

Feb. 4, 2009  
Comet mtg.

## In Vivo Comet Assay: Update on the On-Going Validation Coordinated by JaCVAM

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JEMS/MMS

## Introduction

An *in vivo* rodent alkaline Comet assay is practically used worldwide for detecting genotoxic chemicals, and it is expected as a second *in vivo* genotoxicity test in the revised ICH-S2 guidance.

The assay, however, has not been validated formally, and the international validation study is now on-going coordinated by JaCVAM. The purposes are to validate the *in vivo* Comet assay as a potential predictor of carcinogens and as an alternative follow-up assay to the more commonly used *in vivo* rodent UDS assay.

## Organization Update (in vivo)

### Validation Management Team (VMT)

M. Hayashi (Chair, BSRC)  
R. Corvi (ECVAM)  
M. Honma (NHS)  
L. Schechtman (Consultant)  
R. Tice (NTP/CCVAM)  
Y. Uno (MTPC, JEMS/MMS)

### Secretariat

H. Kojima (NHS/JaCVAM)

### Leading Laboratory

BioReliance (B. Kramanovic, et al.)  
FDSC (K. Yamakage, et al.)  
HLS (B. Burlinson, et al.)  
Merck (R.D. Storer, et al.)

### Consultation Team

N. Asano (JEMS/MMS)  
P. Escobar (Boehringer-Ingelheim)  
D. Lovell (Univ. of Surrey)  
T. Morita (NHS)  
M. Nakajima (BSRC)  
Y. Ohno (NHS/JaCVAM)  
T. Omori (Kyoto Univ.)  
YF. Sasaki (Hachinohe Nat Coll. Tech.)

### Local Committee in JPH

Mainly from JEMS/MMS members

## Progress of Validation Effort (in vivo)

### 2006

Aug.: Kick-off mtg. and Start of 1st validation study with EMS  
Dec.: End of exp. of 1st validation study  
- Study protocol was optimized overall  
- Well-validated data were obtained

### 2007

Apr.: Start of 2nd validation study with EMS and three coded chem.  
Jul.: Announcement of invitation to participate in this validation effort

### 2008

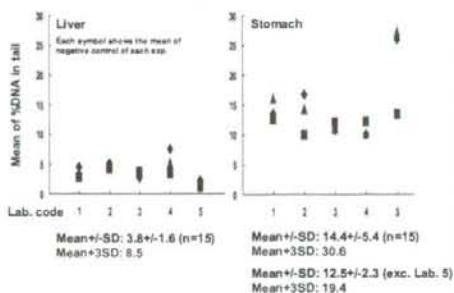
Jan.: Start of exp. to select participants of 4th (definitive) validation study  
Mar.: End of exp. of 2nd validation study  
- Data acceptance criteria were set based on negative & positive cont. data  
- Especially, 2,4- & 2,6-DAT showed a little bit complicated results

May: 3rd validation study with EMS and additional 3 coded chem.  
Aug.: Participants of 4th validation study were selected  
Dec.: End of exp. of 3rd validation study

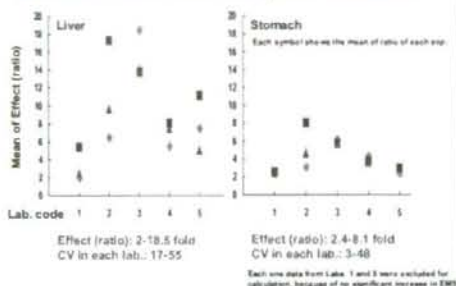
## 2nd Phase Validation Study

- Purpose: by using data of negative control and EMS,
  - ✓ To determine data acceptance criteria
  - ✓ To examine within/between lab variability
- Test compound: EMS\* and Three coded chemicals\*\*
  - \* Each exp. for coded chemicals included EMS group as a positive control, and 3 data of EMS/lab X 5 labs = 15 data were applied to determine data acceptance criteria
  - \*\* Acrylamide, 2,4-diaminotoluene, 2,6-diaminotoluene
- Protocol: version 12
- Result:
  - ✓ Data acceptance criteria (draft) were set
  - ✓ Data of three coded chemicals were obtained

