The JaCVAM / OECD activities on the comet assay

Hajime Kojima
NIHS, JaCVAM, Japan

Today's Topics

1. Summary of *in vivo* comet assay Validation Studies
2. Peer review by the OECD experts
3. General Introduction of OECD TG489
4. *In vitro* comet assay validation study

Summary of *in vivo* comet assay validation Studies

VMT chair: Yoshifumi Uno ((MTPC, JEMS/MMS)

Why Did We Need the Validation Study?

- A reason for extensive use of the comet assay was that U.S. FDA had recommended the assay because the *in vivo* rodent liver UDS assay seemed to be less sensitive for detecting genotoxic chemicals.
- Nevertheless, a positive result of the comet assay had given a critical impact on new chemical development, especially for pharmaceutical candidates.
- Although the assay methodology was scientifically discussed at the IWGT or the ICAW meetings, many researchers in genotoxicity field had yearned to establish the OECD test guideline for regulatory use.
- To establish the test guideline, it was needed to make the robust assay procedures based on validation data. Thus, JaCVAM organized the international validation study, with cooperation of the U.S. NICEATM and ICCVAM, the EURL ECVAM, and the JEMS/MMS.

Process of Our Validation Effort

P1 ***At 5 lead labs with ethyl methanesulfonate (EMS)***

▲ ***Start in Aug.***

P2 ***At 5 lead labs with EMS +3 coded chem.***

Protocol Optimization
Within/Between-Lab Reproducibility

P3 ***Optimized-Protocol Confirmation***
Within/Between-Lab Reproducibility

At 4 lead labs with EMS+3 coded chem.

Lab Recruitment ***Transferability***

Reproducibility **P4-1**

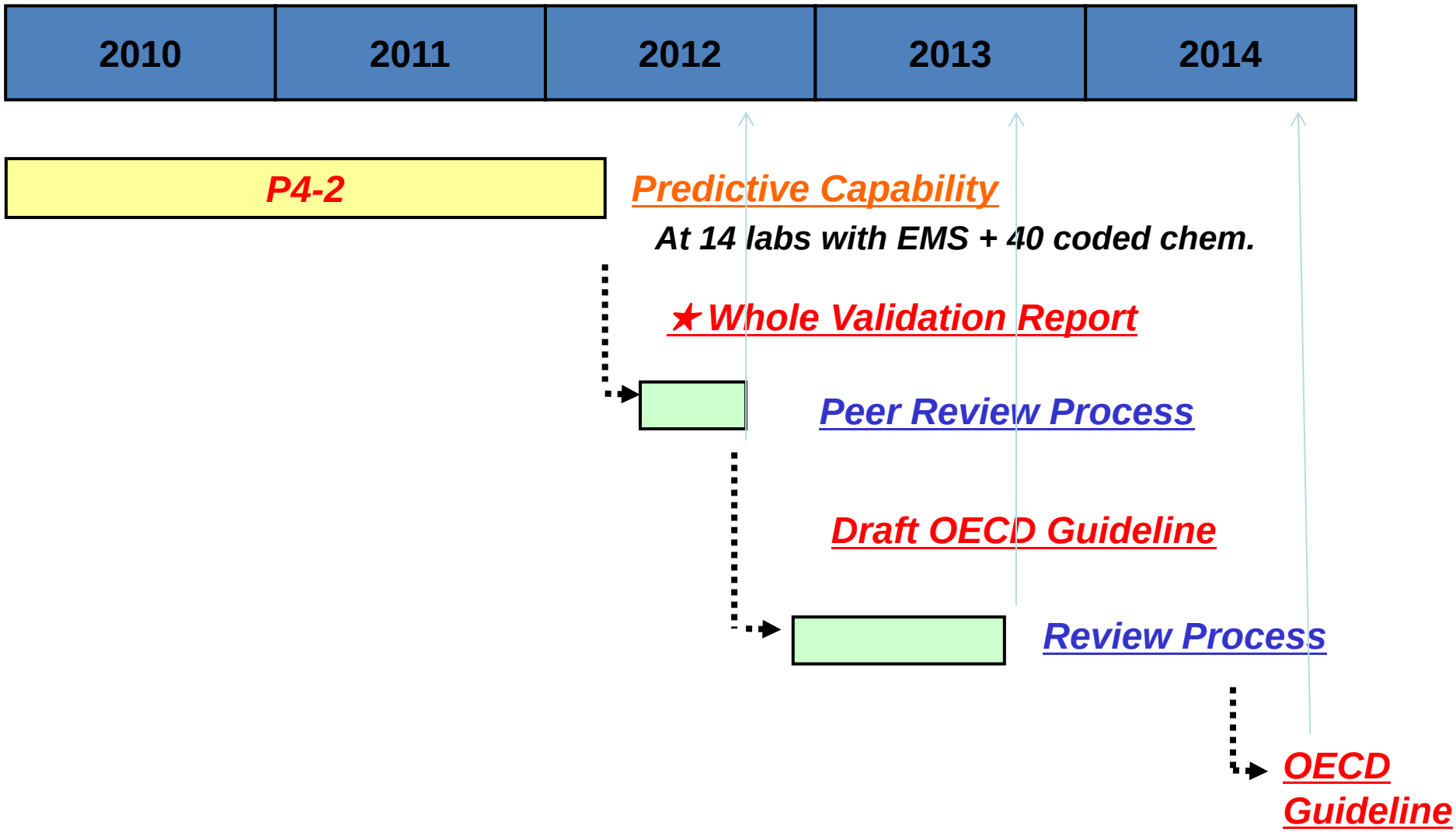
At 13 labs with EMS+4 coded chem.

Predictive Capability **P4-2**

At 14 labs with EMS+40 coded chem.

P: Phase

Process of Our Validation Effort (contd.)



Organization of Validation Study

Validation Management Team (VMT)

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

Consultation Team

N. Asano (Kinki Univ., JEMS/MMS)
P. Escobar (Merck)
D. Lovell (St. George's Univ. of London)
T. Morita (NIHS)
M. Nakajima (Univ. of Shizuoka)
Y. Ohno (NIHS/JaCVAM)
T. Omori (Univ. of Kobe Hospital)

Participant Laboratory (alphabetic order)

1.AstraZeneca (UK) : C. Priestley
2.Bayer Schering Pharma (Germany) : U. Wirnitzer
3.BioReliance* (USA) : K. Pant
4.Covance (UK) : L. Williams
5.Food and Drug Safety Center* (JPN) : K. Yamakage
6.Health Canada (Canada) : J. P. McNamee
7.Huntingdon Life Sciences* (UK) : B. Burlinson
8.Integrated Laboratory System (USA): C. A. Hobbs
9.Janssen R&D (Belgium) : M. De Boeck
10.Merck* (USA) : A. Kraynak
11.LSI Medience (JPN) : H. Takasawa
12.Novartis Pharma (Switzerland) : U. Plappert-Helbig
13.Sumitomo Chemical (JPN) : S. Kitamoto
14.The Institute of Environmental Toxicology (JPN) : K. Wada

