett's one-sided test, $p < 0.05$) in the liver and the stomach at both dose levels of EMS. The magnitude of Effect (diff.) varied between laboratories, e.g. Lab.5 showed approximately 2-fold higher differences between EMS and control responses than Lab.1 at both dose-levels of EMS.

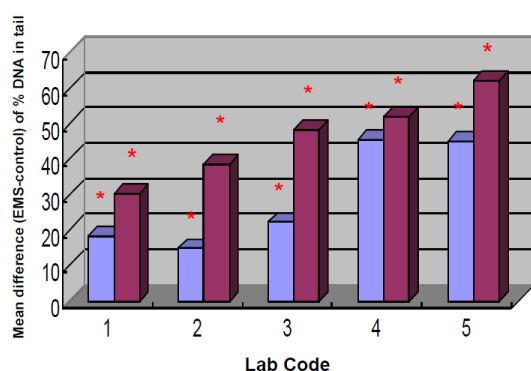


Fig. 1 Effect (diff.) in the liver after treatment with EMS. Each column is calculated with Estimates (diff.) from five animals. Blue and red columns show the results of 100 and 200 mg/kg groups, respectively. Asterisks show statistically significant increases with Dunnett one-sided test ($p < 0.05$).

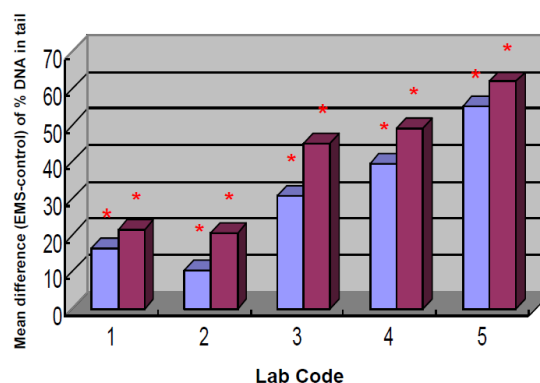


Fig. 2 Effect (diff.) in the stomach after treatment with EMS. Each column is calculated with Estimates (diff.) from five animals. Blue and red columns show the results of 100 and 200 mg/kg, respectively. Asterisks show statistically significant increases with Dunnett one-sided test ($p < 0.05$).

As well as %DNA in tail, Effects (diff.) on tail length and Olive tail moment (Olive, et al., 1990) were also collected and analyzed. Fig. 2A shows Effects (diff.) of the three parameters, %DNA in tail, tail length and Olive tail moment. Dose-dependent slopes of Effects (diff.) are noted in %DNA in tail and Olive tail moment, but not in tail length. The slopes of Effects (diff.) seem more comparable between laboratories in %DNA in tail than Olive tail moment, especially in the stomach.

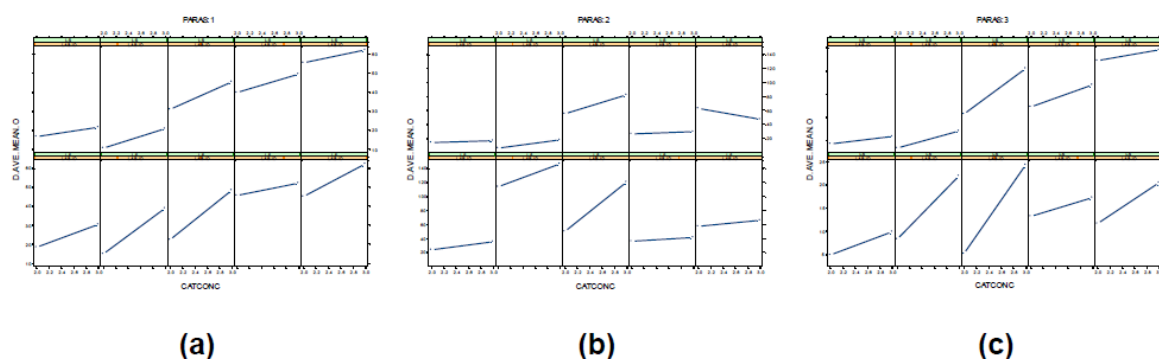


Fig. 2A Comparison of three parameters, (a) %DNA in tail, (b) tail length, and (c) Olive tail moment. Lines in the figures connect Effects (diff.) in the 100 and 200 mg/kg groups of EMS, which show the dose-dependency of responses. The upper five in each figure shows Effects (diff.) in the stomach in five laboratories, and the lower five shows them in the liver.

1-9. Discussion

Since %DNA in tail is considered as the most reliable parameter to show Comet assay results quantitatively (Hartmann, et al., 2003, Kumaravel and Jha, 2006), the VMT mainly focused on the results with this parameter. In addition, %DNA in tail has the advantage that is more easily handled from the viewpoint of statistical analysis than tail length or Olive tail moment, because the maximum value of %DNA in tail is fixed as 100% (cf. the other two parameters may theoretically show infinity).

The magnitude of Effects (diff.) in %DNA in tail seems to vary between laboratories in the liver and the stomach after 2x administration of EMS to animals, but all laboratories showed statistically significant increases in the Effect (diff.). Although the slopes of the responses (i.e. from 100 to 200 mg/kg of EMS) were similar for most laboratories, Laboratory 2 consistently showed a large increase in effect at 200 mg/kg compared to 100 mg/kg in both liver and stomach than the other laboratories. The reasons for this are not known. However, the VMT considered that, since the protocol had not been optimized for validation purposes at this stage, the comparability of results between the laboratories was acceptable. This indicates that the essential methods of the *in vivo* Comet assay are well established (Tice, et al., 2000, Hartmann, et al., 2003, Burlinson, et al., 2007), and, as the protocol-v.4 (and some consensus items) used in this study was prepared in consideration of the previous consensus, the consistency of results obtained was acceptable.

The VMT concluded that, by revision of protocol-v.4, based on comments from the project members, the study protocol could be almost optimized for validation purposes as the Comet assay protocol-v.12 (Appendix 2). In addition, the VMT decided that EMS at 200 mg/kg would be used as a positive control in the further pre-validation and validation studies.

1-10. Further discussion: practical concerns about Comet assay procedures

The following points were practical concerns about Comet assay procedures that had been recognized through the experiments in the 1st phase pre-validation study. A few issues were discussed and resolved in the 2nd international meeting, but others would be resolved through the progress of pre-validation studies.

- 1) Is it necessary to add EDTA into low-melting point agarose gel to reduce the background of negative controls? A consensus of the validation study members was that it would be unnecessary to add EDTA, because the negative control range can be effectively controlled without the addition of EDTA.

- 2) Should the temperature of electrophoresis buffer be below 10°C or room temperature (ca. 20°C)? The validation study members commented that it is possible to effectively control the negative control range at both temperatures, but that positive responses may increase more dramatically at room temperature than below 10°C (increase in sensitivity). One laboratory pointed out a practical issue, namely that the agarose gel often came off the glass slides at room temperature. A consensus of the members was that the experiments in these pre-validation and validation studies should be conducted below 10°C, because this can be defined and controlled more accurately than room temperature, and a more controlled condition is preferable for the purposes of validation.
- 3) Regarding the negative control range in %DNA in tail for the liver and stomach, is a lower value better or should it be a little bit higher? To be discussed later.
- 4) Effect of cytotoxicity, histopathology or neutral diffusion assay? To be discussed later.
- 5) Statistics, and data acceptance criteria. To be discussed later.
- 6) Further reduction of animal use, especially for the positive control group. Currently the ICH-S2(R1) guidance (2012) reveals that it is acceptable not to include a concurrent positive control group in the *in vivo* micronucleus test when there are the sufficient historical control data in the testing facilities. The possibility of implementing this approach in the Comet assay was examined using the data of positive control groups in the 3rd phase pre-validation study.

2. Second Phase pre-validation Study

2-1. Background and Purpose

In the 1st phase pre-validation study, VMT considered that the comet responses induced by EMS in the five leading laboratories were sufficiently comparable to be able to proceed to the next phase. However it was a result from just one trial, and the reproducibility and robustness of positive results with EMS should be verified further by performing experiments with the revised Comet assay protocol-v.12 (Appendix 2). If EMS administration to rats always gave positive results in the liver and the stomach in all testing facilities in all experiments using the revised protocol, the VMT would conclude that the Comet assay protocol-v.12 was acceptable for the definitive validation studies.

The first purpose of the 2nd phase pre-validation study was to optimize the study protocol and to examine the reproducibility and robustness of positive control results with EMS when experiments were conducted in accordance with the Comet assay protocol-v.12. This would examine the acceptability of the protocol-v.12 for the definitive validation studies. The second purpose was to examine the variability of Effects among the five testing facilities, and the range of variability of Effects obtained from this study would be applied as data acceptance criteria for the definitive validation studies. Additional experiments with a small number of coded test chemicals were also to be conducted and to contribute to assessment of the acceptability of protocol-v.12.

The details are described in the 2nd phase pre-validation study plan (Appendix 3).

2-2. Experimental period

May-December, 2007

2-3. Participant laboratories

Five laboratories* participated in the 2nd phase pre-validation study.

* BioReliance (Lab.2), BSRC (Lab.5), FDSC (Lab.4), HLS (Lab.3), Merck (Lab.1)

2-4. Success criteria

- 1) To obtain positive results with the positive control chemical in all testing facilities using protocol-v.12.
- 2) To determine a range of variability of "Effect" so as to be able to apply acceptability criteria in the definitive validation studies.

2-5. Test chemicals, vehicles, dose levels, and administration

EMS, a positive control, was dissolved in physiological saline and dosed at 200 mg/kg on one or two occasions* by oral gavage. Coded test chemicals, namely acrylamide (AA), 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT) were also used in this phase. These coded compounds were administered twice, 21 hours apart, and tissues sampled 3 hours after the second dose. The VMT expected that AA and 2,4-DAT would show weakly positive results at least in the liver, and 2,6-DAT would show a negative result at least in the liver. VMT directed the participating laboratories to use vehicles and dose levels as follows:

- for AA, physiological saline, and 12.5, 25 and 50 mg/kg;
- for 2,4-DAT, 2% tween 80 solution, and the highest dose level of 500 mg/kg in the dose-finding study;
- for 2,6-DAT, corn oil, and the highest dose level of 500 mg/kg in the dose-finding study.

* The difference was caused due to unclear description on the dosing regimen in the protocol. EMS was administered once to rats in all laboratories except for FDSC (Lab. 4).

Data on each coded test compound would be obtained from the five participating laboratories, giving 5 sets of data on each compound. Simultaneously, as each experiment includes negative

(vehicle) and positive control groups, three sets of data from the control groups (one set with each coded chemical) would be obtained from each testing facility, giving a total of 15 sets of vehicle and positive control group data. By using the vehicle/positive control data, the reproducibility and robustness would be examined.

2-6. Results

2-6-1. Vehicle control group

Fig. 3 shows the means of %DNA in tail (Estimate) for the vehicle control groups in the liver. The variability within and between laboratories was quite small. Although the Estimates in Lab.4 seemed to show slight variation between three experiments compared to the other laboratories, the VMT considered that the data indicated acceptable variability for the vehicle control groups because such variation is commonly observed in Comet assay. The mean and standard deviation (S.D.) calculated with the 15 Estimates was $3.8 \pm 1.6\%$. If the vehicle control range is determined as the mean ± 3 S.D., the range is 0 to 8.6%.

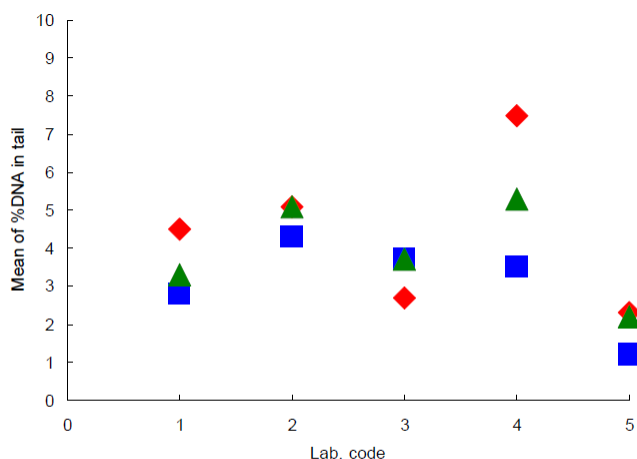


Fig. 3 Mean of %DNA in tail (Estimate) in the vehicle control group in the liver. Each symbol shows the mean of %DNA in tail in five rats per experiment.

Fig. 4 shows the means of %DNA in tail (Estimate) for the vehicle control groups in the stomach. The variability within and between laboratories seems small except for Lab.5. The mean and S.D. calculated with the 15 Estimates are $14.4 \pm 5.4\%$, and if the data from Lab 5 are excluded, the values calculated with the remaining 12 Estimates are $12.5 \pm 2.3\%$. The ranges (mean ± 3 S.D. values) are 0 to 30.6% with 15 Estimates and 5.6 to 19.4% with 12 Estimates. Although the Estimate values in Lab.5 seem to show wider variation between the three experiments than seen in the other laboratories, the VMT considered that such variability would be acceptable, because it occurred for the stomach, where wider variation is commonly seen even in leading laboratories that have sufficient experiences of the Comet assay.

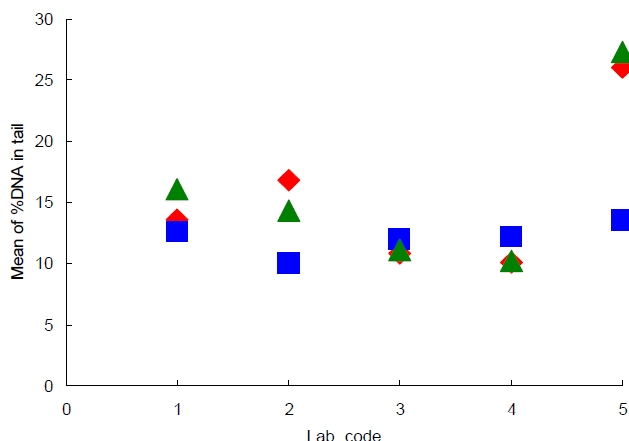


Fig. 4 Mean of %DNA in tail (Estimate) in the vehicle control group in the stomach. Each symbol shows the mean of %DNA in tail in five rats per experiment.

Based on those ranges and above the considerations, the VMT decided that the acceptable vehicle control range would be 1.0 to 8.0% in the liver, and 1.0 to 30.0%, but preferably 1.0 to 20.0%, in the stomach. The reason why the lower limit is 1.0% rather than 0% is to enable the detection of decreases in %DNA in tail by chemical treatment, which would be expected in cross-linker-type genotoxicants.

2-6-2. Positive control group

Figs. 5 and 6 show respectively Effect (diff.) and Effect (ratio) values of mean %DNA in tail between the vehicle control group and the positive control (200 mg/kg EMS) group in the liver. All of the Effect (diff.) and Effect (ratio) values show statistical significance with unpaired t-test (one-sided, $p < 0.05$). The variability between laboratories seems large in both Effect (diff.) and Effect (ratio), i.e. about 40% higher Effect (diff.) in Labs.2 and 3 compared to Lab.1, and about 4-fold higher Effect (ratio) in Lab.3 compared to Lab.1. The VMT determined that these data from leading laboratories with a lot of experiences of Comet assay should be accepted without limitation, as far as the assay procedure was based on our study plan and protocol. Therefore, the minimum values in Effect (diff.) and Effect (ratio) were considered 4.6% and 2-fold in the liver, respectively. To appreciate the variation among three experiments within a given laboratory, the Coefficient of Variation (CV) may be helpful and thus CVs were calculated as follows: for Effect (diff.), 59% (Lab.1), 45% (Lab.2), 1% (Lab.3), 17% (Lab.4) and 25% (Lab.5); and, for Effect (ratio), 55% (Lab.1), 50% (Lab.2), 17% (Lab.3), 19% (Lab.4) and 39% (Lab.5).

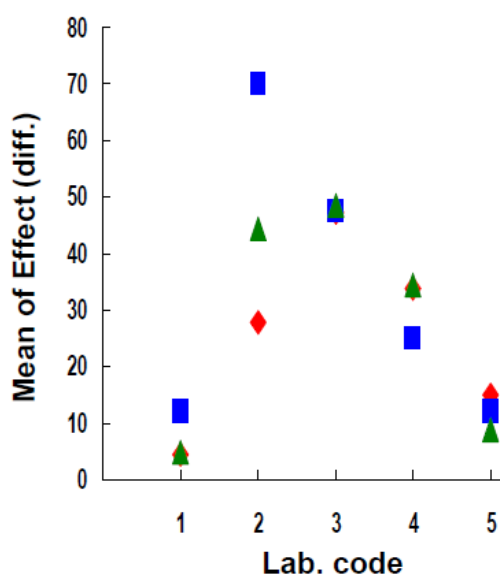


Fig. 5 Effect (diff.) in the liver after treatment with EMS. Each laboratory conducted three independent experiments, and each symbol shows the mean of each experiment. The minimum difference is 5% in Lab.1, and the maximum is 70% in Lab.2.

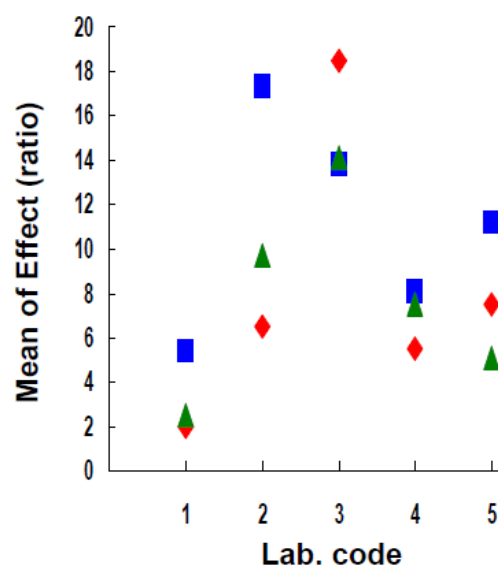


Fig. 6 Effect (ratio) in the liver after treatment with EMS. Each laboratory conducted three independent experiments, and each symbol shows the mean of each experiment. The minimum ratio is 2-fold in Lab.1, and the maximum is 18.5-fold in Lab.3.