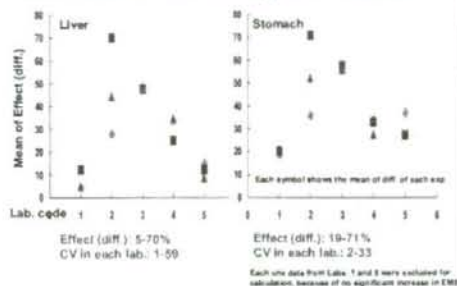
## Negative control ranges (%DNA in tail) set with data of 2nd phase validation



**Effect (ratio, %DNA in tail) and the CV calculated with data of 2nd phase validation**



**Effect (difference, %DNA in tail) and the CV calculated with data of 2nd phase validation**



**Data Acceptance Criteria (draft\*) based on 2nd phase validation study results**

**a. Negative control**

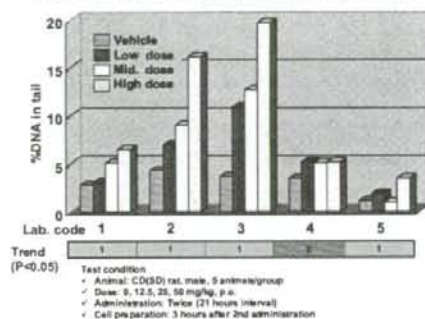
- Mean of %DNA in tail in liver: 1-8%
- Mean of %DNA in tail in stomach: 1-30% (preferably 1-20%)

**b. Positive control: EMS, 200 mg/kg, single (or twice) p.o.**

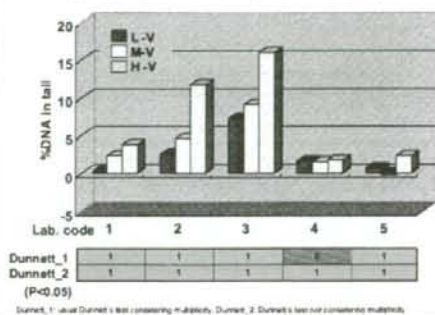
- Effect (ratio of means of %DNA in tail between EMS & vehicle) in liver and stomach: 2-fold or higher
- Effect (difference of means of %DNA in tail between EMS & vehicle) in liver and stomach: 5% or higher
- CV of Effect (ratio) in two or more independent experiments with liver and stomach: 50% or less

\* Data acceptance criteria may be revised based on the 3<sup>rd</sup> phase validation results

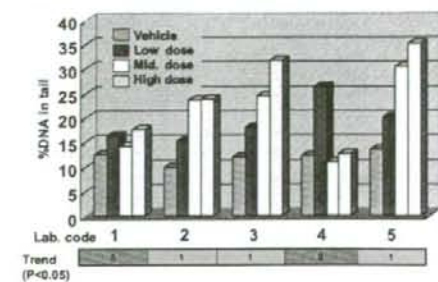
**Mean of %DNA in tail in Liver: Acrylamide**



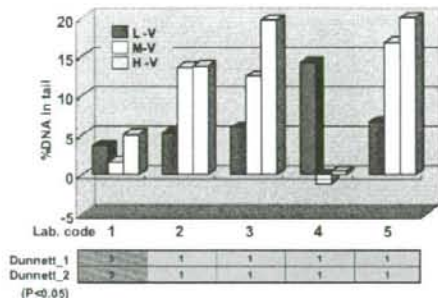
**Effect (diff. of %DNA in tail) in Liver: Acrylamide**



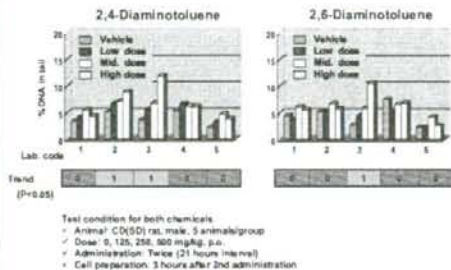
**Mean of %DNA in tail in Stomach: Acrylamide**