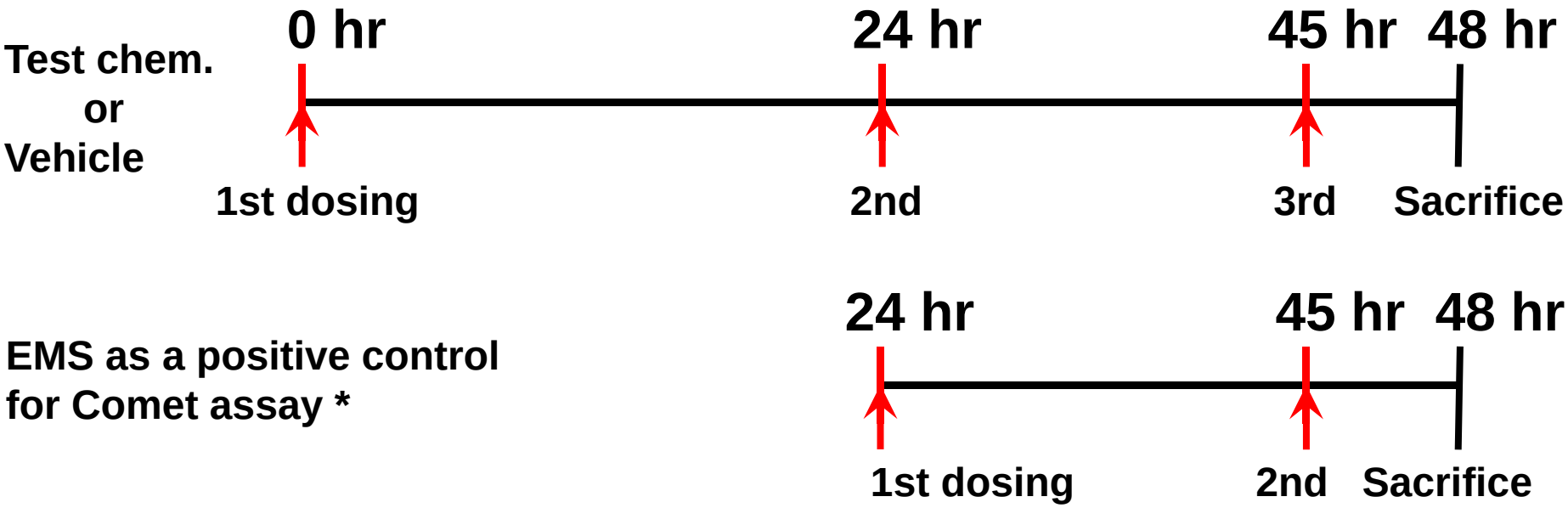
* Lead laboratory

Summary of Study Protocol (v.14.2)

- ✓ **Animal: Crl:CD(SD) male rats, 7-9 weeks old at dosing, 5 rats/group**
- ✓ **Group: vehicle, 3 dose levels of test chemical, and positive control ethyl methanesulfonate (EMS)**
- ✓ **Administration: see the next slide**
- ✓ **Sampling for comet and histopathology: liver and glandular stomach**
- ✓ **Electrophoresis: 0.7 V/cm at approx. 300 mA below 10℃, and at least 20 min duration**
- ✓ **Staining: SYBR gold**
- ✓ **Analysis: 50 comets/slide and 2 slides/animal using an image analyzer system (e.g., Comet IV)**
- ✓ **Primary endpoint: % tail DNA**
- ✓ **Statistics: Dunnett's test (two-sided, $p<0.05$) and linear Trend test (two-sided, $p<0.05$) for Effect (difference of means of % DNA in tail between a negative control group and treatment groups). Student's *t*-test (one-sided, $p<0.025$) for comparison of the positive control to negative control.**

Administration of Test Chemicals

- ✓ In order to combine comet assay with micronucleus (MN) assay, three-times administration of test chemicals was used in the study protocol. MN assay, however, was optional in this validation study.



*** A positive control for MN will be no longer required when considering current ICH-S2 discussion.**

Data Acceptance Criteria

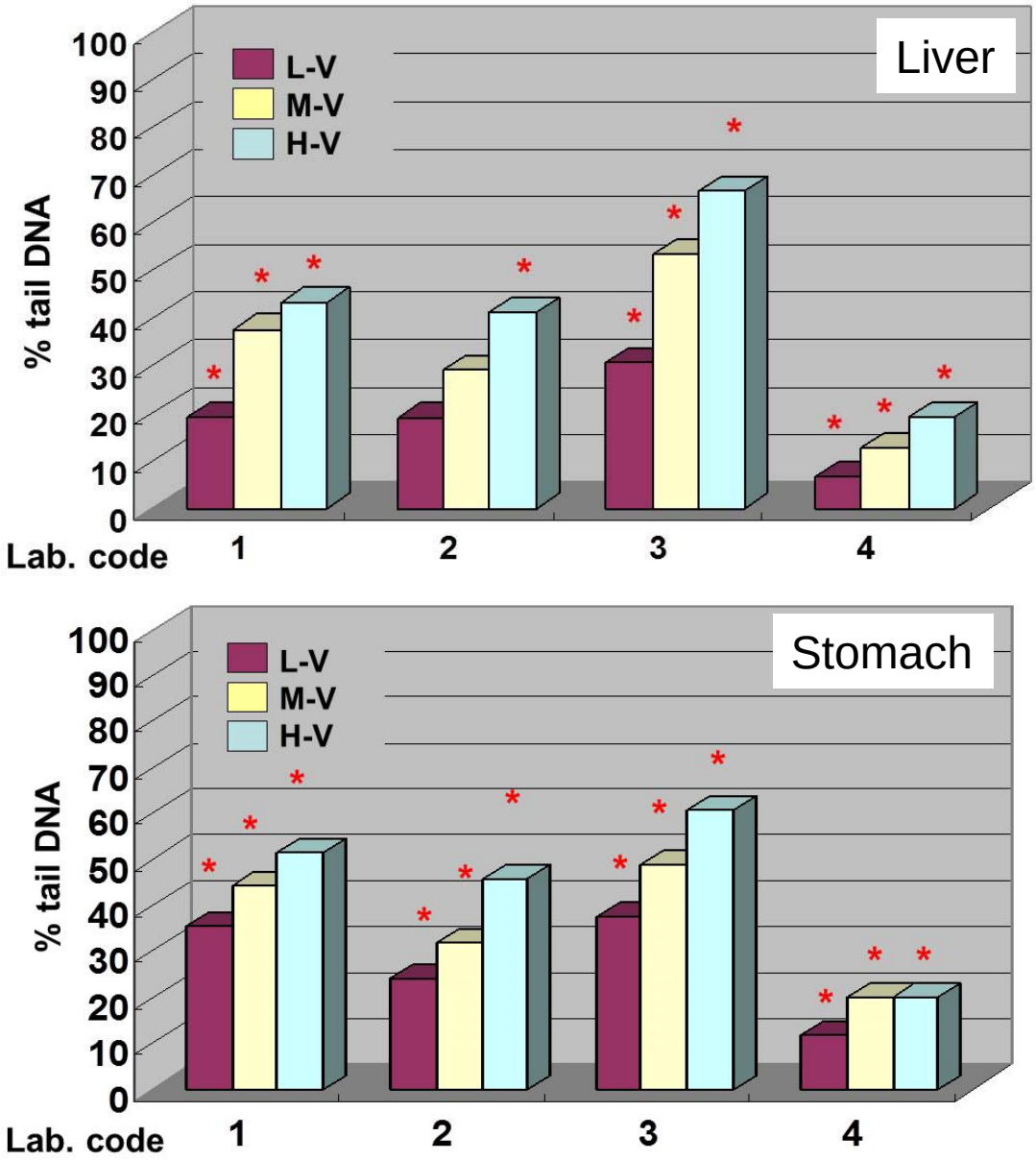
Regarding %DNA in tail,

a. Negative Control
   - The mean in the liver: 1-8%
   - The mean in the stomach: 1-20%*