Figs. 7 and 8 show respectively Effect (diff.) and Effect (ratio) values for % DNA in the tail in the stomach. Effect (diff.) and Effect (ratio) values show statistical significance with unpaired t-test (one-sided, $p < 0.05$) except for each one experiment in Labs.1 and 5. The variability between laboratories seems large in Effect (diff.), i.e. about 30% higher in Labs.2 and 3 compared to Lab.1, but not in Effect (ratio), i.e. about 2-fold higher in Labs.2 and 3 compared to Lab.1. The minimum values of Effect (diff.) and Effect (ratio) with statistical significance were 18.6% and 2.4-fold in the stomach, respectively. Among two or three experiments within each laboratory, the CV were calculated for this trial as follows: for Effect (diff.), 6%* (Lab.1), 33% (Lab.2), 2% (Lab.3), 11% (Lab.4) and 20%* (Lab.5); and, for Effect (ratio), 7%* (Lab.1), 48% (Lab.2), 3% (Lab.3), 10% (Lab.4) and 16%* (Lab.5) (* These CV values were calculated with only two of the experimental data sets because one experimental data set in each of Labs.1 and 5 was excluded due to no statistically significant increase in the EMS group. These failures to induce positive responses in the stomach are discussed later.).

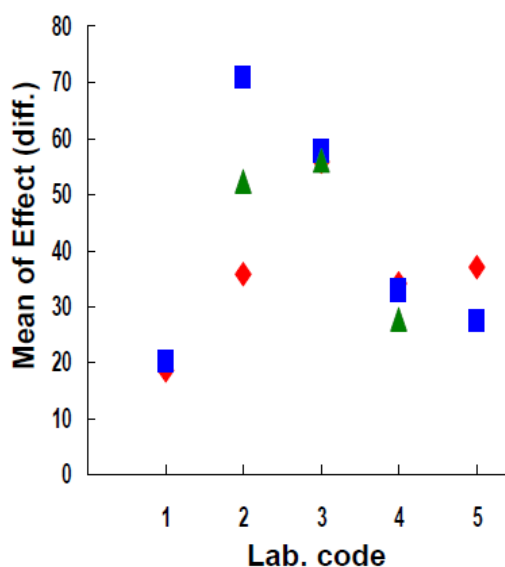


Fig. 7 Effect (diff.) in the stomach after treatment with EMS. Each laboratory conducted three independent experiments, but the data from one experiment in Labs. 1 and 5 are excluded due to no significant increase. Each symbol shows the mean of each experiment. The minimum difference is 19% in Lab.1, and the maximum is 71% in Lab.3.

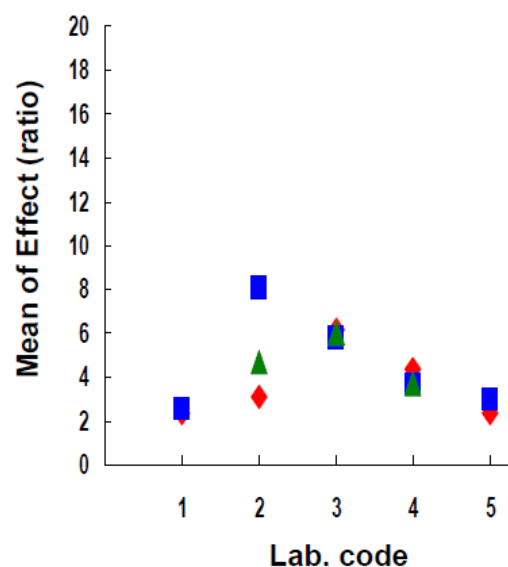


Fig. 8 Effect (ratio) in the stomach after treatment with EMS. Each laboratory conducted three independent experiments, but the data from one experiment in Labs. 1 and 5 are excluded due to no significant increase. Each symbol shows the mean of each experiment. The minimum ratio is 2.4-fold in Lab.1, and the maximum is 8.1-fold in Lab.2.

In consideration of above results, mainly from the liver, VMT decided that the acceptable ranges of Effect (diff.) and Effect (ratio) were 5% or higher and 2-fold or higher, respectively. In addition, the acceptable CV calculated with two or more independent experiments was defined as 50% or less in Effect (ratio), which was set for Effect (ratio) only because Effects (ratio) were relatively higher (i.e. varied) than Effects (diff.). However, the VMT considers that the CV may be less meaningful when calculated with only a few experimental data.

2-6-3. Coded test chemical groups

1) Acrylamide (AA)

Fig. 9 shows Effect (diff.) of mean %DNA in tail between the vehicle (V) control group and the treatment group (low, middle or high – L, M or H) in the liver, i.e., values of L minus V, M minus V, and H minus V for each laboratory, and Fig. 10 shows dose-dependency of the Estimate. Except for Lab.4 (discussed later), all of Effect (diff.) values were statistically significant for at least one dose group with Dunnett's test (one-sided, $p < 0.05$) and the linear trend test was also significant (one-sided, $p < 0.05$).

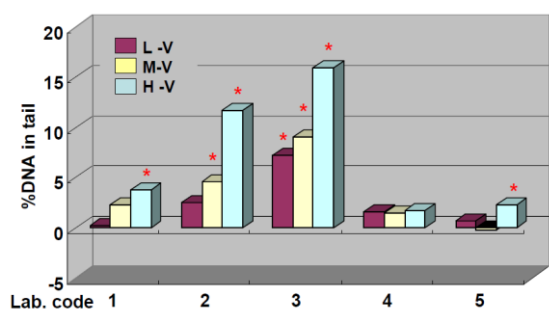


Fig. 9 Effect (diff. of %DNA in tail) in Liver: Acrylamide

* Dunnett (P<0.05; one-sided)
 Test condition
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 12.5, 25, 50 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

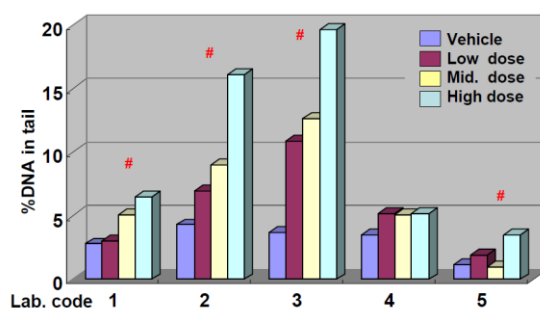


Fig. 10 Mean of %DNA in tail in Liver: Acrylamide

Linear trend (P<0.05)
 Test condition
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 12.5, 25, 50 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

Fig. 11 shows Effect (diff.) of mean %DNA in tail between the vehicle (V) control group and the treatment group (low, middle or high – L, M or H) in the stomach, i.e., values of L minus V, M minus, V, and H minus V for each laboratory, and Fig. 12 shows dose-dependency of the Estimate. Except for Lab.1 (discussed later), all of Effect (diff.) values were statistically significant for at least one dose group with Dunnett's test (one-sided, $p < 0.05$), and, except for Labs.1 and 4 (discussed later), the dose-dependency was demonstrated with statistical significance in the linear trend test (one-sided, $p < 0.05$).

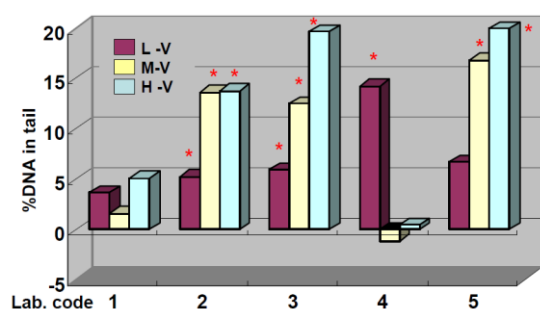


Fig. 11 Effect (diff. of %DNA in tail) in Stomach: Acrylamide

* Dunnett (P<0.05; one-sided)
 Test condition
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 12.5, 25, 50 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

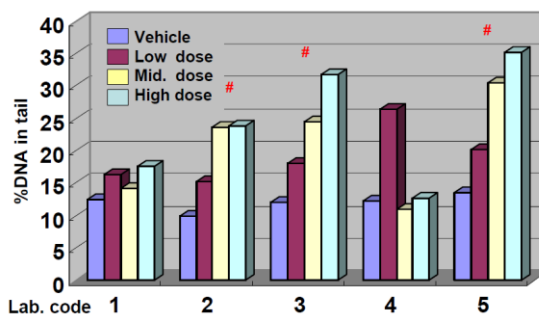


Fig. 12 Mean of %DNA in tail in Stomach: Acrylamide

Linear trend (P<0.05)
 Test condition
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 12.5, 25, 50 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

Although Labs 1 and 4 did not satisfy all of the criteria for a positive response in liver and stomach, based on the judgment criteria of the study protocol, i.e. a statistically significant change in at least one dose group, AA was judged to be positive in the liver and/or the stomach in all laboratories.

2) 2,4-Diaminotoluene (2,4-DAT)

Fig. 13 shows Effect (diff.) of mean %DNA in tail between the vehicle (V) control group and the treatment groups (low, middle or high dose – L, M or H) in the liver, and Fig. 14 shows dose-dependency of the Estimate. Statistically significant increases in Effect (diff.) were observed in the liver in Labs.2, 3 and 5, and the dose-dependency was noted in Labs.2 and 3. The lack of response in Lab 4 is discussed later.

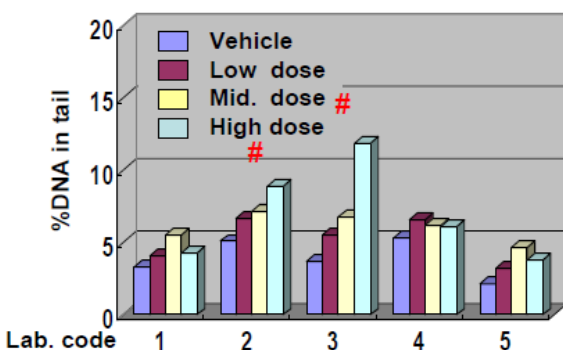
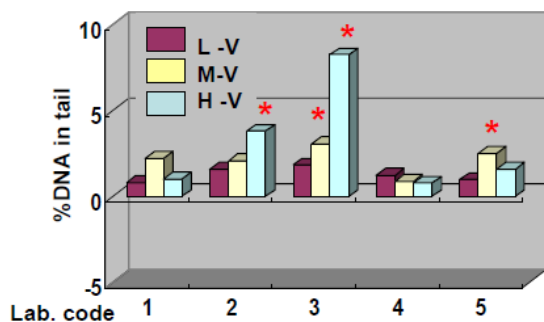


Fig. 13 Effect (diff. of % DNA in tail) in liver: 2,4-DAT

* Dunnett (P<0.05; one-sided)

Fig. 14 Mean of % DNA in tail) in liver: 2,4-DAT

Linear trend (P<0.05)

- Test condition for both chemicals
- ✓ Animal: CD(SD) rat, male, 5 animals/group
 - ✓ Dose: 0, 125, 250, 500 mg/kg, p.o.
 - ✓ Administration: Twice (21 hours interval)
 - ✓ Cell preparation: 3 hours after 2nd administration

- Test condition for both chemicals
- ✓ Animal: CD(SD) rat, male, 5 animals/group
 - ✓ Dose: 0, 125, 250, 500 mg/kg, p.o.
 - ✓ Administration: Twice (21 hours interval)
 - ✓ Cell preparation: 3 hours after 2nd administration

Fig. 15 shows Effect (diff.) of mean %DNA in tail between the vehicle (V) control group and the treatment groups (low, middle of high – L, M or H) in the stomach, and Fig. 16 shows dose-dependency of the Estimate. Since Labs.1 and 5 could not detect EMS (the positive control) as positive, data on 2,4-DAT in the stomach were not accepted in those two laboratories. Statistically significant increases in Effect (diff.) were observed in the stomach in Labs.2 and 3, and the dose-dependency was noted in Lab.3.

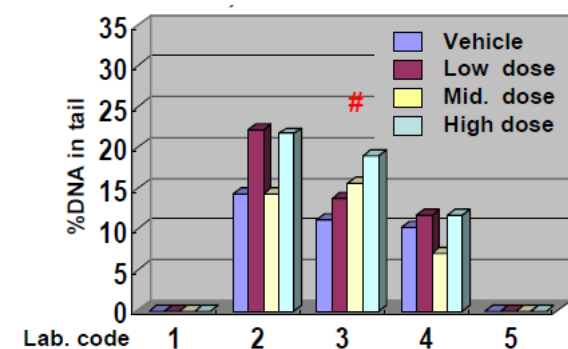
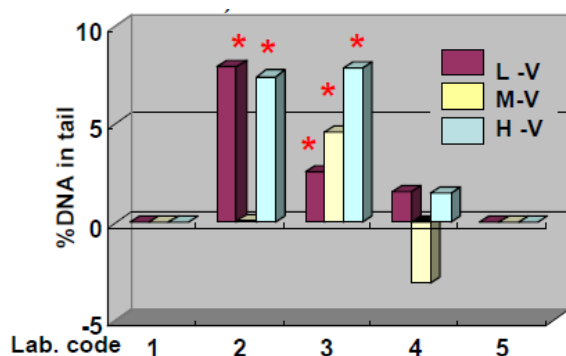


Fig. 15 Effect (diff. of % DNA in tail) in stomach: 2,4-DAT
 * Dunnnett (P<0.05; one-sided)

Fig. 16 Mean of % DNA in tail) in stomach: 2,4-DAT
 # Linear trend (P<0.05)

Test condition for both chemicals
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 125, 250, 500 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

Test condition for both chemicals
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 125, 250, 500 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

Based on the judgment criteria of the study protocol, i.e. a statistically significant change in at least one dose group, 2,4-DAT was judged to be positive in the liver in three of five laboratories and in the stomach in one of three laboratories. The inconsistent results are discussed below.

3) 2,6-Diaminotoluene (2,6-DAT)

Fig. 17 shows Effect (diff.) of mean %DNA in tail between the vehicle (V) control group and the treatment groups (low, middle or high – L, M or H) in the liver, and Fig. 18 shows dose-dependency of the Estimate. Statistically significant increases in Effect (diff.) were observed in the liver in Labs.2 and 3, and the dose-dependency was noted in Lab.3.

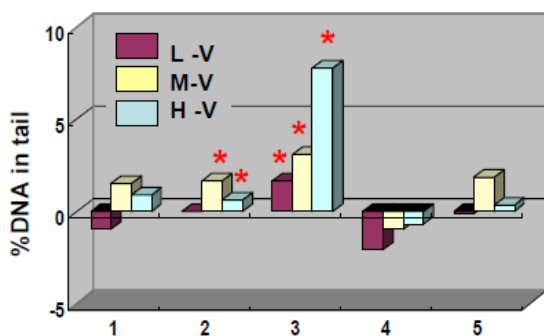


Fig. 17 Effect (diff. of % DNA in tail) in liver: 2,6-DAT

* Dunnett (P<0.05; one-sided)

Test condition for both chemicals
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 125, 250, 500 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

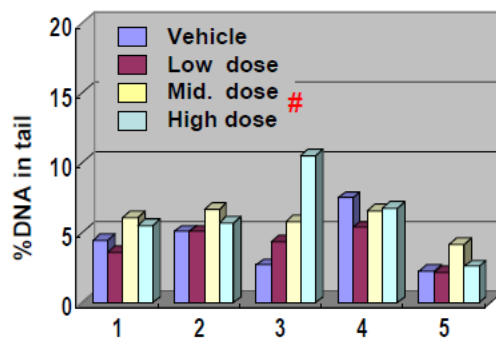


Fig. 18 Mean of % DNA in tail) in liver: 2,6-DAT

Linear trend (P<0.05)

Test condition for both chemicals
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 125, 250, 500 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

Fig. 19 shows Effect (diff.) of mean %DNA in tail between the vehicle (V) control group and the treatment groups (low, middle or high – L, M or H) in the stomach, and Fig. 20 shows dose-dependency of the Estimate. Statistically significant increases in Effect (diff.) were observed in the stomach in Labs.2 and 3 with dose-dependency.

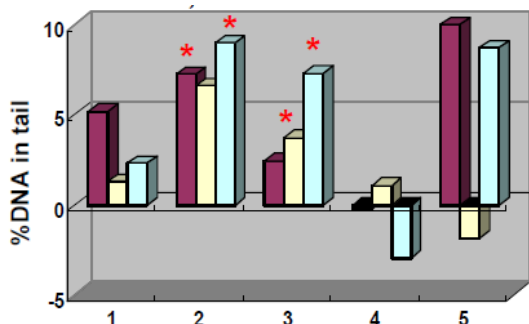


Fig. 19 Effect (diff. of % DNA in tail) in stomach: 2,6-DAT

* Dunnett (P<0.05; one-sided)

Test condition for both chemicals
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 125, 250, 500 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

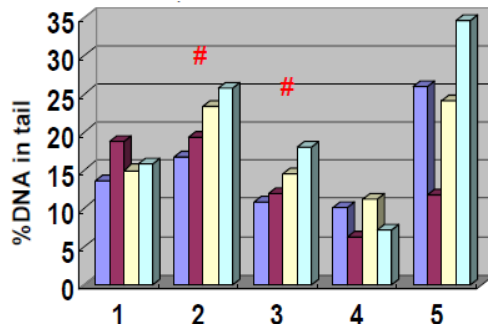


Fig. 20 Mean of % DNA in tail) in stomach: 2,6-DAT

Linear trend (P<0.05)

Test condition for both chemicals
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 125, 250, 500 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

Based on the judgment criteria of the study protocol, i.e. a statistically significant change in at least one dose group, 2,6-DAT was judged to be positive in the liver and the stomach in two of five laboratories. The inconsistency of the results is discussed below.