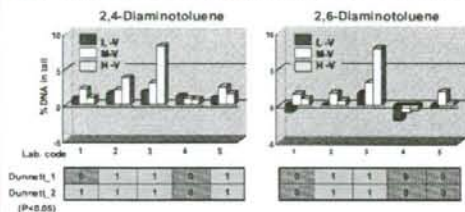
Effect (diff. of %DNA in tail) in Stomach: Acrylamide



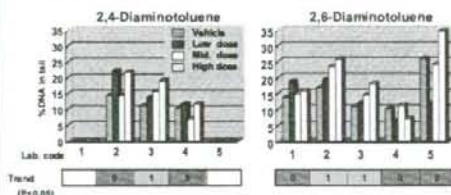
Mean of %DNA in tail in Liver: 2,4- & 2,6-DAT



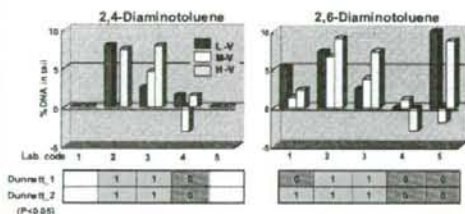
Effect (diff. of %DNA in tail) in Liver: 2,4- & 2,6-DAT



Mean of %DNA in tail in Stomach: 2,4- & 2,6-DAT



Effect (diff. of %DNA in tail) in Stomach: 2,4- & 2,6-DAT



Summary

- My Comments for 2nd phase Validation Data -

- Expected assay results: Acrylamide is clearly but not so strongly positive in both/liver organs. 2,4-DAT and 2,6-DAT are weakly positive and negative in liver, respectively (unknown in stomach).
- Overall, acrylamide would be judged as positive in both organs. This would almost fit the expected assay results, but lab. #4 seems not to detect this chemical as positive.
- In liver, 2,4-DAT seems positive in labs. #2 and 3. 2,6-DAT seems negative except for lab. #3. 2,6-DAT results may almost fit the expected assay results, but 2,4-DAT results may be a little bit unexpected. Overall, this validation study results may be coincident with the rat liver UDS assay results, because 2,4- and 2,6-DAT are reported as weakly positive and negative in the UDS assays, respectively.
- In stomach, 2,4- and 2,6-DAT seem positive in lab. #2 and 3. Both chemicals are mutagens and may have genotoxic potential *in vivo*, and *in vivo* Comet assay may sometimes detect such weakly genotoxic effects.

### **Facilities and Participants of 4th Phase Validation Study**

1. AstraZeneca (UK) : Catherine Smith
2. Bayer Health Care (Germany) : Uta Wirmitzer
3. BioReliance (USA) : Buba Kramanovic
4. Covance (UK) : 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. Johnson & Johnson (Belgium) : Marlies De Boeck
9. Merck (USA) : Richard D. Storer
10. Mitsubishi Chemical Safety Institute (JPN) : Kazunori Narumi
11. Novartis Pharma (Switzerland) : Ulla Plappert-Helbig
12. Sumitomo Chemical (JPN) : Sachiko Kitamoto
13. The Institute of Environmental Toxicology (JPN) : Kunio Wada