     * Preferable range, and 1-30% would be tolerable.

b. Positive Control [EMS, 200 mg/kg, twice (or single) p.o.]
   - Effect (difference of means between EMS & vehicle) in the liver and the stomach: statistically significant increase, and the Effect (difference) is 5% or higher

Results of Phase 3 (pre-validation study)



- The overall magnitude was lower in Lab 4 compared to the other laboratories; this was considered to be due to the shorter electrophoresis duration of 15 min used in this laboratory (cf. the others: 20 min or more).
- The VMT concluded that sufficient pre-validation work had been conducted to be able to define the protocol and success criteria for the main validation study.

Figure legend:

Effect (diff.) of mean % tail DNA between the vehicle (V) control group and the treatment groups (low: 100 mg/kg, middle: 200 mg/kg or high: 300 mg/kg – L, M or H) after treatment with EMS as a coded test chemical. Asterisk (*) indicates statistical significance in Dunnett's test (two-sided, $p<0.05$).

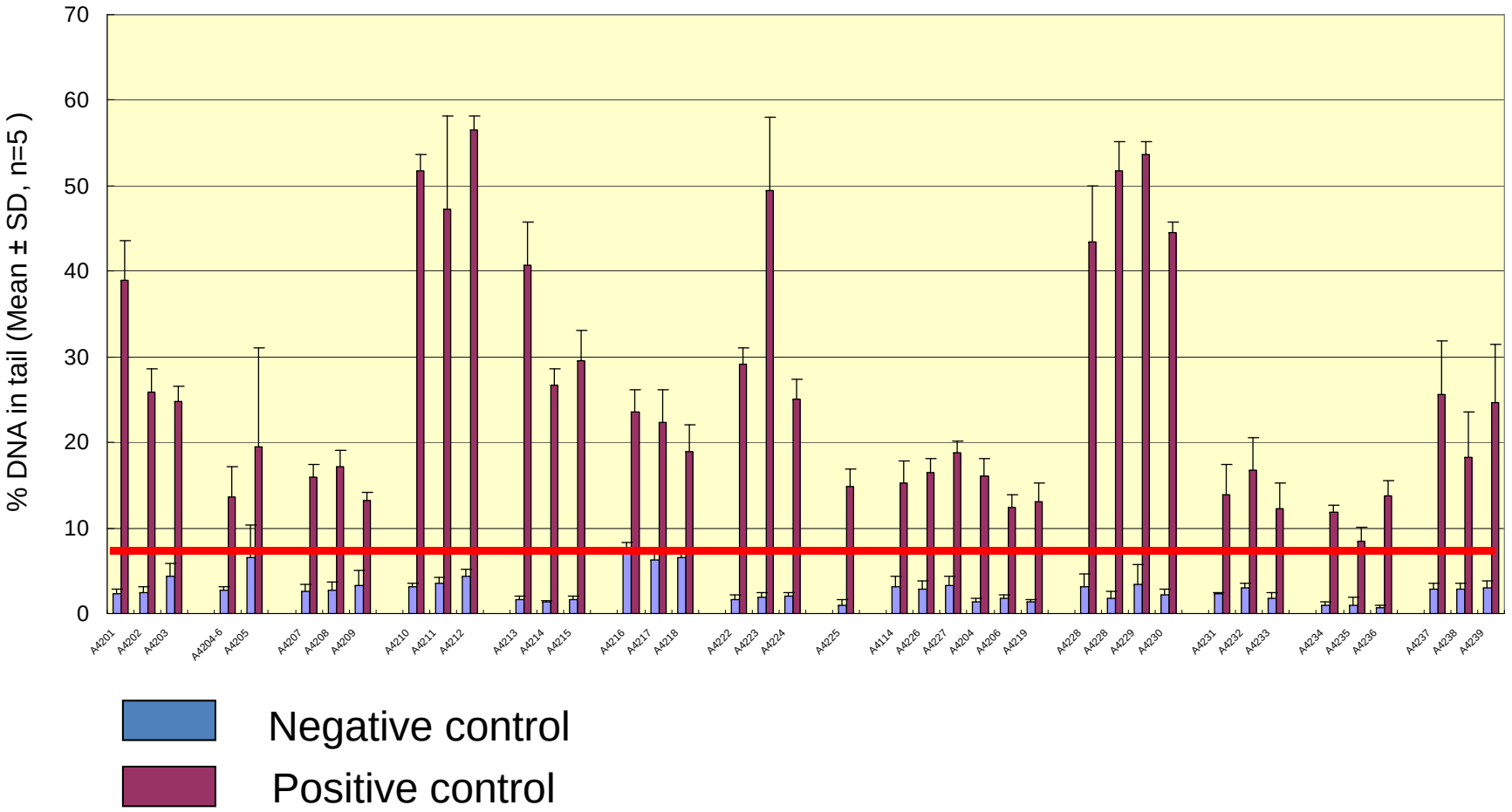
Phase 4-1 Validation Study

- ✓ In the Phase 4-1 validation study, the reproducibility of assay results were confirmed among 13 labs using four coded test chemicals (EMS, MNU, 2-AAF, D-Mannitol) and a positive control EMS when experiments were done with the JaCVAM protocol.
- ✓ The ***within/between-laboratory reproducibility*** of assay results was robustly confirmed in phase 3 & this phase validation study.