2-7. Discussion

1. EMS treatment induced statistically significant increases in Effect (diff.) in the liver in all laboratories, but failed to produce statistically positive responses in the stomach in each one of three independent experiments in two of five laboratories. The number of EMS treatments, dosing on one or two occasions, seemed to have no impact on the magnitude of the responses and did not explain the failures. The failure of 2 laboratories to obtain positive results in the stomach with

EMS was considered to be a critical deviation from one of the two success criteria of the 2nd phase pre-validation study. Thus VMT could not conclude that the Comet assay protocol-v.12 would be acceptable for the definitive validation studies.

2. After discussion with participant laboratories at the 4th international meeting held in March, 2008 about why EMS failed to produce positive results in two of 15 experiments in the stomach, the study protocol was revised to v.13 for a more detailed description of methods. The revised points were as follows: a) voltage of electrophoresis was changed 0.7-1.0 V/cm into 0.7 V/cm; b) preparation timing of cells and slides was defined as cell preparation within 1 hour after animal sacrifice and slide preparation within 1 hour after the cell preparation; c) scoring method of Comet was defined, i.e. approximate 10 areas/slide with 5 cells or less/field of scoring, and any selection bias, overlap counting of cells and edge area observation of slides should be avoided; and d) average DNA migration in the negative control changed from 1-15% to 1-8% for the liver and 1-30% (preferably 1-20%) for the stomach. In addition, it was arranged among the participant laboratories that the electrophoresis duration would be basically 20 minutes in all experiments.
3. The other points discussed at the 4th international meeting were as follows: a) was it needed to increase the dose level of EMS as the positive control in order to get reproducible positive responses more easily? This proposal, however, was not accepted by the VMT, because a threshold response in the positive control group would be preferable to clarify the acceptability of assay results with a test chemical. Thus 200 mg/kg of EMS was considered to be appropriate as the positive control in Comet assay; and b) was the statistical analysis sufficient to call positive responses? When S.D. values were very small, statistical significance was easily observed even if the magnitude of responses was very slight. In such cases, the biological significance of responses might be questionable. In this validation study, however, the VMT decided that positive/negative calls would be simply done with the results of statistical analysis in order to eliminate any subjective biases. After the validation study, the judgment method using only the statistical analysis may be re-evaluated and revised if necessary.
4. In this phase, the VMT tried to examine the three coded chemicals in addition to investigating the reproducibility of responses with the positive control EMS, because the VMT believed that the protocol-v.12 would be optimized for the validation study and good results would be anticipated for EMS. However, as mentioned above, the protocol-v.12 needed to be revised. Therefore, interpretation of the results with the coded test chemicals with the protocol-v.12 could not be clearly made although some discussion might give helpful information to establish the optimized protocol for the definitive validation study.
5. AA gave some positive responses in the liver and/or the stomach in all laboratories, and the results seemed qualitatively coincident between laboratories. However the magnitudes of responses were different between laboratories. 2,4- and 2,6-DAT gave qualitatively inconsistent results between laboratories. The main reasons for the inconsistent responses might be related to some protocol issues, and might be resolved by the modifications introduced into v.13. Other possible reasons may be variation in the systemic exposure levels of these test chemicals and/or the susceptibility of animals used. Since the dose levels of AA were specified by VMT in advance, they may not be appropriate for all laboratories, who might have selected different and more suitable dose levels based on toxic signs in animals. Similar consideration could apply to the results with 2,4- and 2,6-DAT, because VMT specified in advance that the laboratories should use the top dose levels of 500 mg/kg in the dose-finding studies, and subsequently 500 mg/kg was chosen as the top dose for both test chemicals in the Comet assay in all laboratories.
6. AA would be expected to be positive in the liver, but not in the stomach, because genotoxicity of AA is induced by the DNA-reactive metabolite, glycidamide (Kumaravel and Jha, 2006). Four laboratories (except for Lab.4) reported increases in % tail DNA in the liver with statistical significance of both Dunnett's and linear trend tests. Three laboratories (except for Labs. 1 and 4)

also reported statistically significant increases % tail DNA in the stomach with both statistical methods. It was unclear why no increased % tail DNA in the liver was observed in Lab.4. As discussed above, the dose levels and/or susceptibility of animals might be the reasons. Increased % tail DNA was also noted in the stomach at Labs.2, 3 and 5. Since the wide and rapid distribution of AA and/or glycidamide to many tissues has been reported (Miller et al., 1982, Maniere et al., 2005), increased % tail DNA in the stomach might be due to the effects of glycidamide rather than AA itself.

7. Regarding 2,4- and 2,6-DAT, both chemicals essentially exhibit genotoxic activity in other test systems. 2,4- and 2,6-DAT have been often used as reference analogue chemicals to investigate the consistency of results between *in vivo* genotoxicity assays and carcinogenicity studies. It is reported that the *in vivo* UDS assay with rat hepatocytes and the gene mutation assay with transgenic animal models such as MutaTMMouse gave positive results with 2,4-DAT in the liver and negative results with 2,6-DAT (Mirsalis, et al, 1982, Madle, et al., 1994, Barden, et al.,2001, Lambert, et al, 2005). In our pre-validation study, both chemicals revealed positive results in a few laboratories, indicating that the Comet assay is capable of detecting the genotoxic activity of 2,4-DAT, but the inconsistencies and the positive results with 2,6-DAT cannot be explained at the present moment. Since both positive and negative results have also been reported for 2,6-DAT in UDS and Comet assays (Allavena, et al, 1992, Barden, et al.,2001,, Sekihashi et al., 2002, Kirkland and Beevers, 2006), positive findings in a few laboratories in this validation study might not indicate so-called false positive responses. Interestingly, both chemicals gave positive results even in the stomach in two laboratories, whilst it would be expected that metabolic activation would be required to induce the genotoxic activity. The reasons and the toxicological significance are unclear. Further studies would be needed to further investigate the results, especially for 2,4- and 2,6-DAT, after the optimized protocol is available.

8. In conclusion, as the reproducibility of both positive control and coded compounds was not sufficiently achieved in these studies, a further revised protocol-v.13 was prepared, and VMT decided to conduct a 3rd phase pre-validation study over an additional one year beyond the original plan for the international validation study.

3. Third Phase Pre-validation Study

3-1. Background and Purpose

In the 2nd phase pre-validation study, the following problems were identified: 1) EMS treatment induced positive responses for the liver in all (five) leading laboratories through three independent experiments, but failed to produce positive results for the stomach in each one of three experiments conducted in two leading laboratories; and 2) large variation of Effects (diff.) were observed in both organs among five testing facilities. In addition, one of five laboratories (Lab.2) showed large within-laboratory variation of the Effect in both organs.

One of success criteria of the 2nd phase pre-validation study was to obtain positive results in all positive control groups in all testing facilities. Thus the above problems indicated that the Comet assay protocol-v.12 might not be suitable as it was for the further validation studies, at least for the stomach. Based on discussion with the members of VMT, leading laboratories and consultation team including statisticians at the Atagawa meeting (March 13-14, 2008), the Comet assay protocol was revised to version v.13 (Appendix 4) intended to solve above problems. In addition, as draft criteria on data acceptability (see the footnote in section 4-4.) were established in consideration of the data from the 2nd phase pre-validation study, and these would be applied to the laboratory recruitment process for the definitive validation study, it was also necessary to investigate whether or not the draft data-acceptance criteria could be applied to judgment of data reliability in the definitive validation studies.

In this 3rd phase pre-validation study, three coded test compounds were assayed in leading laboratories in accordance with the Comet assay protocol-v.13. The first purpose was to examine the reproducibility and robustness of positive control results with EMS when experiments were conducted in accordance with the Comet assay protocol-v.13, and thereby determine if this version of the study protocol was optimal for the full validation study. Although not a primary purpose of this phase, the data from the 3 coded chemicals were also evaluated to check for reproducibility and the robustness of the revised protocol. The second purpose was to investigate whether or not the draft data-acceptance criteria were suitable to judge reliability of data. These are described at the 3rd phase pre-validation study plan (Appendix 5).

3-2. Experimental period

June-November, 2008

3-3. Participant laboratories

Four laboratories* participated in the 3rd phase pre-validation study. BSRC could no longer participate in the pre-validation study because Dr. Makoto Hayashi, the head of VMT, moved from NIHS to BSRC in April 2008.

* BioReliance (Lab.2), FDSC (Lab.4), HLS (Lab.3), Merck (Lab.1)

3-4. Success criteria

3-4-1 To obtain positive results in all positive control groups in all testing facilities (same as the purpose of 2nd phase pre-validation study).

3-4-2 To confirm that data from all testing facilities can satisfy the draft data-acceptance criteria*.

* Draft data-acceptance criteria

- 1) Negative control: means of %DNA in tail are 1-8% in the liver and 1-30% (preferably 1-20%) in the stomach.
- 2) Positive control EMS, 200 mg/kg, given once p.o.: Effect (ratio of means of %DNA in tail between groups of EMS and vehicle control) is 2-fold or higher in the liver and the stomach; Effect (difference of means of %DNA in tail between groups of EMS and vehicle

control) is 5% or higher in the liver and the stomach; and CV of Effect (ratio of means of %DNA in tail between groups of EMS and vehicle control) is 50% or less in two or more independent experiments with the liver and the stomach.

3-5. Test chemical, vehicle, dose level, and administration

EMS, a positive control, was dissolved in physiological saline and dosed at 200 mg/kg once by oral gavage. In addition to EMS as the positive control, the three coded test chemicals, EMS as a coded test chemical, and *N*-methyl-*N*-nitrosourea (MNU) and D-mannitol (MA) were used. The VMT did not choose the same chemicals as used in the 2nd phase pre-validation study, because the VMT noticed that AA, 2,4-DAT and 2,6-DAT did not give consistent results and therefore might not be appropriate to be used in this critical phase of the pre-validation study. Thus the VMT selected the new coded test chemicals because EMS and MNU are clearly mutagenic (would show clearly positive results in both organs) and MA is clearly a non-mutagen (would show a clearly negative result in both organs). VMT directed testing facilities in advance to use vehicles and dose levels as follows:

- for EMS, physiological saline, and 100, 200 and 300 mg/kg;
- for MNU, water, and 25, 50 and 100 mg/kg;
- for MA, water, and 500, 1000 and 2000 mg/kg.

Four data sets of each coded test compound would be obtained from four testing facilities. Simultaneously, as each experiment includes negative (vehicle) and positive control groups, three data sets of the control groups would be obtained alongside each coded chemical from each testing facility, giving 12 data sets each for vehicle and positive control groups. Again, the main purpose of the 3rd phase pre-validation study was to examine the reproducibility and robustness of positive control results with EMS and not primarily with the coded three chemicals.

3-6. Results

3-6-1. Vehicle control group

Fig. 21 shows the means of %DNA in tail (Estimate) in the vehicle control groups in the liver. The variability within and between laboratories seems small, and all the values were within the acceptable range of 1-8%. The mean and S.D. calculated with the 12 Estimates is $3.1 \pm 1.3\%$. If the vehicle control range is determined as the mean \pm 3S.D., the range is 0 to 7%.

Fig. 22 shows the means of %DNA in tail (Estimate) in the vehicle control groups in the stomach. The variability within and between 3 of the 4 laboratories seems small, but two values in Lab.2 were considerably higher than the others. All the values were within the acceptable range of 1-30%, and the 10 of 12 Estimates were within the preferable range of 1-20%. The mean and S.D. calculated with the 12 Estimates is $11.3 \pm 6.3\%$, and the mean \pm 3S.D. value is 0 to 30.2%. The values calculated with 10 Estimates (i.e. excluding the two high values from Lab.2) are $8.8 \pm 3.0\%$, and the mean \pm 3S.D. value is 0 to 17.8%.

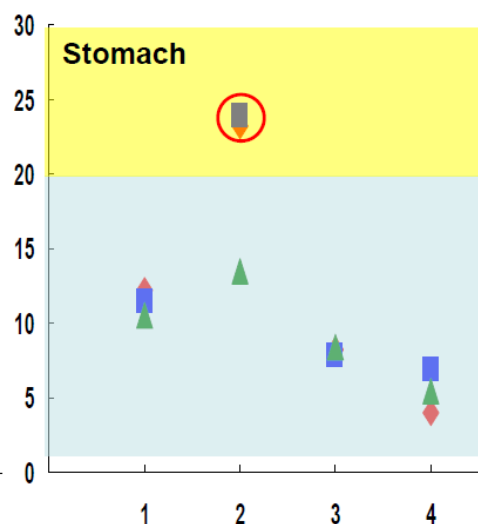
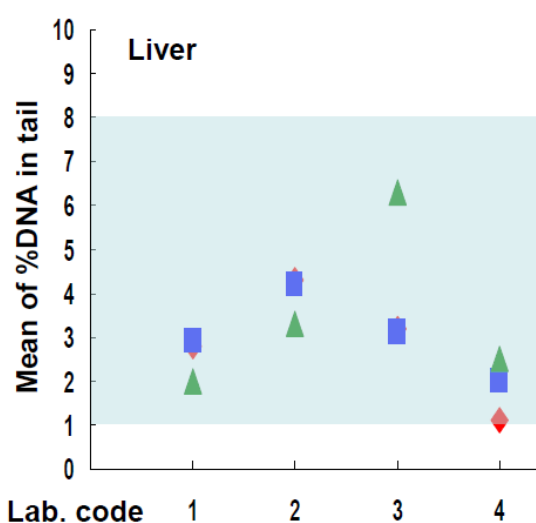


Fig. 21 Negative control ranges (%DNA in tail)

Fig. 22 Negative control ranges (%DNA in tail)

Each symbol shows the mean of negative control of each exp.
 Mean \pm SD: 3.1 \pm 1.3 in liver (n=12); 11.3 \pm 6.3 (n=12) or 8.8 \pm 3.0 (exc. \circ , n=10) in stomach
 Mean + 3SD: 7 in liver; 30.2 or 17.8 in stomach

The above values were quite comparable to those obtained in the 2nd phase pre-validation study. Therefore, VMT considered that the acceptable vehicle control ranges of 1 to 8% in the liver and 1-30% (preferably 1-20%) in the stomach could apply to the further validation study as they were. Again, the reason why the lower limit is 1.0% rather than 0% is to allow detection of decreases in %DNA in tail by chemical treatment, which would be expected with cross-linker-type genotoxicants.

Figs. 23 and 24 show respectively Effect (diff.) and Effect (ratio) values of mean %DNA in tail between the vehicle control group and the positive control EMS group in the liver. All of the Effect (diff.) and Effect (ratio) values were statistically significant with the unpaired t-test (one-sided, $p < 0.05$). Among the three independent experiments within each laboratory, the CV of Effect (ratio) for EMS was 63% in Lab.1 (which is higher than specified in the draft success criteria), 30% in Lab.2, 27% in Lab.3, and 42% in Lab.4. All Effects (ratio) were 2-fold or higher, and Effects (diff.) were 5% or higher (although only for two of the three experiments in Lab.4).

Figs. 25 and 26 show respectively Effect (diff.) and Effect (ratio) values of mean %DNA in tail between the vehicle control group and EMS group in the stomach. All of the Effect (diff.) and Effect (ratio) values were statistically significant with unpaired t-test (one-sided, $p < 0.05$). Among the three experiments within each laboratory, each CV of Effect (ratio) was below the recommended 50% namely 26% in Lab.1, 11% in Lab.2, 13% in Lab.3, and 15% in Lab.4. All Effects (ratio) were 2-fold or higher, and all Effects (diff.) were 5% or higher, and so all exceeded the acceptable range for a positive response.

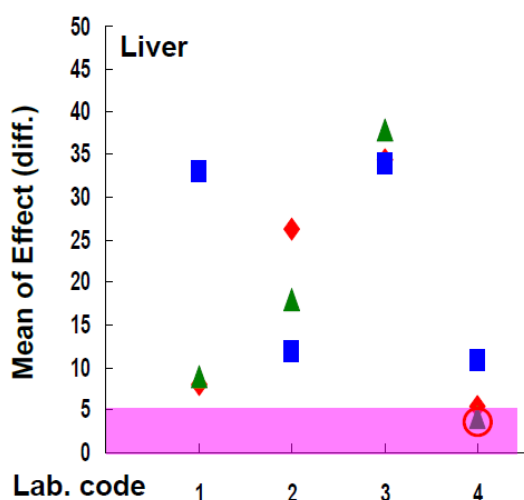


Fig. 23 Effect (difference, %DNA in tail)

Each symbol shows the mean of difference of each experiment. All data met the data acceptance criterion, 5% or higher, except for one experiment (○) at Lab 4.

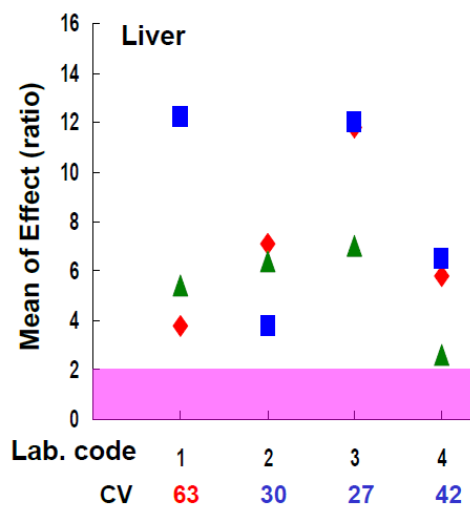


Fig. 24 Effect (ratio, %DNA in tail)

Each symbol shows the mean of ratio of each experiment. All data met the data acceptance criteria, 2-fold or higher of Effect (ratio) and 50% or less of CV, except for CV at Lab 1.