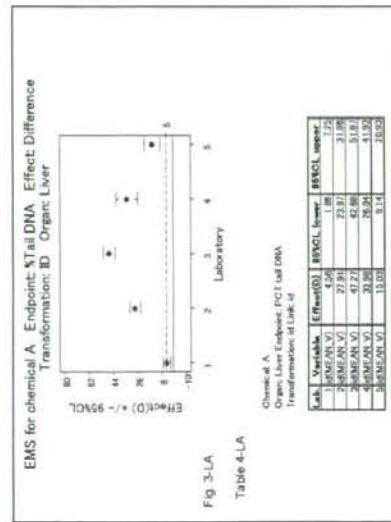
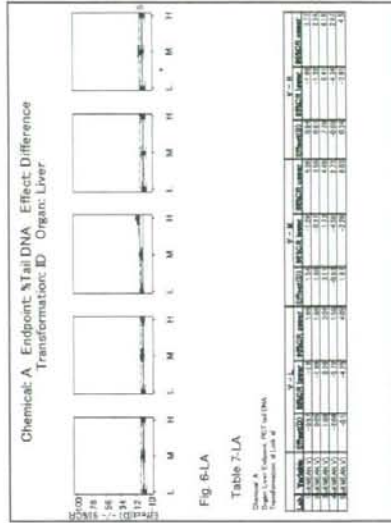
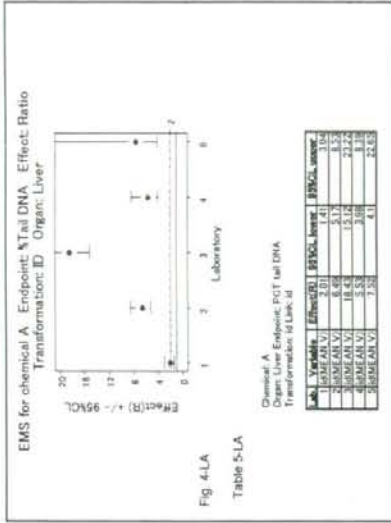
### **Outlines of On-going/Next Phase Validation Studies**

- > Study and purpose:
  - a. 3rd phase validation study: ongoing  
To reconfirm data acceptance criteria based on 2nd phase validation data, and To further optimize the standard protocol
  - b. 4th phase validation study: now planning  
To investigate predictive capacity of genotoxic carcinogens
- > Test compound:
  - a. Coded three chemicals plus EMS in 3rd phase validation
  - b. Coded "the number of 30-50" chemicals in 4th phase validation
- > Participant:
  - a. 4 leading lab for 3rd phase validation
  - b. 4 leading lab plus selected lab (max. 9) for 4th phase validation
- > Method: in accordance with the standard protocol
- > Schedule:
  - a. March/2008 - February/2009 in 3rd phase validation
  - b. Start on 1Q/2009 for 4th phase validation  
Finish by the end of 2010 (tentative)

International validation of the in vitro rodent alkaline comet assay  
for the detection of genotoxic carcinogens

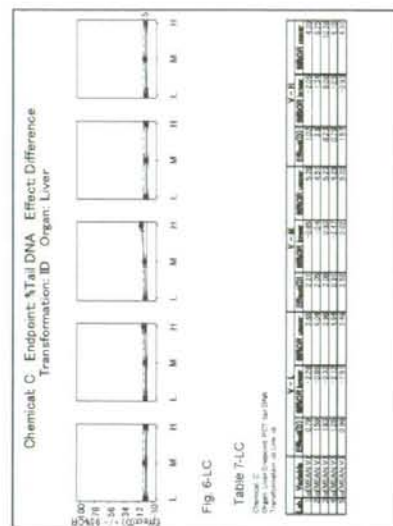
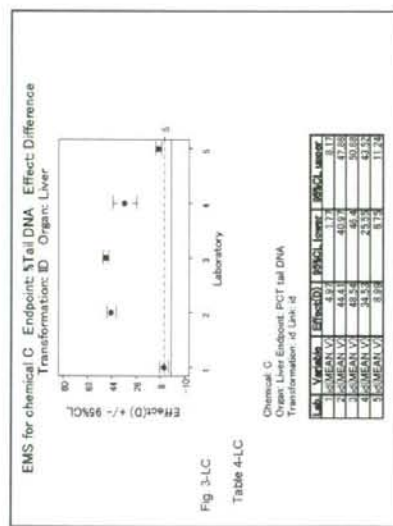
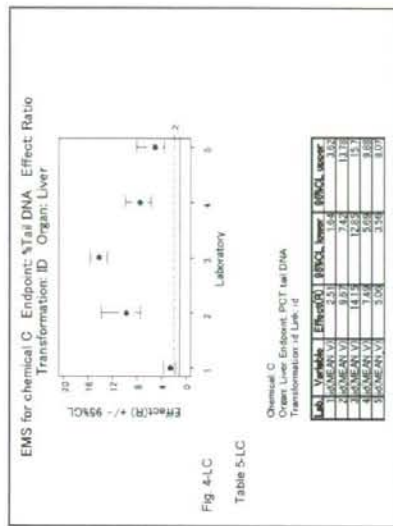
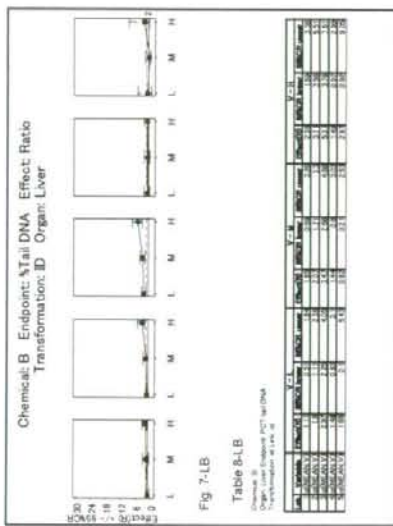
## Re2<sup>nd</sup> phase Figs and tables %tail DNA