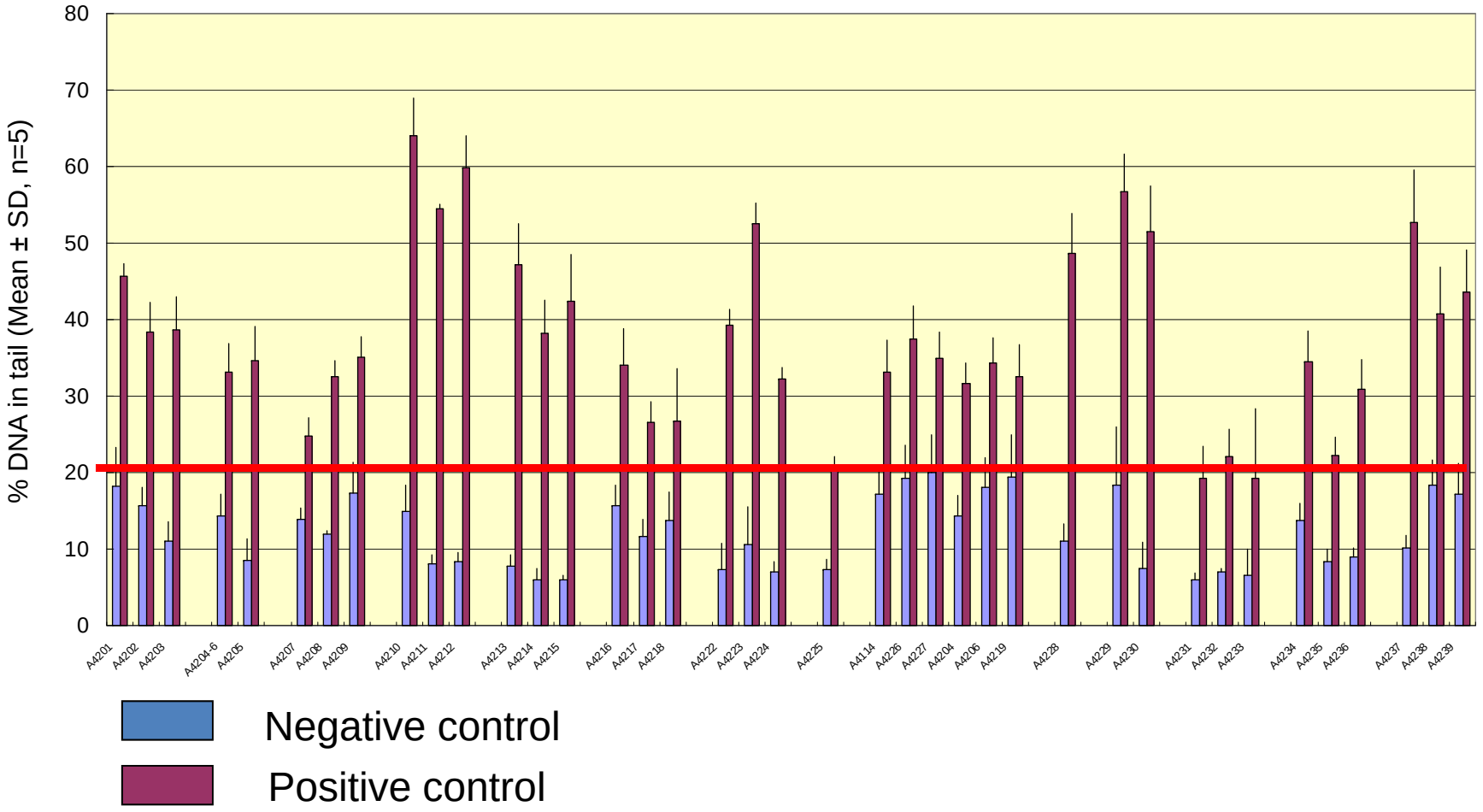
Study Design of Phase 4-2

- ✓ The purpose of the Phase 4-2 validation study was to investigate the predictive capability of comet assay for carcinogenicity of test chemicals with the JaCVAM protocol.
- ✓ 40 test chemicals were selected and assayed, which include different characteristics in chemical classes, i.e.,
  genotoxic carcinogen, genotoxic non-carcinogen, non-genotoxic carcinogen, and non-genotoxic non-carcinogen.
- ✓ Each test chemical was coded and examined in one lab.

Control Values (Liver)



Control Values (Stomach)



Genotoxic Carcinogens: Summary of Results

TEST CHEMICAL	JUDGMENT	NOTE
2-Acetylaminofluorene	Negative	Due to inappropriate dose levels, sampling time, or formulation handling? Positive in UDS assay
Acrylonitrile	Positive (L)	Negative in UDS assay
o-Anisidine	Negative	Due to higher target-organ specificity for urinary bladder?
Azidothymidine	Positive (L)	
Benzene	Negative	Due to aneugen
Busulfan	Negative	Due to cross-linker
Cadmium chloride	Positive (L)	
p-Chloroaniline	Positive (L, S)	
Cisplatin	Positive (L)	
2,4-Diaminotoluene	Positive (L)	
1,2-Dibromoethane	Positive (L, S)	
1,3-Dichloropropene	Positive (L)	Negative in UDS assay
1,2-Dimethylhydrazine 2HCl	Positive (L)	
Hydroquinone	Negative	Due to aneugen
Methyl methanesulfonate	Positive (L, S)	
N-Nitrosodimethylamine	Positive (L)	
4,4'-Oxydianiline	Negative	Due to possibility of goitrogenic effects on rat carcinogenisity?
Sodium arsenite	Equivocal (L)	In two separate experiments
Thioacetamide	Positive (S)	

Genotoxic Non-Carcinogens: Summary of Results

TEST CHEMICAL	JUDGMENT
9-Aminoacridine HCl·H_2O	Negative
p-Anisidine	Negative
2,6-Diaminotoluene	Positive (L)
5-Fluorouracil	Negative
8-Hydroxyquinoline	Negative
p-Phenylenediamine 2HCl	Negative

Note:
Genotoxicity is defined as a positive result in Ames test or standard *in vivo* genotoxicity test such as MN test, i.e. the relevancy to *in vivo* and/or toxicological significance are not always warranted.

- Of the 6 genotoxic non-carcinogens,
  - One (2,6-diaminotoluene: 2,6-DAT) induced a positive % tail DNA response in the liver.
  - Since both positive and negative results have been reported for 2,6-DAT in UDS, MN, and comet assays, this positive result would not indicate a false-positive for carcinogenicity.

Non-Genotoxic Carcinogens: Summary of Results

TEST CHEMICAL	JUDGMENT
Chloroform	Negative
Diethanolamine	Negative
Di(2-ethylhexyl)phthalate	Negative
Ethanol	Negative
Methyl carbamate	Negative
Saccharin	Negative
o-Phenylphenol sodium salt	Negative

- Of the 7 non-genotoxic carcinogens,
  - Chloroform, a hepatotoxicant, induced a significant % tail DNA response in the liver. The increase in liver was considered to be related to increased cytotoxicity, and thus the final judgment was negative.
  - All chemicals in this category were evaluated as negative.

Non-Genotoxic Non-Carcinogens: Summary of Results

TEST CHEMICAL	JUDGMENT
Ampicillin $3H_2O$	Negative
o-Anthranilic acid	Negative
t-Butylhydroquinone	Positive (L)
Ethionamide	Negative
Isobutylaldehyde	Negative
D,L-Menthol	Negative
Sodium chloride	Negative
Trisodium EDTA H_2O	Negative

- Of the 8 non-genotoxic non-carcinogens,
  - Only one -- *t*-Butylhydroquinone (*t*-BHQ) -- was positive.
  - *t*-BHQ was judged positive in the liver based on statistical analysis but was judged to be negative in the testing facility because the increased % tail DNA was within their historical control range.

Conclusions of Validation Study

- The *in vivo* comet assay is highly capable of identifying genotoxic chemicals when it is conducted using the JaCVAM protocol, and therefore it serves as a potentially reliable predictor of rodent carcinogenicity.
- Practically, a combination comet and MN assay would be useful and recommended to assess the *in vivo* genotoxic potential of test chemicals.
- A special issue of Mutation Research on the JaCVAM validation study was published.