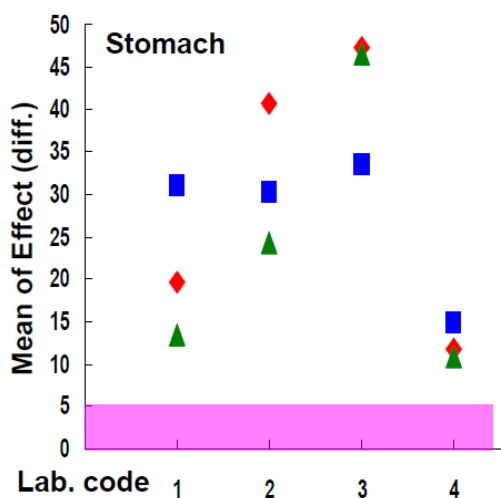


Fig. 25 Effect (difference, %DNA in tail)

Each symbol shows the mean of difference of each experiment. All data met the data acceptance criterion, 5% or higher.

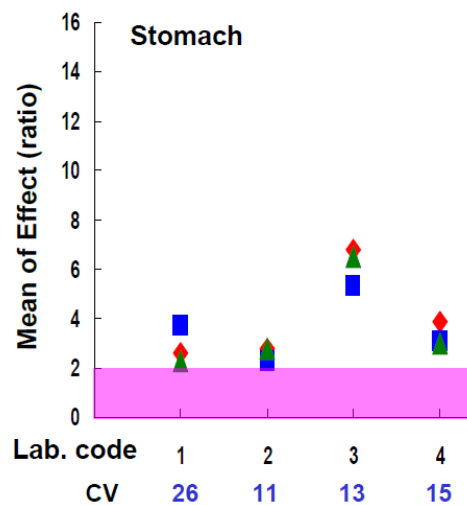


Fig. 26 Effect (ratio, %DNA in tail)

Each symbol shows the mean of ratio of each experiment. All data met the data acceptance criteria, 2-fold or higher of Effect (ratio) and 50% or less of CV.

3-6-2. Coded test chemical groups

1) EMS

Figs 27 shows Effect (diff.) of mean %DNA in tail between the vehicle (V) control group and the treatment groups (low, middle or high – L, M or H) in the liver, and Fig. 28 shows the dose-dependency of the Estimate, i.e., mean of % DNA in tail. Fig. 29 shows Effect (diff.) of mean %DNA in tail between the vehicle (V) control group and the treatment groups (low, middle or high – L, M or H) in the stomach, and Fig. 30 shows the dose-dependency of the Estimate. All of the Effect (diff.) and Estimate values were statistically significant for at least one dose group with Dunnett's test (two-sided, $p < 0.05$) and the linear trend test (two-sided, $p < 0.05$), respectively. The magnitude of responses was lower in Lab.4 compared to the other laboratories, as was seen for the positive control dose of EMS in this lab (see Figs 23-26 above). The reasons are discussed below. Based on the judgment criteria of the study protocol, i.e. a statistically significant change in at least one dose group, EMS was judged to be positive in the liver and the stomach in all laboratories, and the responses had dose-dependency.

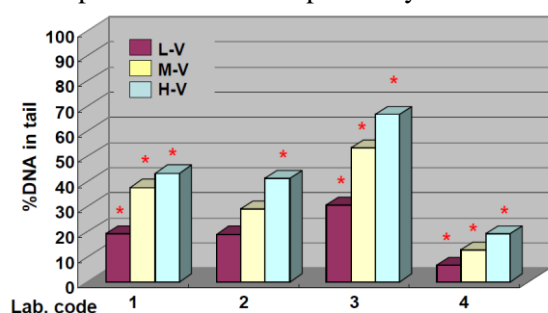


Fig. 27 Effect (diff. of %DNA in tail) in Liver: EMS

* Dunnett ($P < 0.05$; two-sided)

Test condition

- ✓ Animal: CD(SD) rat, male, 5 animals/group
- ✓ Dose: 0, 100, 200, 300 mg/kg, p.o.
- ✓ Administration: Twice (21 hours interval)
- ✓ Cell preparation: 3 hours after 2nd administration

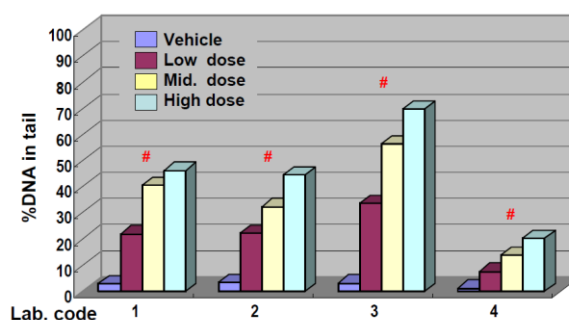


Fig. 28 Mean of %DNA in tail in Liver: EMS

Linear trend ($P < 0.05$; two-sided)

Test condition

- ✓ Animal: CD(SD) rat, male, 5 animals/group
- ✓ Dose: 0, 100, 200, 300 mg/kg, p.o.
- ✓ Administration: Twice (21 hours interval)
- ✓ Cell preparation: 3 hours after 2nd administration

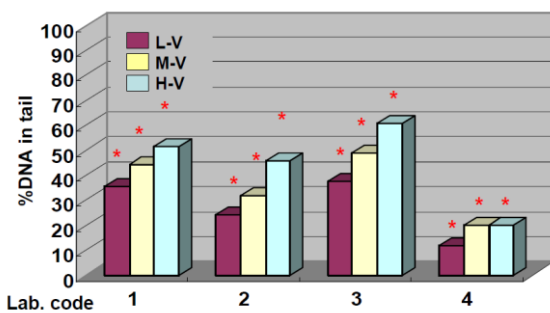


Fig. 29 Effect (diff. of %DNA in tail) in Stomach: EMS

* Dunnett ($P < 0.05$; two-sided)

Test condition

- ✓ Animal: CD(SD) rat, male, 5 animals/group
- ✓ Dose: 0, 100, 200, 300 mg/kg, p.o.
- ✓ Administration: Twice (21 hours interval)
- ✓ Cell preparation: 3 hours after 2nd administration

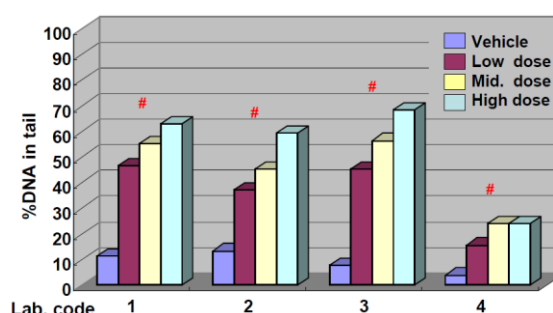


Fig. 30 Mean of %DNA in tail in Stomach: EMS

Linear trend ($P < 0.05$; two-sided)

Test condition

- ✓ Animal: CD(SD) rat, male, 5 animals/group
- ✓ Dose: 0, 100, 200, 300 mg/kg, p.o.
- ✓ Administration: Twice (21 hours interval)
- ✓ Cell preparation: 3 hours after 2nd administration

2) MNU

Fig. 31 shows Effect (diff.) values of mean %DNA in tail between the vehicle (V) control group and the treatment groups (low, middle or high – L, M or H) in the liver, and Fig. 32 shows the dose-dependency of the Estimate. Fig. 33 shows Effect (diff.) of mean %DNA in tail between the vehicle (V) control group and the treatment groups (low, middle or high – L, M or H) in the stomach. Fig. 34 shows the dose-dependency of the Estimate. All of the Effect (diff.) and Estimate values were statistically significant for at least one dose group with Dunnett's test (two-sided, $p < 0.05$) and the linear trend test (two-sided, $p < 0.05$), respectively. In Lab.3, severe cytotoxicity was noted in the 50

and 100 mg/kg groups, and no data were available, but the reasons are not clear. The magnitude of responses was again lower in Lab.4 compared to the other laboratories. Based on the judgment criteria of the study protocol, i.e. a statistically significant change in at least one dose group, MNU was judged to be positive in the liver and the stomach in all laboratories, and the responses had dose-dependency.

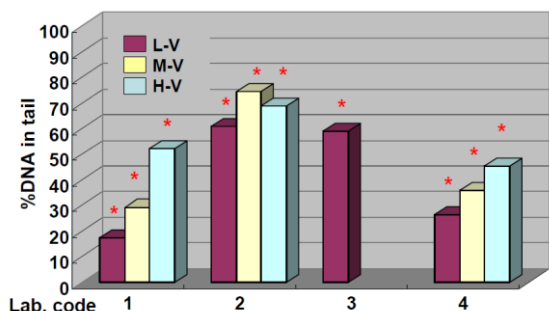


Fig. 31 Effect (diff. of %DNA in tail) in Liver: MNU

* Dunnett (P<0.05; two-sided)
 Test condition
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 25, 50, 100 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

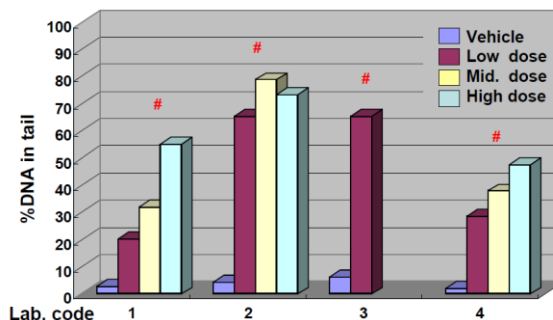


Fig. 32 Mean of %DNA in tail in Liver: MNU

Linear trend (P<0.05; two-sided)
 Test condition
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 25, 50, 100 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

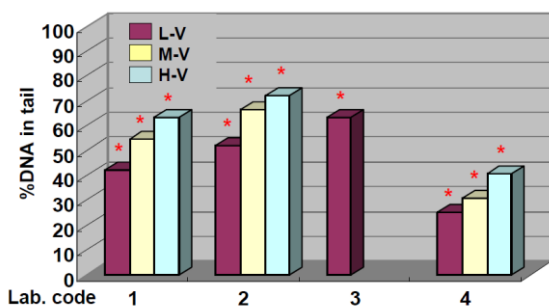


Fig. 33 Effect (diff. of %DNA in tail) in Stomach: MNU

* Dunnett (P<0.05; two-sided)
 Test condition
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 25, 50, 100 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

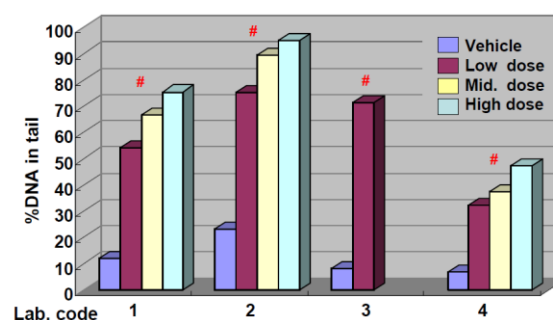


Fig. 34 Mean of %DNA in tail in Stomach: MNU

Linear trend (P<0.05; two-sided)
 Test condition
 ✓ Animal: CD(SD) rat, male, 5 animals/group
 ✓ Dose: 0, 25, 50, 100 mg/kg, p.o.
 ✓ Administration: Twice (21 hours interval)
 ✓ Cell preparation: 3 hours after 2nd administration

3) MA

Fig. 35 shows the Effect (diff.) values of mean %DNA in tail between the vehicle (V) control group and the treatment groups (low, middle or high - L, M or H) in the liver, and Fig. 36 shows the dose-dependency of the Estimate. Fig. 37 shows Effect (diff.) of mean %DNA in tail between the vehicle (V) control group and the treatment groups (low, middle or high - L, M or H) in the stomach. Fig. 38 shows the dose-dependency of the Estimate. None of the Effect (diff.) and Estimate values were statistically significant with Dunnett's test (two-sided, p<0.05) or with the linear trend test (two-sided, p<0.05), respectively. The magnitude of the responses seems slightly higher in Lab.2 compared to the other laboratories, but the reasons are not clear. Based on the judgment criteria of the study protocol, i.e. a statistically significant change in at least one dose group, MA was judged to be negative in the liver and the stomach in all laboratories.

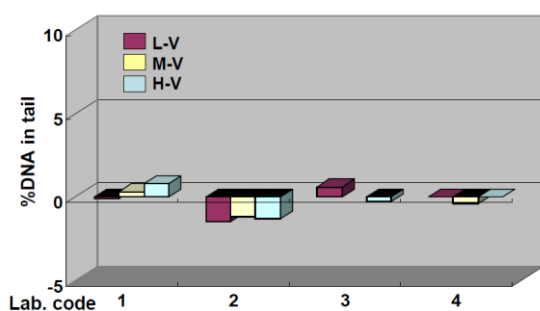


Fig. 35 Effect (diff. of %DNA in tail) in Liver: MA

No statistical significance with Dunnett ($P < 0.05$; two-sided)

Test condition

- ✓ Animal: CD(SD) rat, male, 5 animals/group
- ✓ Dose: 0, 500, 1000, 2000 mg/kg, p.o.
- ✓ Administration: Twice (21 hours interval)
- ✓ Cell preparation: 3 hours after 2nd administration

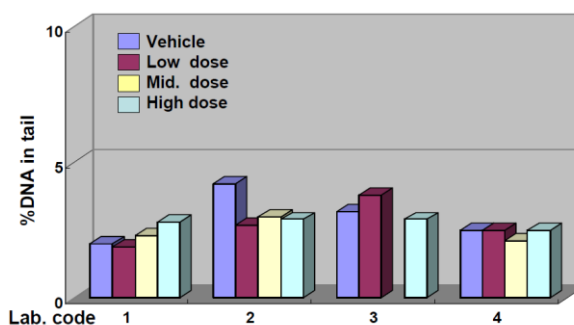


Fig. 36 Mean of %DNA in tail in Liver: MA

No statistical significance with Linear trend ($P < 0.05$; two-sided)

Test condition

- ✓ Animal: CD(SD) rat, male, 5 animals/group
- ✓ Dose: 0, 500, 1000, 2000 mg/kg, p.o.
- ✓ Administration: Twice (21 hours interval)
- ✓ Cell preparation: 3 hours after 2nd administration

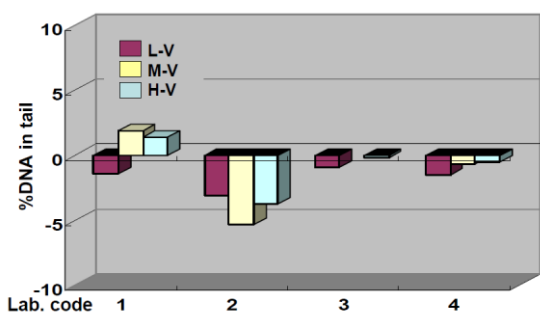


Fig. 37 Effect (diff. of %DNA in tail) in Stomach: MA

No statistical significance with Dunnett ($P < 0.05$; two-sided)

Test condition

- ✓ Animal: CD(SD) rat, male, 5 animals/group
- ✓ Dose: 0, 500, 1000, 2000 mg/kg, p.o.
- ✓ Administration: Twice (21 hours interval)
- ✓ Cell preparation: 3 hours after 2nd administration

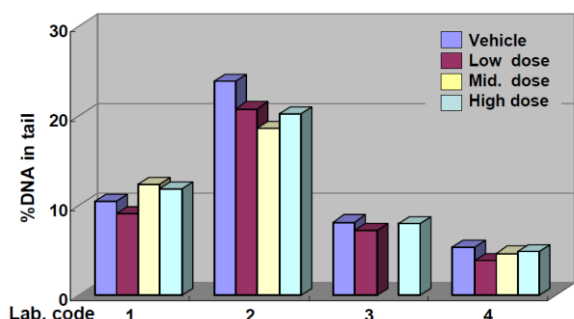


Fig. 38 Mean of %DNA in tail in Stomach: MA

No statistical significance with Dunnett ($P < 0.05$; both sides) and Linear trend ($P < 0.05$)

Test condition

- ✓ Animal: CD(SD) rat, male, 5 animals/group
- ✓ Dose: 0, 500, 1000, 2000 mg/kg, p.o.
- ✓ Administration: Twice (21 hours interval)
- ✓ Cell preparation: 3 hours after 2nd administration

3-7. Discussion

1. EMS treatment as the positive control induced statistically significant increases in Effect (diff.) in both the liver and the stomach in all laboratories. Therefore, the first success criteria were satisfied in this pre-validation study. Although the overall magnitude of responses seemed lower in Lab.4 compared to the other laboratories, and this was considered to be due to the shorter electrophoresis duration of 15 minutes used in Lab.4 (cf. Lab.1: 20 minutes in both organs, Lab.2: 30 minutes in both organs, and Lab.3: 20 minutes in the liver and 30 minutes in the stomach). It was regarded as important to keep constant negative control ranges of 1-8% in the liver and 1-20 or -30% in the stomach by identifying an appropriate duration of electrophoresis rather than applying a fixed duration. The electrophoresis duration was therefore not specified in the protocol-v.13, although it was arranged among the participant laboratories at the 4th international face-to-face meeting (see the section 3-7. discussion of the 2nd phase validation report) that the electrophoresis duration would be basically 20 minutes in all experiments for the 3rd phase pre-validation study. This policy is not changed at the present moment, but VMT also noticed at that time that providing an indication of electrophoresis duration would be useful to obtain more consistent data, and therefore it was defined in a revised protocol-v.14 as "20 minutes" (and the protocol-v.14.2 as "at least 20 minutes" to put the negative control values into the acceptable ranges more certainly). Under the electrophoresis conditions mentioned in the protocol-v.14, an average DNA migration in the negative control would be expected to be 1-8% in the liver and 1-30% (preferably 1-20%) in the stomach. In other words, a laboratory which conducts the *in vivo* Comet assay should set the electrophoresis conditions to put the negative control values into the ranges mentioned above.
2. Almost all data satisfied the second success criteria except for the Effect (diff.) of 4.1% in the

liver in one of three experiments in Lab.4, and the CV of Effect (ratio) of 63% in the liver in Lab.1. However, the former deviation would be most likely be explained by the shorter electrophoresis duration in Lab.4, and the latter was considered to be only a slight deviation from the success criteria of 50% or less. Therefore, VMT considered that the second purpose, namely satisfying the success criteria, was fulfilled in this pre-validation study.