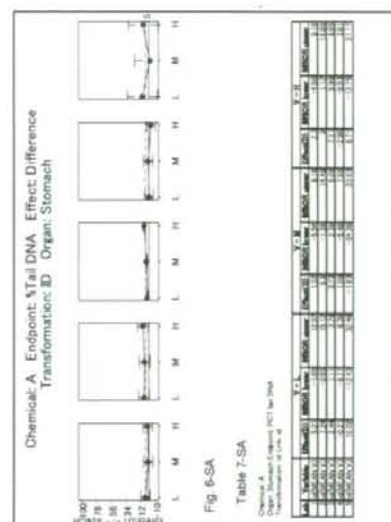
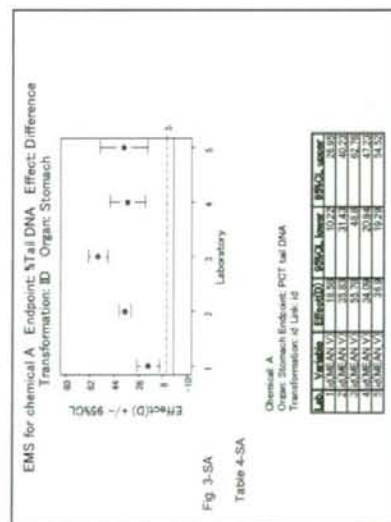
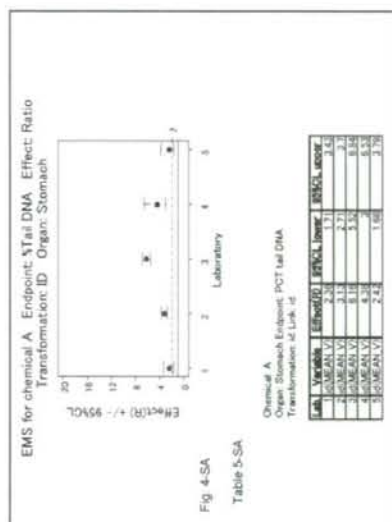
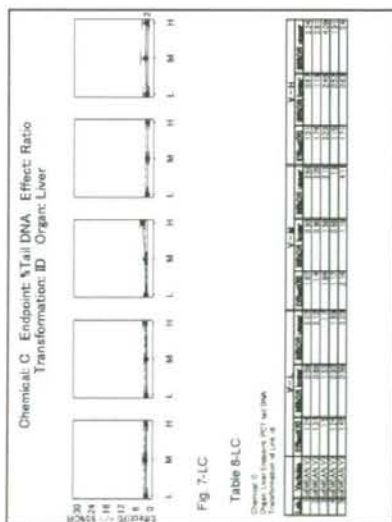
2009/2/5  
Kyoto University School of Public Health  
Takashi Omori