Mutation Research 786–788 (2015) 45–76

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

JaCVAM-organized international validation study of the *in vivo* rodent alkaline comet assay for detection of genotoxic carcinogens: II. Summary of definitive validation study results

Yoshifumi Uno[a,*], Hajime Kojima[b], Takashi Omori[c], Raffaella Corvi[d], Masamistu Honma[b], Leonard M. Schechtman[e], Raymond R. Tice[f], Carol Beevers[g], Marlies De Boeck[h], Brian Burlinson[i], Cheryl A. Hobbs[j], Sachiko Kitamoto[k], Andrew R. Kraynak[l], James McNamee[m], Yuzuki Nakagawa[n], Kamala Pant[o], Ulla Plappert-Helbig[p], Catherine Priestley[q], Hironao Takasawa[r], Kunio Wada[s], Uta Wirnitzer[t], Norihide Asano[u], Patricia A. Escobar[v], David Lovell[w], Takeshi Morita[b], Madoka Nakajima[x], Yasuo Ohno[b], Makoto Hayashi[y]

Peer Review by the OECD Experts

ANNEX 1:
Members of the Peer Review Panel who Submitted Individual Responses to the General Charge Questions

Panel member	Affiliation
Eugenia Cordelli	Laboratory of Toxicology, ENEA, Rome, Italy
Abby Jacobs	Center for Food Safety and Applied Nutrition, US Food and Drug Administration.
Francesco Marchetti	Health Canada
Dan Levy	Center for Food Safety and Applied Nutrition, US Food and Drug Administration.
Birgit Mertens	Scientific Institute of Public Health, Belgium
Veronique Thybaud	Sanofi, France
Paola Villani	Laboratory of Toxicology, ENEA, Rome, Italy

Peer review co- managers: Nathalie Delrue (OECD Secretariat) and Jan van Bethem (RIVM, Netherlands)

10. In addition the Panel considered that this assay detects, in a given tissue, many but not all types of in vivo DNA damage that could potentially result in stable mutations and ultimately cancer or other diseases. Thus, it should not be used as the sole predictor for carcinogenicity, but instead is valuable as part of a battery of tests. The Panel recommends that the regulatory purpose be revised accordingly.

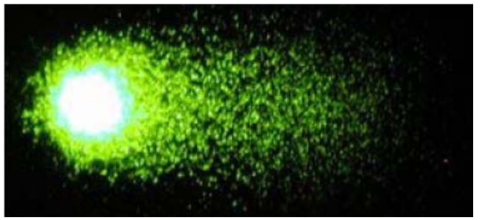
11. The scope of the validation study was limited in terms of tissues analysed, species and gender. As the validation exercise cannot support by itself the broadening of the scope of the Test Guideline, the Panel agreed that there is a need to go to the data from the literature and to check if the published data can support recommendations in the TG to use rodent species other than rats and only one gender (males).

12. Overall the Panel agreed that the validation criteria have been met or partially met and that the information that is missing could be requested from the VMT, collected from the literature, or gained from laboratories that have a long history of using this assay. This additional information may help:
- addressing inter/intra laboratory reproducibility including control levels,
- checking if using the mean or the median for all the data would be helpful to reduce the variations observed between laboratories and thus improve quantitative inter laboratory reproducibility,
- broadening the applicability domain of the assay to classes of chemicals not included or not sufficiently represented in the validation exercise,
- broadening the scope of the TG to other tissues as well as to mice and female animals,
- describing the mechanism underpinning the assay, in particular the link between DNA migration observed in the assay and DNA damage,
- assessing specifically the advantage of the comet assay over the UDS assay.

General Introduction of OECD TG489
- *In vivo* mammalian alkaline comet assay –
(Adopted on September 26, 2014)

Contents of TG489

- Introduction
- Initial considerations and limitations
- Principle of the method
- Verification of laboratory proficiency
- Description of the method
- Procedure
- Data and reporting
- Annexes 1, 2, and 3



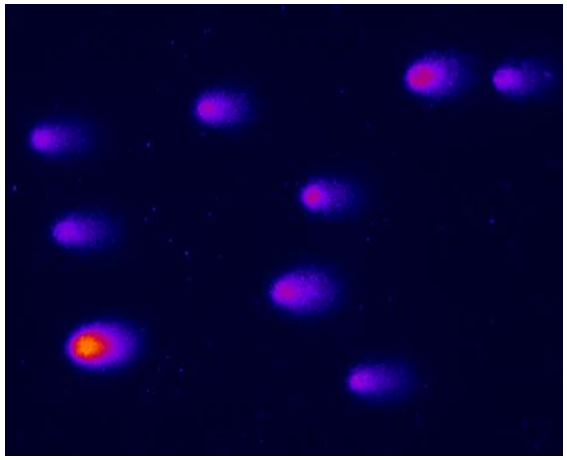
OECD
BETTER POLICIES FOR BETTER LIVES

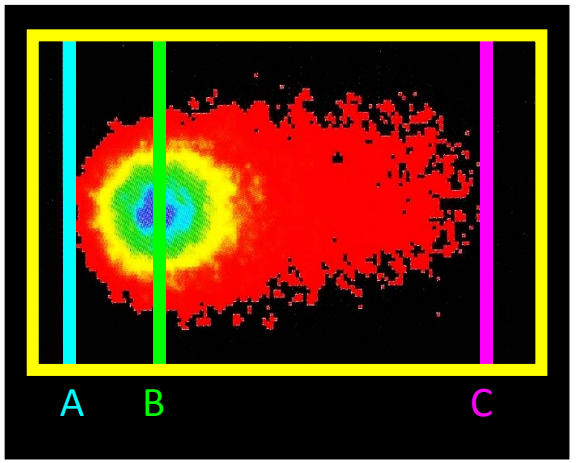
Initial considerations and limitations

The comet assay (is)

- **A method for measuring DNA strand breaks in eukaryotic cells.**
- **Relevant to assess genotoxic hazard.**
- **Can also be integrated with other toxicological studies, or the endpoint can be combined with other genotoxicity endpoints such as micronucleus, to fulfil animal welfare requirements (3Rs principles).**
- **The route of exposure and tissue(s) should be selected based on all available/existing knowledge of the test chemicals, e.g., intended/expected route of human exposure, metabolism.**
- **Most extensively validated in somatic tissues of male rats in collaborative studies. The liver and stomach were used in the JaCVAM trial. (However,) the technique is in principle applicable to any tissue from which analyzable single cell/nuclei suspensions can be derived.**
- **Not considered appropriate to measure DNA strand breaks in mature germ cells, with the standard method described in TG489.**
- **Cannot reliably detect cross-links with the standard experimental conditions.**