3. Regarding the three coded chemicals, all laboratories obtained the expected assay results. The lower magnitude of responses in Lab.4 was considered to be due to the shorter electrophoresis duration as mentioned above.
4. In conclusion, the protocol-v.13 was considered to be a reasonable basis for the further validation studies, because the reproducibility and robustness of the positive control results with EMS were confirmed when the experiments were conducted in accordance with the protocol-v.13. Although the VMT did not determine success criteria for the three coded test chemicals before this phase of the pre-validation study, the reproducibility and robustness of assay results using the protocol-v.13 would be also supported by the data, in that the three coded chemicals, EMS, MNU and MA, gave the expected results. The study protocol was further modified as v.14 (Appendix 6), incorporating additional technical requirements such as electrophoresis duration, in order to obtain more consistent data, and the administration regimen of test chemicals was changed from two times to three times in order to allow the combination of the Comet assay and micronucleus assay. Currently it is required that the 3R's principles of animal use should be strictly considered for toxicity studies, and the ICH-S2(R1) guidance (2012) recommends the combination and/or the integration approach to detect the genotoxic potential of test chemicals. In accordance with the 3R's policy, the administration times were changed in our final validation study protocol. The VMT, however, considered that these modifications to protocol-v.13 did not affect the validity of the data from the 3rd phase pre-validation study with the protocol-v.13 in that acceptable reproducibility within a laboratory and between laboratories had been established for the positive control and 3 coded chemicals. In addition, the draft data-acceptance criteria were considered to be suitable as formal criteria to clarify data reliability in the further validation studies.
5. It was therefore concluded that sufficient pre-validation work had been conducted to be able to define the protocol and success criteria for the main validation study (4th phase) and therefore recruitment of laboratories to participate in the 4th phase was initiated.

4. Recruitment of labs for the definitive (4th phase) validation study

See the report on recruitment process (Appendix 7). Finally, 14 laboratories including four leading laboratories were approved as participants of the definitive (4th phase) validation study.

5. References

- Allavena, A., et al, Evaluation in a battery of *in vivo* assays of four *in vitro* genotoxins proved to be noncarcinogens in rodents. *Teratogen. Carcinogen. Mutagen.*, 12 (1992), 31-41.
- Barden, D.S. et al., Metabolism, toxicokinetics and hemoglobin adduct formation in rats following subacute and subchronic acrylamide dosing. *Neurotoxicology*, 22 (2001), 341-353.
- Burlinson, B., et al, Fourth International Workgroup on Genotoxicity Testing: result of the *in vivo* comet assay workgroup, *Mutat. Res.* 627 (2007), 31-35.
- Hartmann, A., et al., Recommendation for conducting the *in vivo* alkaline Comet assay, *Mutagenesis* 18 (2003), 45-51.
- ICH Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2(R1), ICH harmonized tripartite guideline, 2012.
- Kirkland, D., Beevers, C., Induction of LacZ mutation in Muta Mouse can distinguish carcinogenic from non-carcinogenic analogues of diaminitoluenes and nitronaphthalens. *Mutat. Res.* 608 (2006), 88-96.
- Kumaravel, T. S. , Jha, A. N. , Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals, *Mutat. Res.* 605 (2006), 7-16.
- Lambert, I.B., et al, Detailed review of transgenic rodent mutation assays. *Mutat. Res.*, 590 (2005), 1-280.
- Madle, S., et al., Recommendations for the performance of UDS tests *in vitro* and *in vivo*. *Mutat Res.* 312(1994), 263-85.
- Maniere, I. et al., DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide. *Mutat. Res.*, 580 (2005), 119-129.
- Miller, M.J. et al., Pharmacokinetics of acrylamide in Fisher-344 rats. *Toxicol. Appl. Pharmacol.*, 63 (1982), 36-44.
- Mirsalis, J.C. et al, Detection of genotoxic carcinogens in the *in vivo-in vitro* DNA repair assay. *Environ. Mutagen.*, 4 (1982), 553-562.
- Olive, P. L., Banath, J. P. , Durand, R. E., Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the “comet” assay, *Radiat. Res.* 122 (1990), 86-94.
- Rothfuss, A., et al., Collaborative study on fifteen compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies. *Mutat. Res.*, 702, 40-69, 2010
- Sekihashi, K., et al., Comparative investigations of multiple organs of mice and rats in the comet assay, *Mutat. Res.* 517 (2002), 53-74.
- Tice, R. R., et al., Single cell gel/Comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing, *Environ. Mol. Mutagen.* 35 (2000), 206-221.

PHASE 1-3, APPENDIX 1

**TESTING PROCEDURE OF IN VIVO ALKALINE COMET ASSAY FOR THE
INTERNATIONAL VALIDATION STUDIES**

(DRAFT, VER. 4)

Issued by: the management team of the international validation studies of in vivo alkaline Comet assay

Date: August 15, 2006

A. PURPOSE OF THIS DOCUMENT

This document is provided to clarify the outline of testing procedure of in vivo alkaline Comet assay for the international validation studies. A study protocol will be provided in each testing facility based on the instruction of this document.

B. ASSURANCE OF DATA QUALITY

The study will be conducted under the spirit of Good Laboratory Practice in each site. Consistency between raw data and a final report should be assured in the responsibility of each testing facility. Management team will review the consistency if necessary.

C. ANIMAL WELFARE

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

D. TESTING PROCEDURE

1. MATERIALS AND METHODS

1.1. Test substances and positive/negative controls

Test substance

Test substances will be supplied to each testing facility from the management team. Test substance names may be concealed.

Test substance preparation

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

Positive control

Ethyl methanesulfonate (EMS, CAS No. 62-50-0) will be used as a positive control.

EMS will be supplied to each testing facility from the management team.

EMS will be dissolved in physiological saline just before administration.

Negative control (solvent/vehicle)

Solvents/vehicles for test substance preparation will be used as negative controls. An appropriate solvent/vehicle for each test substance may be instructed by the management team. When no 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.

1.2. Test animals

Species

Rat or Mouse

Sex

Male

Strain

Rat: CrI:CD(SD) or Mouse: CrI:CD1 (ICR)

Source

Charles River, Inc.

Justification of the strain selection

These strains are commonly used for safety assessment studies.

Age

At the time of purchase: 7 weeks of age

At the time of dosing: 8 weeks of age

Body weight

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

Number of animals in each dose group at each sampling time

4 animals

Animal maintenance

Animals will be reared under appropriate housing and feeding conditions according to the standard operating procedures (SOP) in each testing facility. For reference, the housing conditions in An-Pyo Center are described in sections 1.2.9.1-1.2.9.3.

1.2.9.1 Housing condition

Animals will be housed in an animal room (Room No. XXX: W 3.5 × D 5.5 × H 2.5 m, 48.1 m³) under the conditions as follows.

Temperature: 24.5±2.5°C (comment: 22±3°C would be preferable)

Relative humidity: 55±20% (comment: 50±20% would be preferable)

Ventilation: 18 times/h

Lighting: 12 hours light/dark cycle (light on: 7 a.m., light off: 7 p.m.)

One to 3 animals will be housed in a zafone™ (Lab Products, Inc.) animal cage (W: XX × D: YY × H: ZZ cm, cage space ABC cm³) with bedding (ALPHA-dri™: Shepherd Specialty Papers, Inc.) setting on the Micro-Isolator™ System rack (Lab Products, Inc.) with an automatic watering system.

1.2.9.2 Housing condition

Animals will be fed *ad libitum* with a commercially available pellet diet, (name).

1.2.9.3 Water

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

Animal quarantine and acclimation

Animals will be quarantined and acclimated for at least 5 days prior to the start of the study according to the SOP in each testing facility. For reference, the methodologies in An-Pyo Center are described below:

Upon arrival at the testing facility, each animal will be monitored once a day for at least 5 days to ensure that they are healthy and growing normally; and animals will be acclimated to the laboratory environment during this period. Animals with any abnormalities will not be used in this study.

Animal identification and group assignment

Animals will be identified uniquely and assigned to groups according to the SOP in each testing facility. For reference, the methodologies in An-Pyo Center are described below:

Each animal will individually be identified by clipping unique patterns and by attaching a cage label showing the temporary animal ID-No. to each cage during the quarantine period. Animals will randomly be assigned to groups using a stratified body weight procedure just before dosing. All animals will individually be identified by pen marks on the tail and housed in cages each with a label showing the experiment No., the animal ID-No., etc. after assignment to groups. Extra animals will be euthanized by CO₂ inhalation.

1.3. Preparation of reagent solution

Following solutions will be prepared freshly for use and used within one week.

1.0% (w/v) agarose gel for the bottom layer

Agarose will be dissolved at 1.0% (w/v) in Dulbecco's phosphate buffer by heating in a microwave. This solution will be used just after preparation.

1.0% (w/v) low-melting agarose gel

Low-melting agarose will be dissolved at 1.0% (w/v) in Dulbecco's phosphate buffer by heating in a microwave. This solution will be kept at 37-45°C until use.

Lysing solution

The lysing solution consists of 100 mM EDTA, 2.5 M sodium chloride and 10 mM tris hydroxymethyl aminomethane in purified water and is adjusted to pH 10.0 with 1 M sodium hydroxide or hydrochloric acid. This solution will be refrigerated until use. 1 % (v/v) of triton-X and 10 % (v/v) DMSO will be added to this solution immediately before use and the complete lysing solution will be refrigerated for at least 30 minutes prior to slide addition.

Alkaline solution for unwinding and electrophoresis

The alkaline solution consists of 300 mM sodium hydroxide and 1 mM EDTA in purified water, pH >13. This solution will be refrigerated until use.

Neutralization solution

The neutralization solution consists of 0.4 M tris hydroxymethyl aminomethane in purified water and is adjusted to pH 7.5 with hydrochloric acid. This solution will be refrigerated until use.

Mincing and/or homogenization buffer

The mincing and/or homogenization buffer consists of 20 mM EDTA and 10% DMSO in HBSS (Ca⁺⁺, Mg⁺⁺ free) and is adjusted to pH 7.5 with 1 M sodium hydroxide. This solution will be refrigerated until use.

Staining solution

The staining solution consists of 20 µg/mL ethidium bromide in purified water.

1.4. Comet assay procedure**Experimental design**

| Compound | Dose (mg/kg) | Number of animals |
|----------------------------|----------------------------|-------------------|
| Vehicle (negative control) | 0 | 4 |
| EMS (positive control) | 250 for rat, 400 for mouse | 4 |
| Test substance | Low dose* | 4 |
| Test substance | High dose** | 4 |

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

**High dose level may be instructed by the management team. When no instruction from the management team, the high dose level 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. The limit dose is 2000 mg/kg when a test compound has no toxicity.

Tissue samples will be obtained at 3 hours after dosing (2-4 hours after dosing will be acceptable). When negative responses are identified in all analyzed tissues at this sampling time, an additional experiment will be performed under the same experimental design described above and tissue samples will be obtained at 24 hours after dosing (23-25 hours after dosing will be acceptable). In this additional experiment, tissue samples from EMS group will be obtained at 3 hours after dosing (2-4 hours after dosing will be acceptable) as a positive control.

Administration to animals

The test substance will be usually administered once to animals orally by gavage. Routes of exposure other than oral will be acceptable, but the i.p. route will be prohibited. The dosage volume will be usually set at less than 0.1 mL per 10 g body weight on the basis of the animal weight just before administration.

Measurement of body weight and examination of animal conditions

Individual body weight will be measured on the day of receipt, at the end of the acclimation period, and before administration (at the time of grouping). In addition, individual body weight will be measured before tissue removal only when tissue samples will be obtained at 24 hours after dosing.

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.

Tissue sampling

Animals will be anesthetized with ether or a proper anesthetic at 3 and/or 24 hours after dosing, and the liver and the stomach* will be removed following exsanguination from the abdominal aorta. Tissues will be placed into ice-cold mincing and/or homogenization buffer and stored on ice. Tissues will be washed with the cold mincing and/or homogenization buffer to remove blood. A part of each tissue will be cut and stored for histopathological examination according to the SOP in each testing facility.

* According to the minimal requirement of the recommendation by Hartmann et al., 2003 (need to be discussed which tissues should be assessed).

Preparation of cell nuclei or single cells

The liver and the stomach will be handled as follows:

Liver: The liver will be cut with a pair of fine scissors into about 5 millimeters cubic pieces on a plastic dish and washed in the cold mincing and/or homogenization buffer until as much blood has been removed as possible. 2-3 mL of the cold mincing and/or homogenization buffer will be added into the dish. Then single cells or cell nuclei will be prepared by using either of the methods described in sections 1.4.5.1-1.4.5.4. The management team will indicate which method each testing facility should use.

Stomach: The forestomach will be removed and discarded. The glandular section will be cut open and washed free from food using the cold mincing and/or homogenization buffer. The stomach will be then placed into a plastic dish, covered with about 2 mL of the cold mincing and/or homogenization buffer and incubated on ice for about 15 minutes. After incubation, the stomach will be removed and the surface epithelia will be gently scraped two times using the back of a scalpel blade. This layer will be discarded and the gastric mucosa rinsed with the cold mincing and/or homogenization buffer. 2-3 mL of the cold mincing and/or homogenization buffer will be added to a clean plastic dish and the stomach epithelia will be carefully scraped 4-5 times with the back of a scalpel blade to release the cells. Then single cells or cell nuclei will be prepared by using either of the methods described in

sections 1.4.5.1-1.4.5.4. The management team will indicate which method each testing facility should use.

Mincing method (to obtain single cells)

The cut liver will be minced with a pair of fine scissors to release the cells, and the cells will be collected into a plastic tube using a disposable pastette (pipette). The stomach cells will be collected into a plastic tube using a disposable pastette (pipette). The obtained cell suspensions will be preserved on ice until preparation of gel embedding slides.

Homogenization method (to obtain nuclei)

The cut or scraped tissue solution will be transferred into a homogenization tube and then gently homogenized once using a Dauns loose-type homogenizer. The homogenate will be carried through a tube with a nylon mesh membrane (pore size: 150 μm ; synthetic fiber nylon filter) to remove remaining tissue mass. The obtained nucleus suspensions will be preserved on ice until preparation of gel embedding slides.

Mesh membrane method for the liver* (maybe to obtain nuclei)

The cut liver will be placed on a nylon mesh membrane (pore size: 150 μm) held over a 50-mL tube. The cell mass will be carried through the tube with a plunger of a disposable syringe, and 2-3 mL of the ice-cold mincing and/or homogenization buffer will be flashed into the tube. The obtained cell or nucleus suspensions will be preserved on ice until preparation of gel embedding slides.

*Need to be discussed about the necessity of investigation of this method because the comparison of cells and nuclei would be sufficient to perform the mincing method and the homogenization method.

Digestive enzyme method* (to obtain single cells)

*Need to be discussed about the necessity of investigation of this method because the comparison of cells and nuclei would be sufficient to perform the mincing method and the homogenization method. In addition, this method may not be popular now.

Slide preparation

Three slides per tissue per animal will be prepared (and scored). A frosted-end glass slide will be pre-coated with 1.0% agarose solution prior to use. The nucleus or cell suspension (75 μL) prepared in section 1.4.5. and 1.0% low-melting agarose gel (75 μL) will be mixed, and then 75 μL of cell/agar mixture will be dispensed onto the pre-coated slide and cover with a clean slide glass. The layer will be solidified for at least 15 minutes, and then the clean slide glass will be carefully removed. 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 to remove residual detergent and salts prior to alkali unwinding step.

Unwinding and electrophoresis

The slides will be placed onto a platform of submarine-type electrophoresis unit (a re-circulating type may be preferable) containing a chilled electrophoresis solution. The slides from each treatment group will be electrophoresed all together to avoid any positional effects. If the electrophoresis cannot be done at the same time, at least one slide from each treatment group will be included for each electrophoresis and slides will be randomly distributed in a platform.

Electrophoresis solution will be poured until the surfaces of the slides will be completely covered with the solution. The nucleoids will be left to be unwinded for 20 minutes. After alkali unwinding, the slides will be electrophoresed for 15 minutes from 0.7 to 1 V/cm, with accompanying amperage of 0.25-0.30 A.

Electrophoresis solution should be maintained a constant temperature below 5°C during electrophoresis. 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.

Neutralization and dehydration of slides

After completion of electrophoresis, the slides will be immersed in the neutralization buffer for 10 to 20 minutes. All slides will be dehydrated by immersing the slides into absolute ethanol ($\geq 99.6\%$) up to 10 minutes.

DNA staining, comet visualization and analysis

After all slides are coded, they will be examined by the masking method according to the SOP in each testing facility. The slides will be stained with 50 μL of the staining solution and the comets will be visualized using a fluorescence microscope at magnification between 200X to 400X. The comets will be measured via a digital (e.g. CCD) camera linked to an image analyzer system.

Fifty cells or nuclei in migrating image per slide will be analyzed. Heavily damaged cells exhibiting a specific microscopical image (commonly referred to as hedgehogs) consisting of small or non-existent head and large, diffuse tails potentially represent dead or dying cells and should be excluded from data collection.

Percentage of DNA in tail will be calculated using an image analyzer system. Following parameters may be calculated as well but not essential: tail length, the Olive tail moment, and the categorization of comets into different 'classes' of migration.