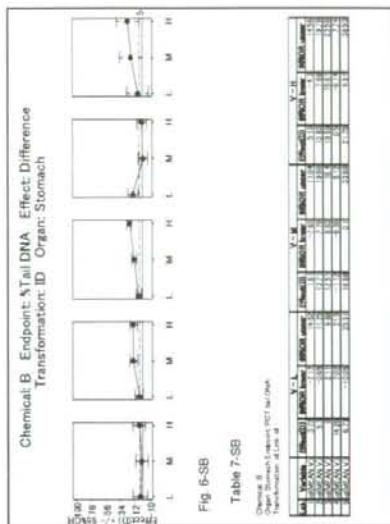
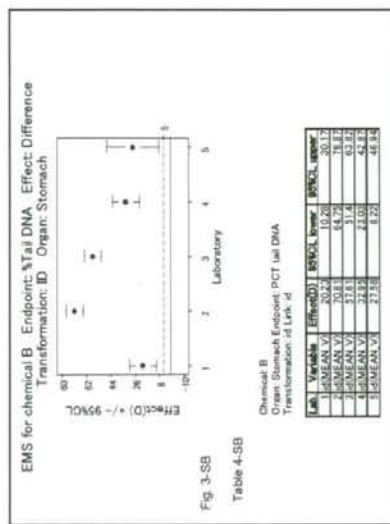
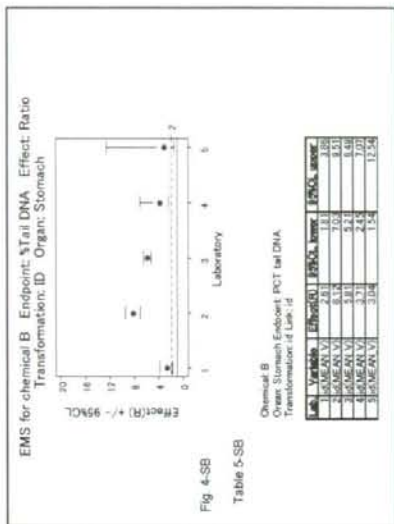
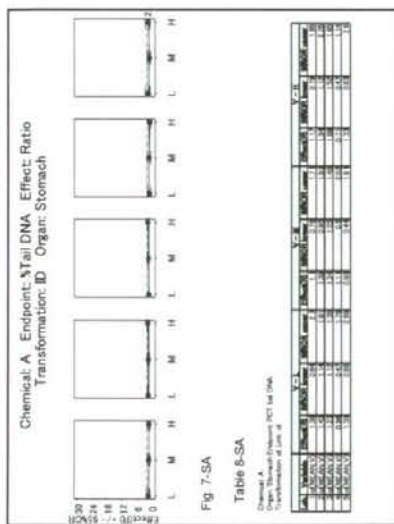
















### *In Vivo* Comet Assay: 3rd Phase Validation Study

### *Purpose of 3rd Phase Validation Study*

In the 2nd phase validation study, two issues were noted:

1. EMS treatment failed to produce positive results for the stomach in each one of three experiments done in two labs. These facts deviated from one of success criteria of the 2nd phase validation study, i.e. positive results were obtained in all EMS groups in all testing facilities.
2. Large variation of Effects (difference of the averages between groups of negative-control and EMS) were noted among five facilities (also noted in three coded chemicals). In addition, one and three of five labs showed large within-laboratory variation of the Effect in the liver and in the stomach, respectively.

### *Purpose of 3rd Phase Validation Study (cont.)*

These issues indicated that the protocol-v.12 might not be suitable as it is for the further validation studies, at least for the stomach. Based on discussion at Atagawa mtg. (March 13-14, 2008), the protocol revised to v.13 intended to solve above problems.

The first purpose of 3rd phase validation study was to examine reproducibility and robustness of positive control results with EMS when experiments were conducted in accordance with the protocol-v.13. This means to check acceptability of the protocol-v.13 for further validation studies, too.

### *Purpose of 3rd Phase Validation Study (cont.)*

In addition, it was also needed to check whether or not draft data-acceptance criteria established with the data of 2nd phase validation study could be applied to judgment of data reliability in the future validation studies.

The second purpose was to investigate whether or not the draft data-acceptance criteria would be suitable to judge reliability of data from testing facilities.