In vitro comet assay validation study





Masamitsu Honma

National Institute of Health Sciences

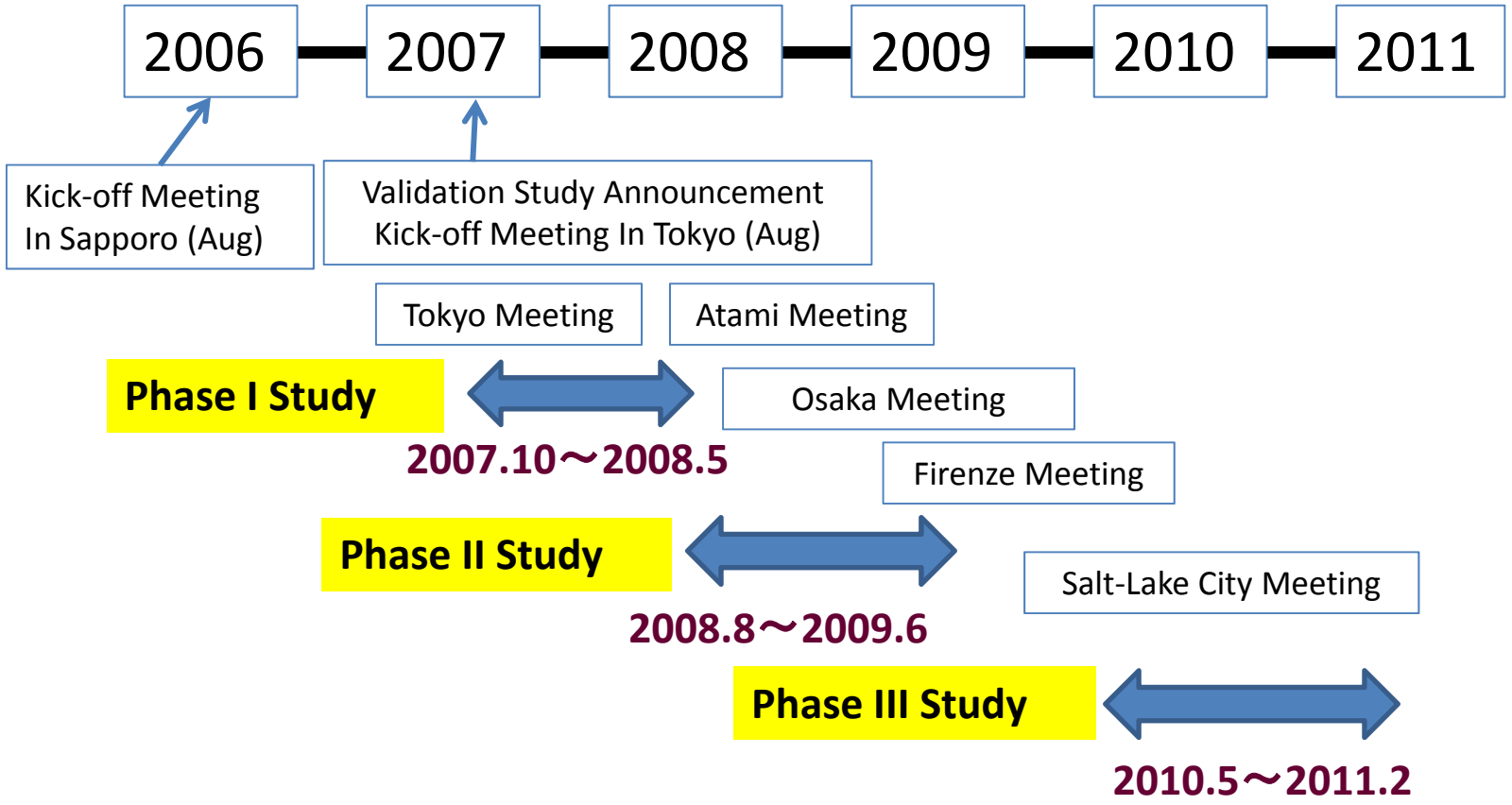
Purpose of the Validation Study

In order to establish a robust *in vitro* comet assay protocol and to make consensuses for evaluation and interpretation of the Comet results (including cytotoxicity), leading laboratories conduct the *in vitro* comet assay for several genotoxic or non-genotoxic chemicals. The management members review and validate the comet results with the consultation of experts.

Form the studies, we pursuit the possibility of the in vitro comet assay as alternative for other *in vitro* or *in vivo* genotoxicity tests.

To be robust *in vitro* comet assay protocol.

Action of the *In Vitro* Pre-Validation Study



Organization of Validation Study

Validation Management Team (VMT)

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

Consultation Team

N. Asano (Kinki Univ., JEMS/MMS)
D. Lovell (St. George's Univ. of London)
T. Morita (NIHS)
Y. Ohno (NIHS/JaCVAM)
T. Omori (Univ. of Kobe Hospital)
S. Hoffman (Consultant)
S. Hann (KFDA)
Y. Seo (Donggulk Univ.)
G. Spite (Ulm Univ.)
A. Collins (Univ. of Oslo)

Participant Laboratory

1. BioReliance (USA) : K. Pant
2. Food and Drug Safety Center (JPN) : K. Yamakage
3. Huntingdon Life Sciences (UK) : B. Burlinson
4. KIT (Korea) : M.K. Chung
5. Merck (USA) : P.Escober

30

Specific Issues of the *In Vitro* Comet Assay Protocol

1. **Cells, Cell lines**
   - **TK6 human lymphoblastoid cells**
2. **Duration of treatment with chemicals**
   - **4h**
3. **Cytotoxicity**
   - **Cytotoxic parameter, level of cytotoxicity**
4. **Metabolic activation**
   - **S9 condition**
5. **Statistical analysis**