Histopathology

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.

STATISTICS

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 a data transformation, such as logit or probit transformation, or over-dispersion for between individuals variation. Since it is not yet well known that which approach is adequate, we can't yet recommend something for statistical methods. Therefore we will also need to examine the performance of several approaches for statistical test through this study.

DATA AND REPORTING

3.1 Treatment of results

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

3.2 Evaluation and interpretation of results

A change in the percentage of DNA in tail will be determined by using the statistical method described in section 2. A positive response is defined as a statistically significant change in the percentage of DNA in tail in a single dose group at least 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 histopathology.

Positive results indicate that the test substance induce DNA damage in vivo 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.

3.3 Study report

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

3.3.1 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.

3.3.2 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.

3.3.3 Regents to prepare reagent solutions

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

3.3.4 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; conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), 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/nucleus 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 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.

3.3.5 Results

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

3.3.6 Discussion of the results and/or conclusion, if necessary

4 ARCHIVES AND REVIEW

The study report and all raw data (including slide samples) 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

- R.R. Tice et al., Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.*, 35, 206-221, 2000.
- A. Hartmann et al., Recommendation for conducting the *in vivo* alkaline Comet assay. *Mutagenesis*, 18(1), 45-51, 2003.
- A. Hartmann et al., 4th International Workgroup on Genotoxicity Testing: result of the in vivo comet assay workgroup (in preparation).

E. Statistical analysis

For purpose 1, the variance for inter-facility variation of each group by applying multilevel model to percentage of DNA in tail will be calculated.

For purpose 2, the ratio of the weighted average adjusted facility and individual animal for percentage of DNA by the mincing method to that by the homogenization method.

Appendix

Data sheet for 1st International pre-validation study of *in vivo* alkaline comet assay (Phase 1 trial)

Laboratory:

Test substance:

(Chemical

Animal ID:

| | 1 | 2 | 3 | 4 |
|-----------------------------|----|----|----|----|
| Corn oil | | | | |
| | 5 | 6 | 7 | 8 |
| EMS Low dose | | | | |
| | 9 | 10 | 11 | 12 |
| EMS High Dose | | | | |
| | 13 | 14 | 15 | 16 |
| Test substance Low dose | | | | |
| | 17 | 18 | 19 | 20 |
| Test substance High Dose | | | | |

Name:

Date for recording data in this file:

Comment:

| | | Organ: Liver | | | | | | | | | Parameter: Tail length | | |
|-------|------------|-----------------------------|---|---|---|---|---|---|---|---|-------------------------------|---|---|
| | | Corn oil (negative control) | | | | | | | | | | | |
| Group | Animal No. | | | | | | | | | | | | |
| | Slide No. | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| | # of Cells | | | | | | | | | | | | |
| | 1 | | | | | | | | | | | | |
| | 2 | | | | | | | | | | | | |
| | 3 | | | | | | | | | | | | |
| | 4 | | | | | | | | | | | | |
| | 5 | | | | | | | | | | | | |
| | 6 | | | | | | | | | | | | |
| | 7 | | | | | | | | | | | | |
| | 8 | | | | | | | | | | | | |
| | 9 | | | | | | | | | | | | |
| | 10 | | | | | | | | | | | | |
| | 11 | | | | | | | | | | | | |
| | 12 | | | | | | | | | | | | |
| | 13 | | | | | | | | | | | | |
| | 14 | | | | | | | | | | | | |
| | 15 | | | | | | | | | | | | |
| | 16 | | | | | | | | | | | | |
| | 17 | | | | | | | | | | | | |
| | 18 | | | | | | | | | | | | |
| | 19 | | | | | | | | | | | | |
| | 20 | | | | | | | | | | | | |
| | 21 | | | | | | | | | | | | |
| | 22 | | | | | | | | | | | | |
| | 23 | | | | | | | | | | | | |
| | 24 | | | | | | | | | | | | |
| | 25 | | | | | | | | | | | | |
| | 26 | | | | | | | | | | | | |
| | 27 | | | | | | | | | | | | |
| | 28 | | | | | | | | | | | | |
| | 29 | | | | | | | | | | | | |
| | 30 | | | | | | | | | | | | |
| | 31 | | | | | | | | | | | | |
| | 32 | | | | | | | | | | | | |
| | 33 | | | | | | | | | | | | |
| | 34 | | | | | | | | | | | | |
| | 35 | | | | | | | | | | | | |
| | 36 | | | | | | | | | | | | |
| | 37 | | | | | | | | | | | | |
| | 38 | | | | | | | | | | | | |
| | 39 | | | | | | | | | | | | |
| | 40 | | | | | | | | | | | | |
| | 41 | | | | | | | | | | | | |
| | 42 | | | | | | | | | | | | |
| | 43 | | | | | | | | | | | | |
| | 44 | | | | | | | | | | | | |
| | 45 | | | | | | | | | | | | |
| | 46 | | | | | | | | | | | | |
| | 47 | | | | | | | | | | | | |
| | 48 | | | | | | | | | | | | |
| | 49 | | | | | | | | | | | | |
| | 50 | | | | | | | | | | | | |

PHASE 1-3, APPENDIX 2**INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (VERSION 12)**

Issued by: the Validation Management Team (VMT)

Date: December 11, 2006 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: CrI: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 (Cambrex AG5897) 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 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 (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) | 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: 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. 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, 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. Tissues samples will be also collected from the same liver lobe and the minimal possible portion for stomach in case of the histopathological examination (see section 1.1.12.).

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 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, 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. 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 1-15% (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 ($\geq 99.6\%$) 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 (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 between 200X to 400X. For each sample (animal/tissue), fifty comets cells per slide will be analyzed, with 2 slides scored per sample. 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, 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].

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 ($\geq 99.6\%$) 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 (e.g., 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. add more information about classification and clarification and more pictures

1.4.12 Histopathology to revise

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. 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. 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 Treatment of results

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

3.2 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.

3.3 Study report

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

3.3.1 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.

3.3.2 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.

3.3.3 Reagents to prepare reagent solutions

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

3.3.4 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.

3.3.5 Results

Signs of toxicity, including histopathology in the appropriate tissue(s) if applicable; individual and mean/median values for DNA migration (and ranges) and % cells with low molecular weight DNA and % hedgehogs in individual tissue, animal, and group; concurrent positive and negative control data; and statistical evaluation.

3.3.6 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.

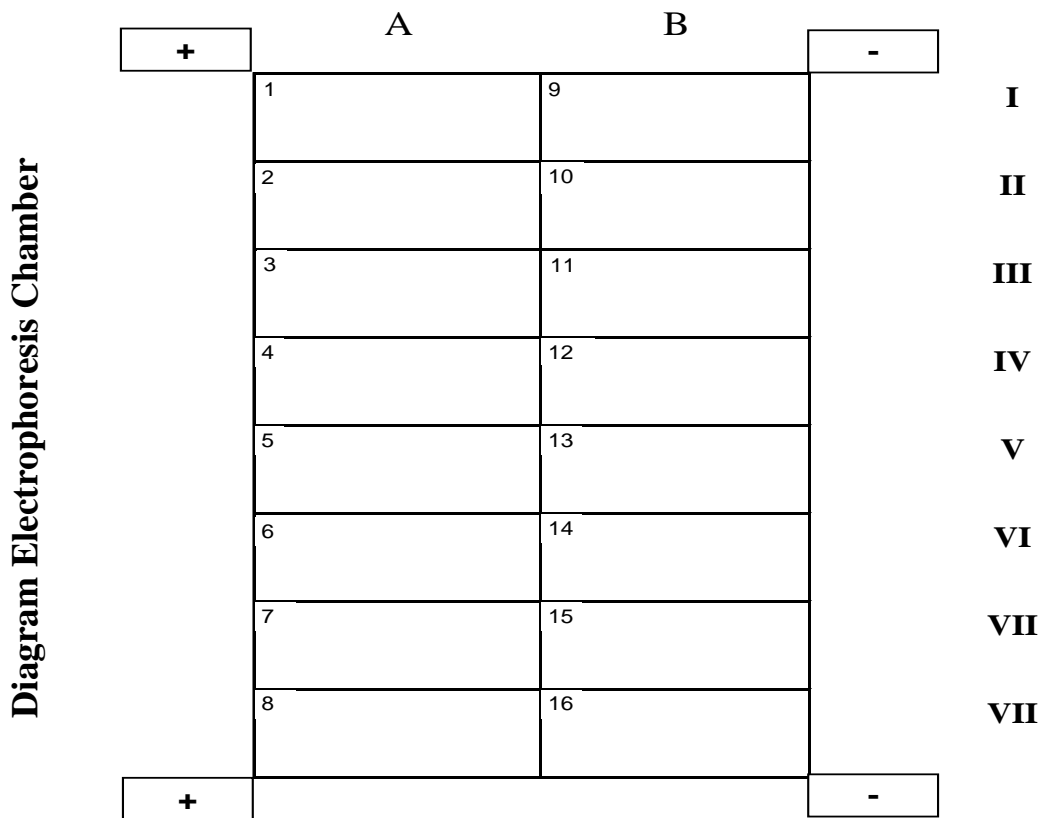
5 REFERENCES

- 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:

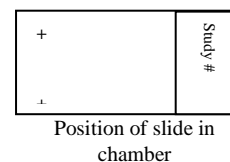
SLIDES UNWINDING & ELECTROPHORESIS RECORDING SHEET

| 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. | | | | | |



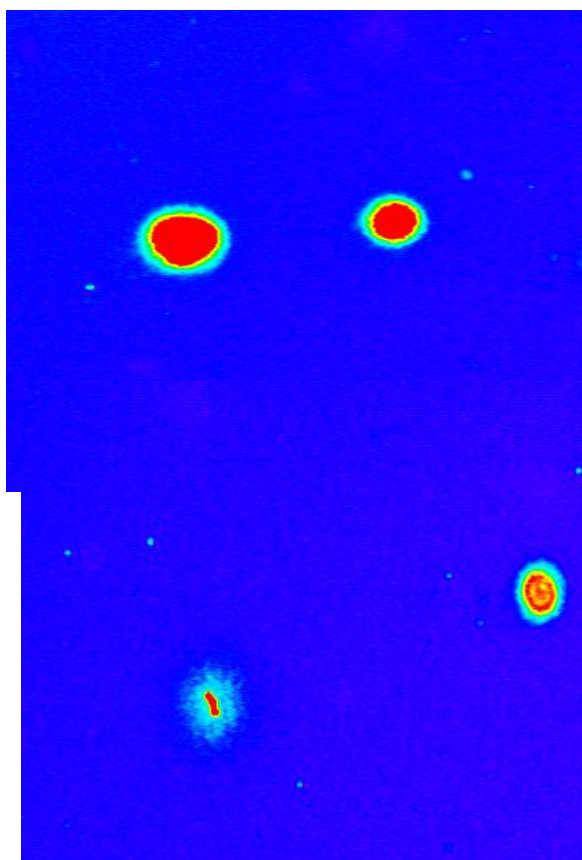
Appendix 1
RED(+)

BLACK(-)

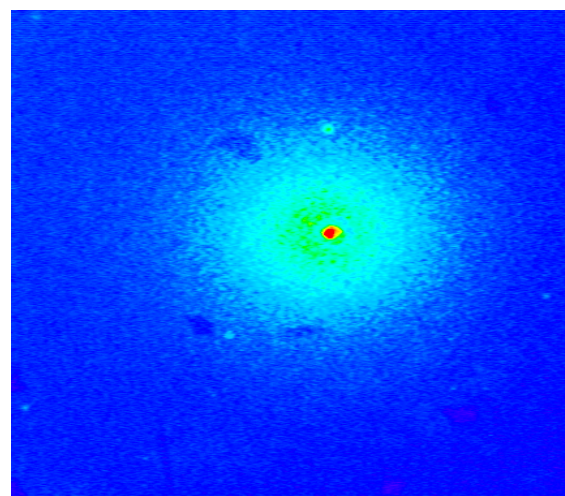


Photographs of cells with diffuse DNA indicative of low molecular weight DNA. Comet slides were removed from lysis after one to 24 hours, rinsed to remove detergents, stained with SYBRGreen, and examined at 250X magnification. Control cells with high molecular weight DNA are in picture A. The remaining 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.

A



B



PHASE 1-3, APPENDIX 3

INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS - Supplementary Protocol for 2nd Pre-validation Study -

Issued by: the Validation Management Team (VMT)

Date: April 8, 2007

June 8, 2007 (added section E. 2. 2-1. and 2-2.)

PURPOSE OF THIS DOCUMENT

This document is provided trial by trial as a supplement of study protocol to clarify the purpose, the schedule, and the specific notes of each trial of an international validation study to evaluate the ability of the *in vivo* rodent alkaline Comet.

STUDY TITLE

2nd pre-validation study of international validation of the *in vivo* rodent alkaline Comet assay for the detection of genotoxic carcinogens (abbreviation: 2nd pre-validation study of *in vivo* Comet assay)

BACKGROUND AND PURPOSE OF THIS STUDY

The previous pre-validation study with EMS has been finished successfully, and VMT considers that data from five leading laboratories would be well validated. However this is a result from one trial, and reproducibility and robustness of positive testing-results with EMS, a positive control of further validation studies, should be verified furthermore under a condition that experiments are conducted based on the comet assay protocol-version 12. That is, when EMS administration to rats always gives positive results in the liver and the stomach in all testing facilities in all experiments of this study, VMT will be able to conclude that the Comet assay protocol-version 11 is acceptable for definitive validation studies.

In data analysis process of the previous pre-validation study with EMS, three conceptual key terms, i.e. "Endpoint", "Estimate", and "Effect" were 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.

In this study, three coded test compounds (e.g. two are expected as weakly-positive results, and one is expected as a negative result) will be assayed in five leading laboratories in accordance with the Comet assay protocol-version 11. Five data of each test compound will be obtained from five testing facilities. Simultaneously, as each experiment includes a positive control group, three data of positive control groups will be obtained from each testing facility, and finally 15 data of positive control groups will be available.

The first purpose of this study is to examine reproducibility and robustness of positive control results with EMS when experiments are conducted in accordance with the Comet assay protocol-version 12, and this means to examine acceptability of the Comet assay protocol-version 12 for definitive validation studies. The second purpose is to examine variation of "Effects" among five testing facilities, and a range of variation of "Effect" obtained from this study will be applied as acceptable variation criteria in definitive validation studies.

SCHEDULE

~April 8, 2007: Fixation of this supplementary protocol in VMT

~April 15, 2007: Delivery of protocol-version 11 and supplementary protocol to testing facilities

~May 30, 2007: Delivery of test compounds; Fixation of study protocol in each testing facility

May ~ December, 2007: Experimental period (Data on each test compound will be submitted to VMT ASAP when available. At least 2 test compound data will be submitted to VMT by the end of September)

December 31, 2007: Deadline of all data submission to VMT

~March 31, 2008: Finalization of data analysis

SPECIFIC NOTES

1. SUCCESS CRITERIA

1-1. To obtain positive results in all positive control groups in all testing facilities.

1-2. To determine a range of variation of "Effect" to apply as acceptable variation criteria in definitive validation studies.

2. OTHERS

2-1. Dose selection of three coded test compounds

The dose levels of one of three compounds will be directed by VMT. VMT will inform an appropriate individual within the organization who is not involved in the study, and then the individual will inform you of the dose levels. The other two compounds will be determined based on the limited dose finding studies in each laboratory. The dose finding studies will be conducted with the highest dose level of 500 mg/kg.

2-2. Solvent/vehicle

One compound which dose levels are directed by VMT will be dissolved in physiological saline. VMT will also inform the solvent/vehicles for the other two compounds.

PHASE 1-3, APPENDIX 4

**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 31, 2008 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 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. (Notes: we will decide the appropriate number of animals/group 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. (Notes: will likely need to specify shelf life for some solutions as we reconcile lab-specific protocols.)

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) | 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: 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. 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. 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 a scalpel blade or a Teflon scraper. 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 scraper 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 (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 (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 0.30 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 . 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 ($\geq 99.6\%$) 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 (Notes: to be re-evaluated after statistical analysis). Approximately 10 areas/slide should be observed at 5 cells or less/field (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 (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, 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]. (Notes: at Atagawa meeting held on March 13-14, 2008, there were some discussions about necessity of tail length and Olive tail moment. 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 side test is also applied for data analysis.

3. DATA AND REPORTING

3.1 Treatment of results

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

3.2 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.

3.3 Study report

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

3.3.1 Test substance and positive/negative controls

Identification; CAS number; supplier; lot number; physical nature and purity; physicochemical 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.

3.3.2 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.

3.3.3 Reagents to prepare reagent solutions

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

3.3.4 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.

3.3.5 Results

Signs of toxicity, including histopathology in the appropriate tissue(s) if applicable; individual and mean/median values for DNA migration (and ranges) and % cells with low molecular weight DNA and % hedgehogs in individual tissue, animal, and group; concurrent positive and negative control

data; and statistical evaluation.

3.3.6 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.