### *3rd Phase Validation Study*

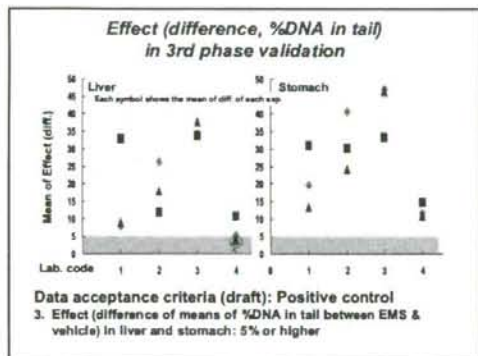
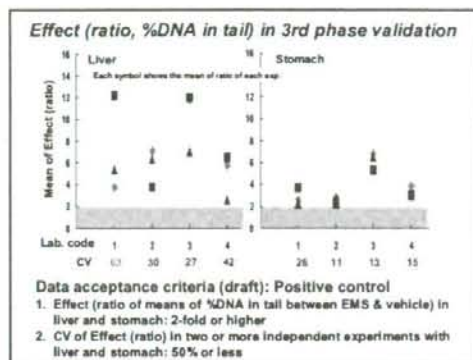
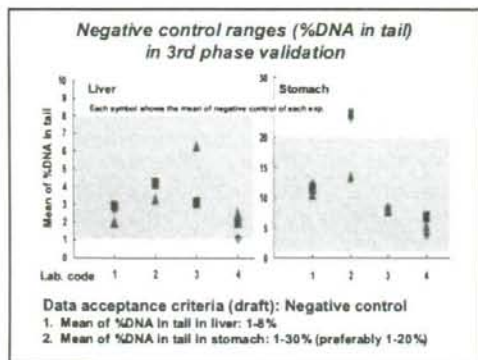
- > Purpose: by using data of negative control and EMS,
  - ✓ To examine within/between lab variability
  - ✓ To check the draft data acceptance criteria
- > Test compound: EMS\* and Three coded chemicals\*\*
  - \* Each exp. for coded chemicals included EMS group as a positive control, and 3 data of EMS/lab X 5 labs = 15 data were available
  - \*\* EMS: 100, 200, 300 mg/kg (vehicle: saline)  
N-Methyl-N-nitrosourea (MNU): 25, 50, 100 mg/kg (vehicle: water)  
D-Mannitol: 500, 1000, 2000 mg/kg (vehicle: water)
- > Protocol: version 13
- > Result: to be discussed
  - ✓
  - ✓

### *Data Acceptance Criteria (draft\*)*

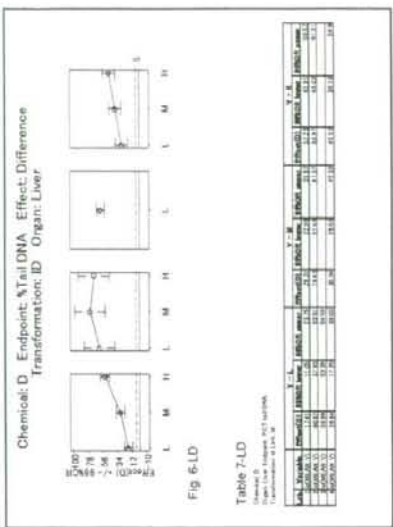
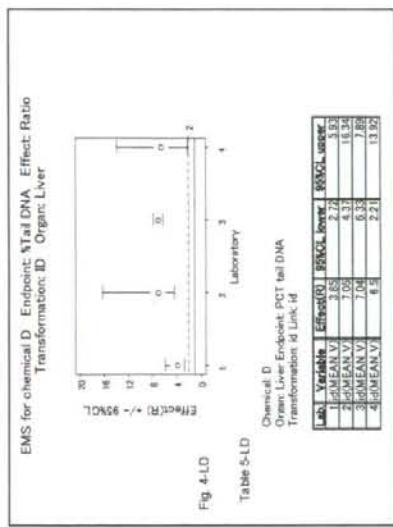
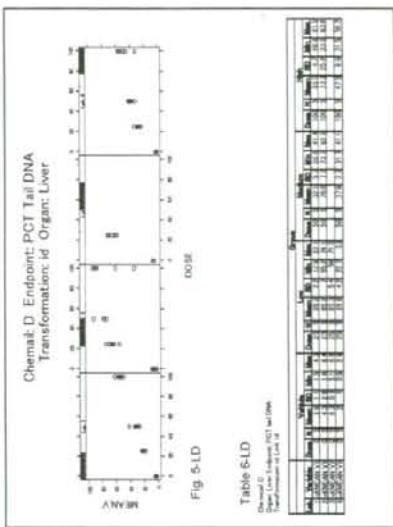
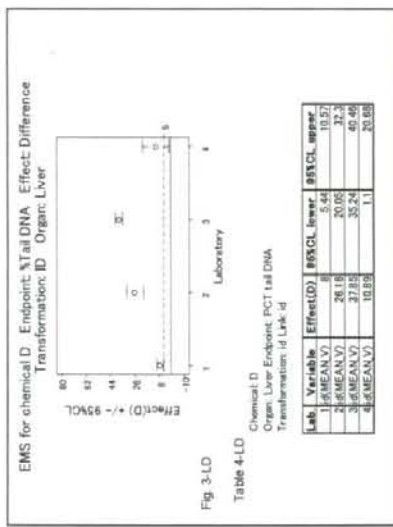
*based on 2nd phase validation study results*