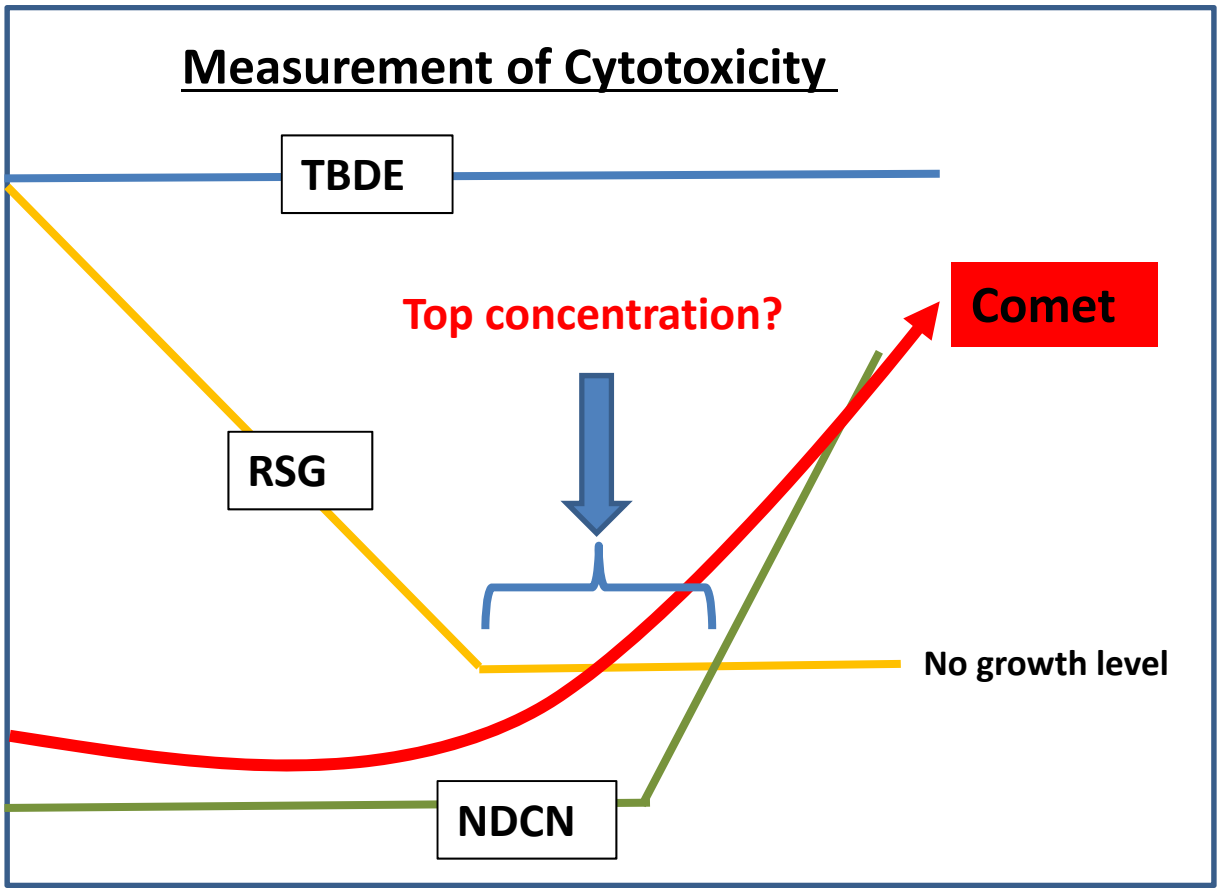
Basic Protocol for Comet Assay

*** In vitro alkaline Comet assay protocol is identical to the in vivo one after cell preparation.**

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

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

Problems and their Resolution (1)



TBDE does not generally change just after the treatment.

RSG may be useless to find the top dose, because the comet does not sometimes appear under the lowest RSG (no cell growth).

NDCN may be useful as a cytotoxic parameter, although it steeply appears.

- It is difficult to find the top concentration in the *in vitro* comet assay. The comet assay might be done regardless of cytotoxicity, and then acceptable data would be considered later.

Problems and their Resolution (2)

- **S9**
  - ***In vitro* comet assay dose not work well for chemicals requiring metabolic activation under S9, although other genotoxicity tests (MN) appropriately work in the same condition.**
  - **Genotoxic chemicals requiring metabolic activation (CP, DMN) yielded very weak positive response even with S9 in the phase II study.**

➢ Need confirmation by other chemicals.

Summary of Pre-Validation Study

Dose selection

- **Wide-range dose selection regardless of cytotoxicity is acceptable.**

Criteria for evaluating doses

- **Cell growth is not applicable.**
- **Is 20% NCDN OK as top concentration?**
- **Is NDCN not comet? Some NDCN may be a strong comet shape?**

Effect of S9

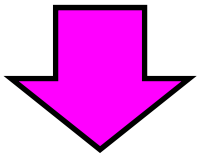
- **S9 works for 2AA, but the positive responses accompany severe cytotoxicity and many NDCN.**

Positive control

- **EMS(500ug/ml) is appropriate positive control (except for one lab).**

Conclusion in the Pre-Validation Study

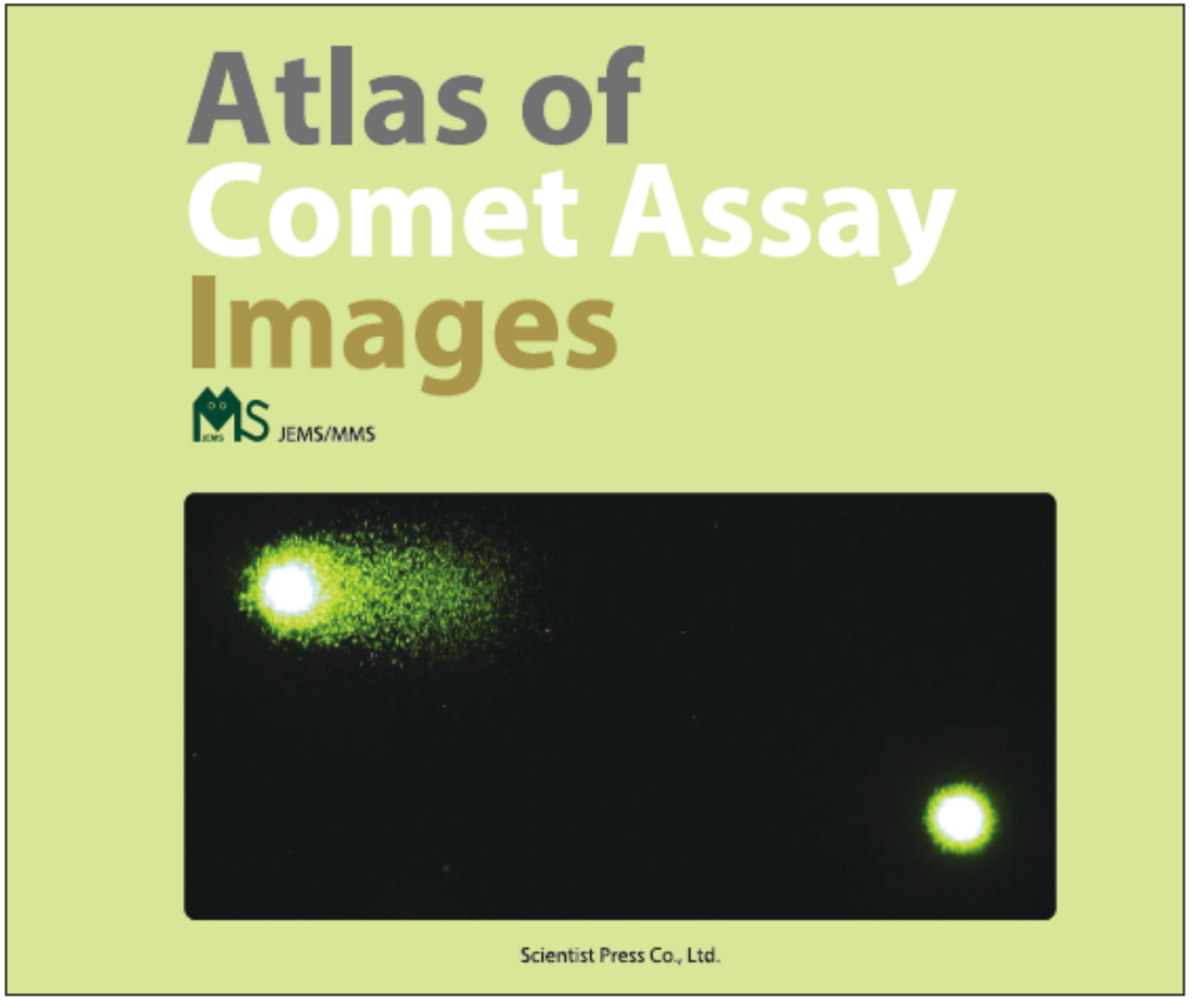
- **The *in vitro* comet response based on NCDC is more reproducible than the cell growth in inter-laboratory analysis.**



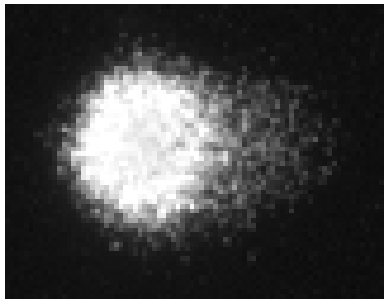
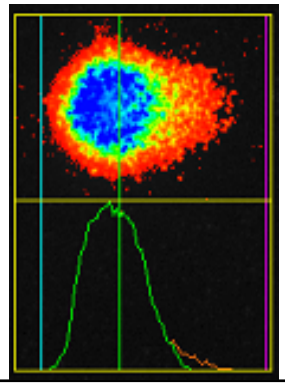
Wide-range dose setting and choosing top dose by appropriate NDCN level (>20%) could be recommended in the *in vitro* comet assay.