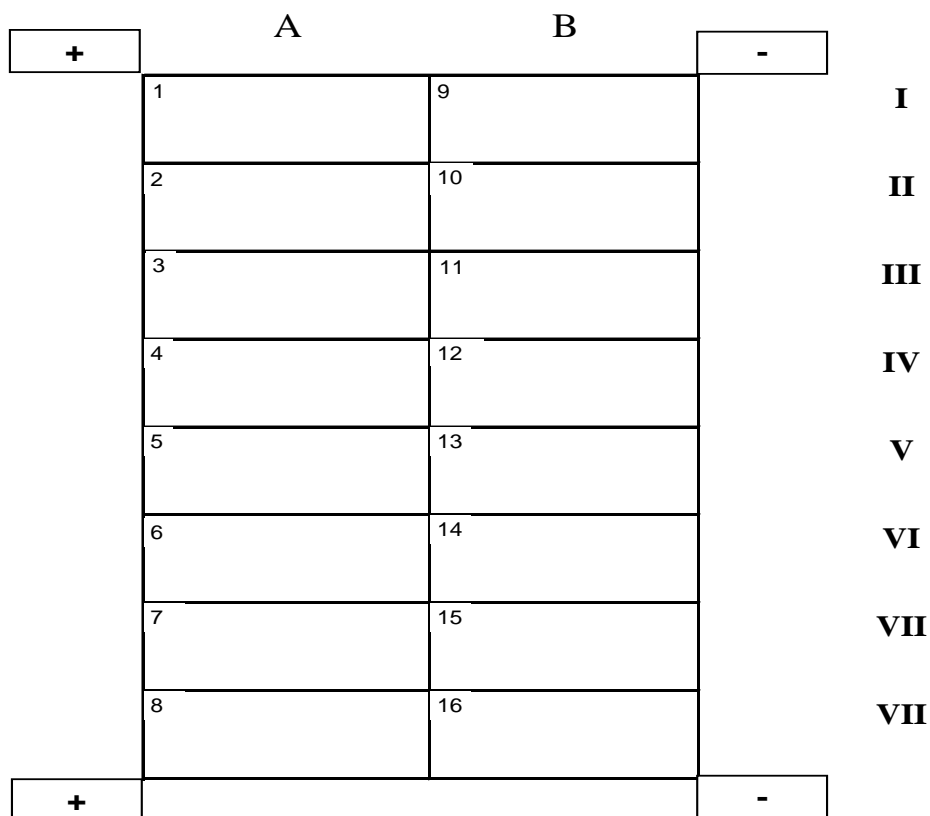
5. REFERENCES

- 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. | | | | | |

Diagram Electrophoresis Chamber



RED(+)

BLACK(-)



Position of slide in chamber

PHASE 1-3, APPENDIX 5

**INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY
FOR THE DETECTION OF GENOTOXIC CARCINOGENS
- Study Plan for 3rd Phase Validation Study -**

Issued by: the Validation Management Team (VMT)

Date: March 31, 2008

Revised #1: October 2, 2009

A. PURPOSE OF THIS DOCUMENT

This document is provided trial by trial as a supplement of study protocol to clarify the purpose, the schedule, and the specific notes of each trial of an international validation study to evaluate the ability of the *in vivo* rodent alkaline Comet.

B. STUDY TITLE

3rd phase validation study of international validation of the *in vivo* rodent alkaline Comet assay for the detection of genotoxic carcinogens (abbreviation: 3rd phase validation study of *in vivo* Comet assay)

C. BACKGROUND AND PURPOSE OF THIS STUDY

In the 2nd phase validation study of *in vivo* Comet assay, following problems were clarified: 1) EMS treatment induced positive responses for the liver in all (five) leading laboratories through three independent experiments, but failed to produce positive results for the stomach in each one of three experiments conducted in two leading laboratories; and 2) large variation of Effects (difference of an average of Estimate between a negative control group and an EMS treatment group) were observed among five testing facilities. In addition, one and three of five laboratories showed large within-laboratory variation of the Effect in the liver and in the stomach, respectively.

One of success criteria of the 2nd phase validation study of *in vivo* Comet assay was to obtain positive results in all positive control groups in all testing facilities. Thus the above problems indicate that the comet assay protocol-version 12 may not be suitable as it is for the further validation studies, at least for the stomach. Based on discussion with the members of VMT, leading laboratories and consultation team including statisticians at Atagawa meeting (March 13-14, 2008), the comet assay protocol has been revised to version 13 intended to solve above problems. In addition, tentative criteria on data acceptability applied in the laboratory selection process for the future validation (see section E.2.) have been established in consideration of the data from the 2nd phase validation study of *in vivo* Comet assay. It is also necessary to investigate whether or not the tentative data-acceptance criteria can be applied to judge data reliability in the future validation studies.

In this 3rd phase validation study of *in vivo* Comet assay, two or three coded test compounds will be assayed in leading laboratories in accordance with the Comet assay protocol-version 13. The first purpose is to examine reproducibility and robustness of positive control results with EMS when experiments are conducted in accordance with the Comet assay protocol-version 13, and this means to examine acceptability of the Comet assay protocol-version 13 for further validation studies. The second purpose is to investigate whether or not the tentative data-acceptance criteria are suitable to judge reliability of data.

D. SCHEDULE

~March 31, 2008: Fixation of this study plan in VMT

~April 15, 2008: Delivery of protocol-version 13 and study plan to testing facilities

~May 31, 2008: Delivery of test compounds; Fixation of study protocol in each testing facility

June ~ November, 2008: Experimental period (Data on each test compound will be submitted to VMT ASAP when available)

November 30, 2008: Deadline of all data submission to VMT

~February 28, 2009: Finalization of data analysis

E. SPECIFIC NOTES

1. SUCCESS CRITERIA

- 1-1. To obtain positive results in all positive control groups in all testing facilities.
- 1-2. To confirm that data from all testing facilities can satisfy the tentative data-acceptance criteria.

2. TENTATIVE DATA-ACCEPTANCE CRITERIA*

2-1. Negative control

Means of %DNA in tail are 1-8% in the liver and 1-30% (preferably 1-20%) in the stomach.

2-2. Positive control in case of EMS, 200 mg/kg, once (or twice) p.o.

Effect (ratio of means of %DNA in tail between groups of EMS and vehicle control) is 2-fold or higher in the liver and the stomach; Effect (difference of means of %DNA in tail between groups of EMS and vehicle control) is 5% or higher in the liver and the stomach; and CV of Effect (ratio of means of %DNA in tail between groups of EMS and vehicle control) is 50% or less in two or more independent experiments with the liver and the stomach.

* Data-acceptance criteria may be revised based on the 3rd phase validation study results, especially for criteria on the positive control, because the dose level may change to 300 mg/kg as from the 3rd phase validation study in order to obtain clearer positive results.

3. OTHERS

3-1. Dose selection of three coded test compounds

The dose levels of all compounds will be directed by VMT. VMT will inform an appropriate individual within the organization who is not involved in the study, and then the individual will inform you of the dose levels.

3-2. Solvent/vehicle

VMT will inform the solvent/vehicles for all compounds later.

3-3. Voltage in electrophoresis

The slides will be electrophoresed at 0.7 V/cm with a constant voltage at approximately 0.30 A.

4. TENTATIVE JUDGMENT CRITERIA ON CODED TEST CHEMICAL DATA

When the following three requirements are fulfilled, the coded test chemical will be judged as "positive" in the organ: Effect (ratio of means of %DNA in tail between groups of coded test chemical and vehicle control) is 2-fold* or higher in the liver and/or the stomach; Effect (difference of means of %DNA in tail between groups of coded test chemical and vehicle control) is 5%* or higher in the liver and/or the stomach; and statistically significant difference in means of %DNA in tail is noted with the Dunnett test ($p < 0.05$, both side) between groups of coded test chemical and vehicle control in the liver and/or the stomach.

Otherwise the coded test chemical will be judged as "negative" in the organ.

* These values may be revised based on the reanalysis results of the 2nd phase validation study data.

PHASE 1-3, APPENDIX 6

**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 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.

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: CrI: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. (Notes: we will decide the appropriate number of animals/group 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. (Notes: will likely need to specify shelf life for some solutions as we reconcile lab-specific protocols.)

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) | 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: 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). This regimen will enable us to detect comet and micronucleus at the same time. EMS will be administered once orally by gavage at 3 hours before animal sacrifice. 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. 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. 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 (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 (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 0.30 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 . 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 (Notes: to be re-evaluated after statistical analysis). Approximately 10 areas/slide should be observed at 5 cells or less/field (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 cannot properly score them (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, 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]. (Notes: at Atagawa meeting held on March 13-14, 2008, there were some discussions about necessity of tail length and Olive tail moment. 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 side test is also applied for data analysis.

3. DATA AND REPORTING

3.1 Treatment of results

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

3.2 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.

3.3 Study report

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

3.3.1 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.

3.3.2 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.

3.3.3 Reagents to prepare reagent solutions

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

3.3.4 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.

3.3.5 Results

Signs of toxicity, including histopathology in the appropriate tissue(s) if applicable; individual and mean/median values for DNA migration (and ranges) and % cells with low molecular weight DNA and % hedgehogs in individual tissue, animal, and group; concurrent positive and negative control data; and statistical evaluation.

3.3.6 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.

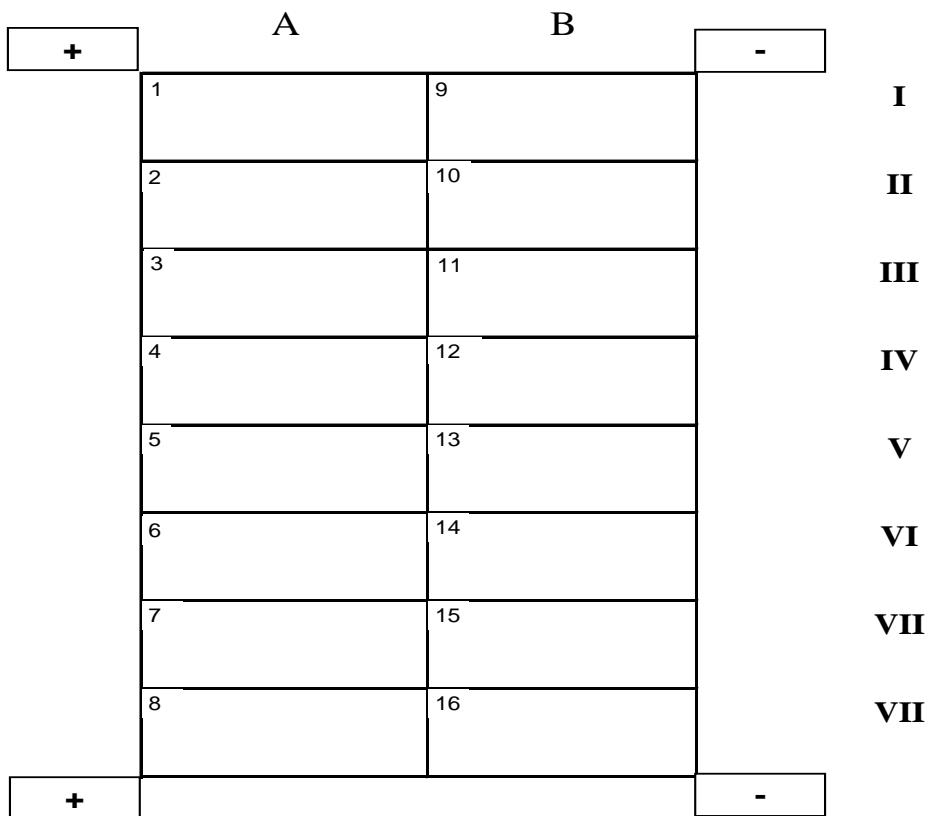
5. REFERENCES

- 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**

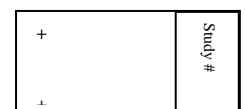
| Electrophoresis Run # | | | | Initials & Date | |
|---|-------|-------|-----|-----------------|--|
| Approximate alkaline electrophoresis buffer volume in chamber | | | | | |
| Unwinding | | | | | |
| Time | Total | Start | End | | |
| | | | | | |
| Buffer Temperature | | | | | |
| Electrophoresis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

Diagram Electrophoresis Chamber



RED(+)

BLACK(-)



Position of slide in chamber

APPENDIX 7

Final report on the recruitment of laboratories for participation in the 4th phase international *in vivo* Comet assay validation study (Draft)

Prepared: Yoshifumi Uno, Ph.D., D.V.M.

Date: November 30, 2009

1. Introduction

This report is provided to show the process of laboratory recruitment for the 4th phase (definitive) international validation study of *in vivo* rodent Comet assay, the data obtained from candidate laboratories, and the analysis results on their data. This report (or summary of this report) may be released in the future to clarify the laboratory recruitment processes (in case of release, laboratory names will be coded).

2. Advance judgment: *in vivo* Comet assay experience

The validation management team (VMT) requested laboratories considering participation in this validation study to provide the historical negative and positive control data of the *in vivo* rodent Comet assay generated in their laboratories only from the most recent five or more different studies to the VMT, using a fixed form. Copies of the standard detailed protocols used in the laboratories were requested also. The VMT also requested copies of any relevant publications supporting the investigator's/laboratory's experience with this *in vivo* test method. The VMT reviewed the submitted information to ensure that the investigator/laboratory had sufficient experience with this test method to participate in the validation effort. For laboratories that could not provide *in vivo* rodent Comet assay data generated from at least five different studies, VMT requested them to demonstrate, as best they could, technical competency with the *in vivo* Comet assay, e.g., letter of recommendation from a lead laboratory in this field or from a scientific organization such as JEMS/MMS.

Fifteen laboratories (Annex 1) responded to "the invitation to participate in an international *in vivo* Comet assay validation studies" issued by the VMT (Annex 2). Six of the fifteen laboratories submitted the requested data and/or published papers to the VMT. The VMT reviewed the submitted materials, and considered these six laboratories to have sufficient experiences with the *in vivo* rodent Comet assay (laboratory code: Lab.1-6). Four of the fifteen laboratories could not submit the requested data because of fewer experiences, but provided letters of recommendation from a lead laboratory in this field or from JEMS/MMS. The VMT considered these four laboratories demonstrated technical competency with the *in vivo* Comet assay (Lab.7-10). Regarding the other five laboratories, one was still doing dose-dependent experiments with EMS as well as the 1st phase pre-validation study in order to obtain JEMS/MMS recommendation at that moment, but finally gave up the participation (Lab.11); one gave up the participation due to a technical issue, in that they could not obtain stable negative control data (Lab.12); one declined to participate in this validation effort, because they could not adhere to the standardized protocol as they wanted to use the Trevigen gel slides for Comet assay (Lab.13); one did not have sufficient background data with the Comet assay but still has many concerns about the international validation study (Lab.14); and one had provided no response after the first contact (Lab.15).

3. Submission of the Comet assay data on coded chemicals to the VMT

Since Labs.1-10 were provisionally approved for participation in this validation effort, the VMT provided these laboratories with two coded chemicals (code: Black, Yellow) and a positive control EMS to test in the *in vivo* Comet assay in accordance with standardized protocol version v.12 developed during the pre-validation efforts.

The laboratories were requested to follow the standard protocol rigorously. Laboratories were required to submit the results of those tests using forms provided to the VMT by May 2008 in our original plan, but

the deadline was extended to July 2008, because some laboratories required more time in order to balance the validation work with their own work (note: the deadline for Lab.4 was further extended to September 2008).

Data-acceptance criteria were discussed at the Atagawa meeting in March 2008 and determined tentatively as follows: A. negative control: means of %DNA in tail are 1-8% in the liver and 1-30% (preferably 1-20%) in the stomach; B. positive control EMS, 200 mg/kg, once (or twice) p.o.: a) effect (ratio of means of %DNA in tail between groups of EMS and vehicle control) is 2-fold or higher in the liver and the stomach, b) effect (difference of means of %DNA in tail between groups of EMS and vehicle control) is 5% or higher in the liver and the stomach, and c) CV of Effect (ratio of means of %DNA in tail between groups of EMS and vehicle control) is 50% or less in two or more independent experiments with the liver and the stomach. These draft data-acceptance criteria were applied to the data from candidate laboratories in order to judge the acceptability of laboratories.

Nine laboratories out of 10 submitted the data required, and the VMT determined which laboratories could participate in the 4th phase validation study. The section 4 of this report shows the summary of data and the analysis results. Section 5 reveals the VMT judgment.

4. Summary of data on negative and positive controls and the analysis results

Table 4-1. Data from Labs.1-6

| Lab. Code | Organ | Exp. No. | Negative control: Mean, SD | Positive control: Mean, SD | Effect (ratio) | Effect (difference) | CV for Effect (ratio) |
|-----------|---------|----------|----------------------------|----------------------------|----------------|---------------------|-----------------------|
| 1 | Liver | Black | 2.2, 0.7 | 28.8, 4.8 | 13.0 | 26.5 | 1.4 |
| | Liver | Yellow | 1.6, 0.6 | 20.6, 2.0 | 13.2 | 19.0 | |
| | Stomach | B | 12.1, 3.3 | 38.3, 4.5 | 3.2 | 26.2 | 7.1 |
| | Stomach | Y | 11.4, 3.8 | 40.0, 6.6 | 3.5 | 28.6 | |
| 2 | Liver | B & Y | 16.2, 8.5 | 31.2, 6.5 | 1.9 | 15.0 | ND |
| | Stomach | B & Y | 18.6, 3.2 | 41.1, 6.0 | 2.2 | 22.4 | ND |
| 3 | Liver | B | 8.7, 1.7 | 28.8, 4.6 | 3.3 | 20.1 | 27.1 |
| | Liver | Y | 3.4, 0.5 | 16.4, 7.1 | 4.9 | 13.1 | |
| | Stomach | B | 8.3, 4.4 | 30.0, 7.3 | 3.6 | 21.6 | 2.1 |
| | Stomach | Y | 6.0, 2.3 | 21.0, 3.8 | 3.5 | 15.0 | |
| 4 | - | - | - | - | - | - | - |
| 5 | Liver | B | 10.7, 3.2 | 32.5, 23.1 | 3.1 | 21.9 | 44.1 |
| | Liver | Y-1 | 6.1, 2.3 | 17.3, 7.4 | 2.8 | 11.2 | |
| | Liver | Y-2 | 1.5, 0.7 | 8.8, 0.9 | 5.9 | 7.3 | |
| | Stomach | B | 12.3, 5.6 | 40.7, 17.6 | 3.3 | 28.3 | 37.4 |
| | Stomach | Y-1 | 13.2, 6.7 | 26.9, 12.4 | 2.0 | 13.6 | |
| | Stomach | Y-2 | 3.2, 1.3 | 14.2, 2.6 | 4.5 | 11.1 | |
| 6 | Liver | B | 4.3, 1.4 | 19.0, 1.8 | 4.4 | 14.6 | 33.9 |
| | Liver | Y | 3.0, 0.6 | 21.5, 3.3 | 7.2 | 18.5 | |
| | Stomach | B | 5.9, 0.4 | 24.0, 4.1 | 4.0 | 18.0 | 3.6 |
| | Stomach | Y | 6.9, 1.2 | 26.5, 2.3 | 3.8 | 19.6 | |

Notes:

- 1) Red letter means that the value fails to meet the draft data-acceptance criteria.
- 2) Since Lab.2 examined two coded compounds in one experiment, they submitted control data of one negative and one positive. Thus CV can not be calculated.
- 3) Finally Lab.4 did not submit the data although the deadline was prolonged until September, 2008 (and no reply against reminder e-mails).
- 4) Lab.5 repeated the experiment for Yellow.

Table 4-2. Data from Labs.7-10

| Lab. Code | Organ | Exp. No. | Negative control: Mean, SD | Positive control: Mean, SD | Effect (ratio) | Effect (difference) | CV for Effect (ratio) |
|-----------|---------|----------|----------------------------|----------------------------|----------------|---------------------|-----------------------|
| 7 | Liver | EMS-1 | 2.5, 0.9 | 16.4, 2.1 | 6.7 | 14.0 | 24.4 |
| | Liver | EMS-2 | 1.1, 0.7 | 9.9, 1.6 | 8.7 | 8.8 | |
| | Liver | Black | 1.8, 0.5 | 11.1, 1.4 | 6.0 | 9.3 | |
| | Liver | Yellow | 1.2, 0.9 | 12.5, 3.2 | 10.3 | 11.3 | |
| | Stomach | EMS-1 | 9.0, 1.1 | 34.7, 7.5 | 3.8 | 25.7 | 46.9 |
| | | EMS-2 | 5.1, 2.0 | 46.8, 4.9 | 9.2 | 41.7 | |
| | | B | 9.8, 6.3 | 37.7, 6.8 | 3.8 | 27.9 | |
| | | Y | 6.5, 3.3 | 31.8, 5.0 | 4.9 | 25.2 | |
| 8 | Liver | EMS-1 | 2.5, 0.7 | 19.5, 4.6 | 7.8 | 17.0 | 36.1 |
| | Liver | EMS-2 | 4.4, 1.3 | 13.4, 3.3 | 3.1 | 9.0 | |
| | Liver | Black | 2.8, 0.5 | 20.4, 2.6 | 7.2 | 17.6 | |
| | Liver | Yellow | 2.3, 0.8 | 12.5, 4.0 | 5.5 | 10.2 | |
| | Stomach | EMS-1 | 6.9, 3.1 | 33.7, 7.9 | 4.9 | 26.8 | 29.0 |
| | | EMS-2 | 6.0, 1.2 | 17.5, 0.6 | 2.9 | 11.5 | |
| | | B | 5.6, 1.1 | 29.3, 5.0 | 5.2 | 23.7 | |
| | | Y | 5.2, 1.8 | 16.5, 2.8 | 3.2 | 11.3 | |
| 9 | Liver | EMS-1 | 5.4, 1.5 | 43.3, 8.4 | 8.0 | 37.9 | 53.4 |
| | Liver | EMS-2 | 4.4, 1.2 | 57.8, 4.9 | 13.3 | 53.4 | |
| | Liver | Black | 2.7, 0.9 | 75.7, 6.1 | 28.5 | 73.1 | |
| | Liver | Yellow | 3.4, 0.7 | 52.8, 8.1 | 15.3 | 49.3 | |
| | Stomach | EMS-1 | 9.9, 1.4 | 50.7, 6.3 | 5.1 | 40.8 | 17.3 |
| | | EMS-2 | 15.7, 4.2 | 59.6, 5.9 | 3.8 | 43.8 | |
| | | B | 14.6, 3.1 | 80.8, 2.5 | 5.5 | 66.2 | |
| | | Y | 13.3, 3.2 | 55.8, 6.1 | 4.2 | 42.4 | |
| 10 | Liver | B | 4.3, 1.0 | 11.6, 1.9 | 2.7 | 7.3 | ND |
| | Liver | Y | 4.2, 1.0 | | | | |
| | Stomach | B | 6.5, 1.6 | 20.2, 1.7 | 3.1 | 13.6 | ND |
| | Stomach | Y | 5.5, 0.6 | | | | |

Notes:

- 1) Red letter means that the value fails to meet the draft data-acceptance criteria.
- 2) Labs.7-9 additionally examined EMS two times to obtain JEMS/MMS recommendation.

- 3) Since Lab.10 examined two coded compounds in one experiment, they submitted two negative (two vehicle) control data and one positive control data. Thus CV cannot be calculated.

5. Judgment of acceptability to participate in the 4th phase validation study

Labs.1, 6, 7, and 8 were fully approved to participate in the 4th phase validation study, because they could submit the data that completely met the tentative data-acceptance criteria.

Labs.3, 5, and 9 were accepted to participate in the 4th phase validation study, because they could submit the data that almost met the draft data-acceptance criteria (the deviation seems minimal).

Discussion was needed for Labs.2 and 10. These laboratories examined two coded chemicals in one experiment, and thus there is one set of data for the positive control EMS and their data could not be applied to calculation of CV as one of the draft data-acceptance criteria. Their higher experimental performance, i.e. so many organ samples were prepared in only one experiment, was not anticipated. Since we did not request them in advance to conduct two separate experiments with two coded chemicals, it seemed difficult to request them to submit another set of data on the negative control and EMS. In addition, their procedure of experiments was preferable from the viewpoint of 3R's, and the inability to calculate CV with two sets of data was not considered a critical deficiency. Therefore, we judged their data on the draft data-acceptance criteria (except the CV) and on their experiences with the Comet assay until now.

In Lab.2, the negative control data are slightly higher and varied in the liver, and the mean value, 16.2%, does not meet the tentative data acceptance criterion, 1-8% in the liver. In addition, the effect (ratio) value, 1.9, does not reach the tentative data-acceptance criterion, 2-fold or higher. This laboratory seems to have extensive experience with the Comet assay using the collagenase liver perfusion technique to prepare hepatocytes, but they have fewer experiences with the mincing method described in our standard protocol. Finally, we requested Lab.2 to submit additional two sets of data on the negative control and the positive control EMS at 200 mg/kg by December, 2008.

In Lab.10, all the data submitted met the tentative data-acceptance criteria except the CV. This laboratory seemed to have fewer experiences because they could not submit their background data in advance, but, in contrast, this laboratory also seemed to have sufficient experimental competence when considering they could examine two coded chemicals in one experiment with two negative controls (60 animals/experiment). Finally, we asked Lab.10 again whether or not they could submit their background data because they might conduct the Comet assay a few times before the experiment with two coded chemicals. If they could submit additional background data, we would review them and then discuss the acceptability of Lab.10. If not, we should request Lab.10 to submit one additional set of data on the negative control and the positive control EMS at 200 mg/kg by December, 2008.

6. Additional data from Labs. 2 and 10, and judgment of their acceptability

As mentioned above, Labs. 2 and 10 were requested to submit additional data to the VMT. Since Lab.10 could not submit additional background data, they submitted one additional set of data on the negative control and the positive control EMS at 200 mg/kg (Table 5, blue letters). Both laboratories were accepted to participate in the 4th phase validation study, because they could submit the data that almost met the draft data-acceptance criteria (there were deviations, but they seemed minimal).

Table 6. Labs.2 & 10: summary of additional data (blue letters), and the analysis results

| Lab. Code | Organ | Exp. No. | Negative control: Mean, SD | Positive control: Mean, SD | Effect (ratio) | Effect (difference) | CV for Effect (ratio) |
|-----------|---------|----------|----------------------------|----------------------------|----------------|---------------------|-----------------------|
| 2 | Liver | B&Y | <u>16.2</u> , 8.5 | 31.2, 6.5 | <u>1.9</u> | 15.0 | ND |
| | Stomach | B&Y | 18.6, 3.2 | 41.1, 6.0 | 2.2 | 22.4 | ND |
| | Liver | EMS-1 | 8.0, 3.3 | 22.9, 3.8 | 2.9 | 14.9 | 21.6 |
| | Liver | EMS-2 | <u>8.6</u> , 1.8 | 18.0, 1.5 | 2.1 | 9.4 | |
| | Stomach | EMS-1 | 11.3, 1.0 | 31.2, 6.8 | 2.8 | 19.9 | 32.9 |
| | Stomach | EMS-2 | 14.8, 1.0 | 25.4, 4.4 | <u>1.7</u> | 10.6 | |
| 10 | Liver | B | 4.3, 1.0 | 11.6, 1.9 | 2.7 | 7.3 | 6.7 |
| | Liver | Y | 4.2, 1.0 | - | - | - | |
| | Liver | EMS | 5.3, 0.6 | 12.9, 0.6 | 2.4 | 7.6 | |
| | Stomach | B | 6.5, 1.6 | 20.2, 1.7 | 3.1 | 13.6 | 32.5 |
| | Stomach | Y | 5.5, 0.6 | - | - | - | |
| | Stomach | EMS | 9.3, 2.0 | 18.0, 3.2 | <u>1.9</u> | 8.7 | |

Underlined figures: the value fails to meet the tentative data-acceptance criteria.

7. Additional participation of Lab.16

In April, 2009, Dr. Raymond Tice, a member of VMT, asked whether it would be acceptable for Lab.16 to participate additionally in this validation study, even though the 1st step of the 4th phase was already under way. The VMT considered that Lab.16 has sufficient experiences and historical data on *in vivo* rodent Comet assay. In order to examine the performance of Lab.16, the VMT requested Lab.16 to submit one set of data on the negative control and the positive control EMS at 200 mg/kg. The experiment was conducted in accordance with the protocol version 14. Lab.16 submitted the data on the negative control and the positive control EMS at 25, 50, 100 and 200 mg/kg (Table 6: data in 200 mg/kg). Lab.16 was accepted to participate in the 2nd step of the 4th phase validation study, because they could submit the data that met the draft data-acceptance criteria except CV (not calculated).

Table 6. Lab.16

| Lab. Code | Organ | Exp. No. | Negative control: Mean, SD | Positive control: Mean, SD | Effect (ratio) | Effect (difference) | CV for Effect (ratio) |
|-----------|---------|----------|----------------------------|----------------------------|----------------|---------------------|-----------------------|
| 16 | Liver | - | 2.7, 0.7 | 18.0, 1.4 | 6.6 | 15.2 | ND |
| | Stomach | - | 6.7, 1.4 | 39.8, 3.9 | 5.9 | 33.1 | |

8. Conclusion

Nine laboratories were accepted to participate in the 1st step of the 4th phase validation study. Ten laboratories were accepted to participate in the 2nd step of the 4th phase validation study.

ANNEX 1: LIST OF LABORATORY CODE AND LABORATORY NAME (REPRESENTATIVE)

- Lab.1: AstraZeneca (Catherine Pritestley)
- Lab.2: Bayer HealthCare (Uta Wirmitzer)
- Lab.3: Covance (Carol Beevers, Lucinda Williams)
- Lab.4: GlaxoSmithKline (Jonathan Howe)
- Lab.5: Johnson & Johnson (Marlies De Boeck)
- Lab.6: Novartis Pharma (Ulla Plappert-Helbig)
- Lab.7: Sumitomo Chemical (Sachiko Kitamoto)
- Lab.8: Mitsubishi Chemical Safety Institute (Kazunori Narumi)
- Lab.9: The Institute of Environmental Toxicology (Kunio Wada)
- Lab.10: Health Canada (James McNamee)
- Lab.11: (Not disclosed)
- Lab.12: (Not disclosed)
- Lab.13: (Not disclosed)
- Lab.14: (Not disclosed)
- Lab.15: (Not disclosed)
- Lab. 16: Integrated Laboratory Systems, Cheryl A. Hobbs

ANNEX 2: INVITATION TO PARTICIPATE IN AN INTERNATIONAL

In Vivo Comet Assay Validation Studies

The Japanese Center for the Validation of Alternative Methods (JaCVAM) is organizing an international validation study of the *in vivo* Comet assay, in cooperation with the U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Centre for the Validation of Alternative Methods (ECVAM), and the Mammalian Mutagenicity Study Group (MMS)/Japanese Environmental Mutagen Society (JEMS). The purpose of this validation study is to evaluate the ability of the *in vivo* Comet assay to identify genotoxic chemicals as a potential predictor of rodent carcinogenicity.

Efforts to organize this validation study have been in progress since August 2006. The 1st phase, successfully completed in late 2006, was a pre-validation study with ethyl methanesulfonate (EMS), the proposed positive control. This phase, using five laboratories with extensive experience with this test method, was conducted to establish a common protocol and to work out data acquisition and reporting requirements. Currently, the 2nd phase of this validation effort is in progress; the purpose of this phase of the pre-validation study is to evaluate the utility and reproducibility among laboratories of the defined protocol in the same five laboratories using three coded chemicals, with EMS as the positive control. The main validation studies are expected to start in July 2008 and will involve testing a larger number of chemicals in more laboratories. The structure of this stage of the validation effort has not yet been finalized, but tentatively, over 40 coded chemicals will be examined during a two-year period with the expectation that each participating laboratory will test three or four coded chemicals per year.

The validation management team (VMT) would like to invite interested scientists to join in the validation of this important test method. Dr. Makoto Hayashi serves as the chair of the VMT and Dr. Hajime Kojima serves as the project coordinator. The prerequisites to participate include the ability to: 1) adhere to a defined protocol, 2) conduct GLP compliant studies, 3) collect DNA migration data and images using an image analyzer system, and 4) provide for consideration by the VMT historical *in vivo* Comet assay data on at least five chemicals. Even if these requirements cannot all be fulfilled, the VMT will consider participation in the validation study on a case-by-case basis. All candidate laboratories will need to demonstrate proficiency with the assay using a coded chemical provided by the VMT before being considered as a prospective participant in the validation study. Coded compounds will be provided for testing but other financial resources will not be provided.

To indicate your willingness to participate in this international validation study, please read the following information and contact Dr. Hajime Kojima. Also, please do not hesitate to contact him by email if you have any questions.

Project Coordinator:

Hajime Kojima, Ph.D.,
JaCVAM, Division of Pharmacology, Biological Safety Research Center,
National Institute of Health Sciences
1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
+81-3-3700-9874
h-kojima@nihs.go.jp

VMT Chair:

Makoto Hayashi, D.Sc.

Head, Division of Genetics and Mutagenesis
National Institute of Health Sciences
1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
+81-3-3700-9872
hayashi@nihs.go.jp

**Requirements for the consideration for participation in the
international *in vivo* Comet assay validation study**

To be considered for participation in this validation study, please provide the following information to Dr. Kojima.

1. *In vivo* Comet assay experience

Please provide the historical NEGATIVE AND POSITIVE CONTROL DATA of the *in vivo* rodent Comet assay generated in your laboratory only from the most recent five or more different studies to the VMT, using the attached form. A copy of the standard detailed protocol used in your laboratory in these studies is requested also. The VMT also requests copies of any relevant publications supporting the investigator's/laboratory's experience with this *in vivo* test method. The VMT will review the submitted information to ensure that the investigator/laboratory has sufficient experience with this test method to participate in the validation effort.

Laboratories that cannot provide *in vivo* rodent Comet assay data generated from at least five different studies will still be considered for participation in this validation study. In this situation, you are requested to demonstrate, as best you can, technical competency with the *in vivo* Comet assay (e.g., letter of recommendation from a lead laboratory in this field or from a scientific organization such as JEMS/MMS).

2. Submission of the Comet assay data on a coded chemical to the VMT

If you have been provisionally approved for participation in this validation effort, the VMT will provide you with a coded chemical to test in the *in vivo* Comet assay in accordance with a standardized protocol developed for this validation effort. The laboratory will be requested to follow the standard protocol rigorously. You would then be required to submit the results of this test using forms provided to the VMT by February 2008 (an earlier submission is preferred). The VMT will review the data and inform you by May 2008 as to whether or not you have been accepted as a participant in the validation study.

Attached form: Historical Data Sheet on the *In Vivo* Comet Assay
[Please fill in a separate sheet for each study]

Name of Principal Investigator:

Affiliation:

Address:

Telephone number:

E-mail address:

Please answer the following questions (a detailed protocol for these studies must be provided)

1. GLP facility? : Yes / No but accept protocol and raw data audits by the VMT
2. Information on image analyzer system:
3. Animal species (strain, sex): Rat () / Mouse ()
If you have historical data of both species, please submit rat data only.
4. Organs examined: Liver / Glandular stomach / Others ()
5. Cell preparation method: Mincing / Homogenizing / Others ()
6. Historical data: Please fill in the columns of the following tables with “mean \pm S.D. (n=numbers of animals examined)”. If you also have the data in another format (e.g., graph), please provide.

A. Negative control data

Vehicle name:

(You may add columns and fill them separately for each vehicle)

| Organ | %DNA in tail | Tail length (μ m) | Olive tail moment |
|-------------------|--------------|------------------------|-------------------|
| Liver | (n=) | (n=) | (n=) |
| Glandular stomach | (n=) | (n=) | (n=) |
| | | | |

B. Positive control data

Chemical name:

(You may add columns and fill them separately for each positive control)

| Organ | %DNA in tail | Tail length (μ m) | Olive tail moment |
|-------------------|--------------|------------------------|-------------------|
| Liver | (n=) | (n=) | (n=) |
| Glandular stomach | (n=) | (n=) | (n=) |
| | | | |