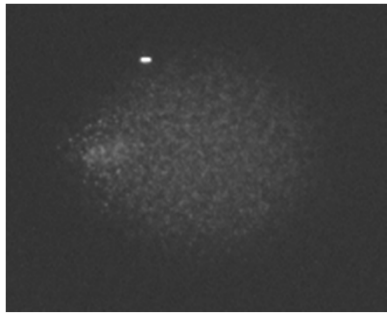
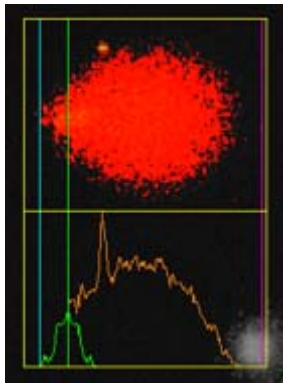
The optimal protocol had no agreements in the pre-validation study.

Just Information…



Price: JPY 3888
How to get it…. see next page.

No.13	
Comet image	**Analytical image**
	
Tail % Intensity (% tail DNA)	**1.9**
Scorable	

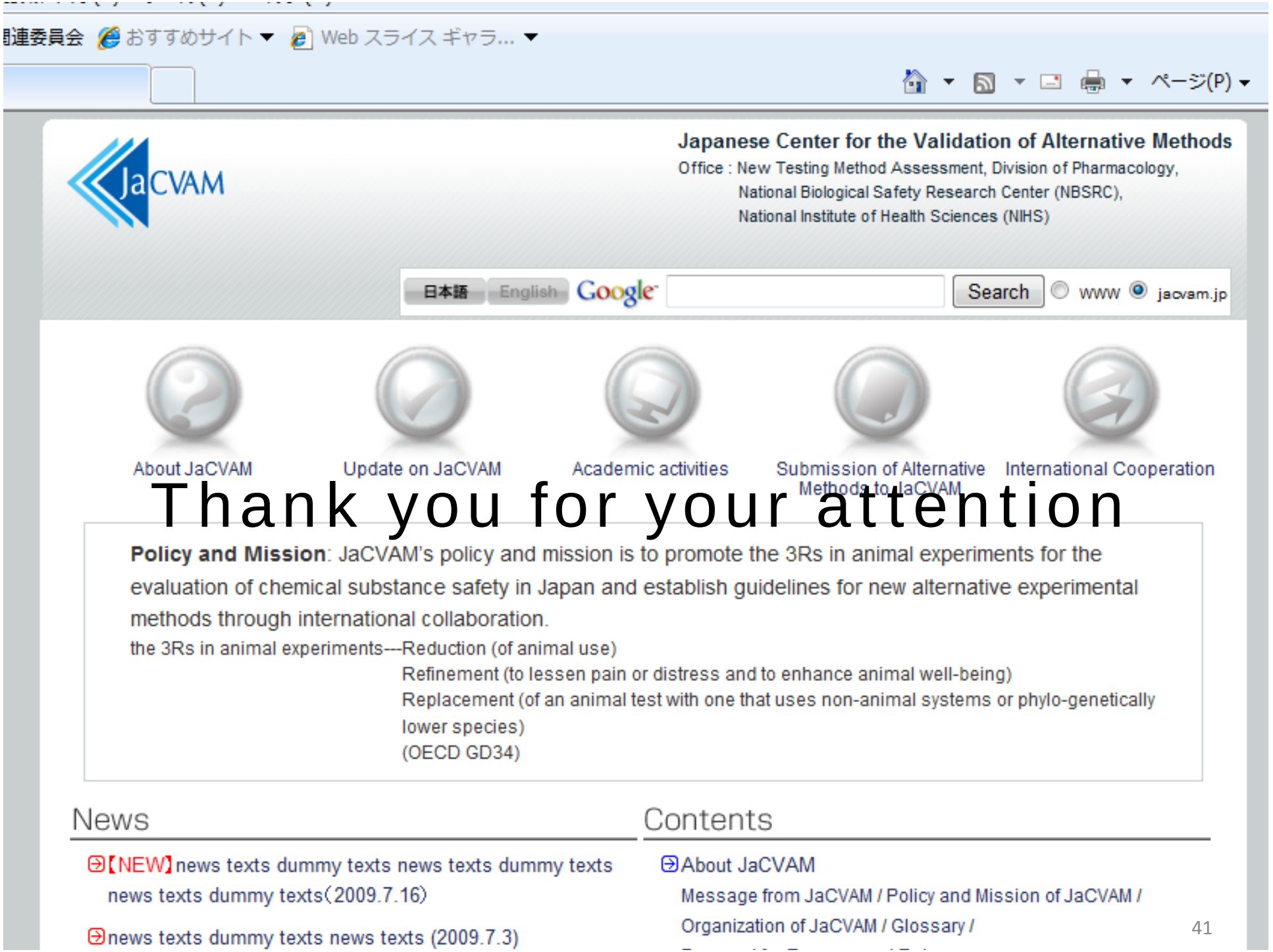
No.74	
Comet image	**Analytical image**
	
Tail % Intensity (% tail DNA)	**90.2**
Hedgehog	

OECD TG489, ANNEX 3
Current Limitations of the Assay

Due to the current status of knowledge, several limitations are associated with the *in vivo* comet assay. It is expected that these limitations will be reduced or more narrowly defined as there is more experience with application of the assay to answer safety issues in a regulatory context.

Acknowledgements

On behalf of the comet assay international validation management team, I appreciate the all participants and consultants involved with adapting TG 489.
This validation study was supported by the MHLW funding in Japan.


連委員会
おすすめサイト
Web スライス ギャラ...
ページ(P)
JaCVAM
Japanese Center for the Validation of Alternative Methods
Office : New Testing Method Assessment, Division of Pharmacology,
National Biological Safety Research Center (NBSRC),
National Institute of Health Sciences (NIHS)
日本語
English
Google
Search
www
jacvam.jp
About JaCVAM
Update on JaCVAM
Academic activities
Submission of Alternative Methods to JaCVAM
International Cooperation
Thank you for your attention
Policy and Mission: JaCVAM's policy and mission is to promote the 3Rs in animal experiments for the evaluation of chemical substance safety in Japan and establish guidelines for new alternative experimental methods through international collaboration.
the 3Rs in animal experiments---Reduction (of animal use)
Refinement (to lessen pain or distress and to enhance animal well-being)
Replacement (of an animal test with one that uses non-animal systems or phylo-genetically lower species)
(OECD GD34)
News
【NEW】news texts dummy texts news texts dummy texts news texts dummy texts(2009.7.16)
news texts dummy texts news texts (2009.7.3)
Contents
About JaCVAM
Message from JaCVAM / Policy and Mission of JaCVAM / Organization of JaCVAM / Glossary /