- a. Negative control
  - Mean of %DNA in tall in liver: 1-8%
  - Mean of %DNA in tall in stomach: 1-30% (preferably 1-20%)
- b. Positive control: EMS, 200 mg/kg, single (or twice) p.o.
  - Effect (ratio of means of %DNA in tall between EMS & vehicle) in liver and stomach: 2-fold or higher
  - Effect (difference of means of %DNA in tall between EMS & vehicle) in liver and stomach: 5% or higher
  - CV of Effect (ratio) in two or more independent experiments with liver and stomach: 50% or less

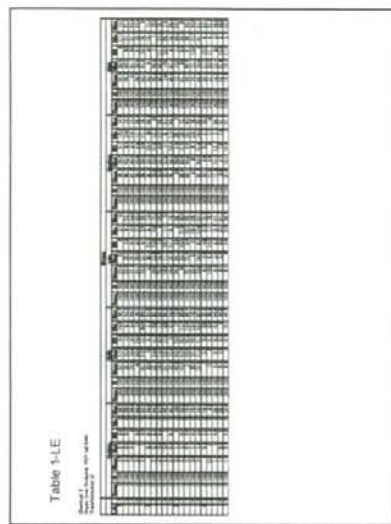
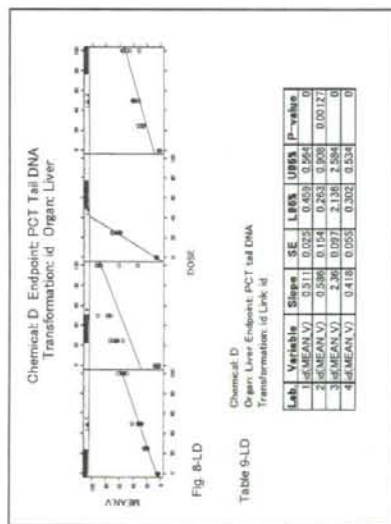
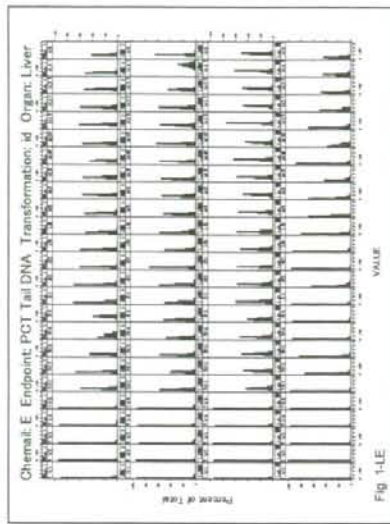
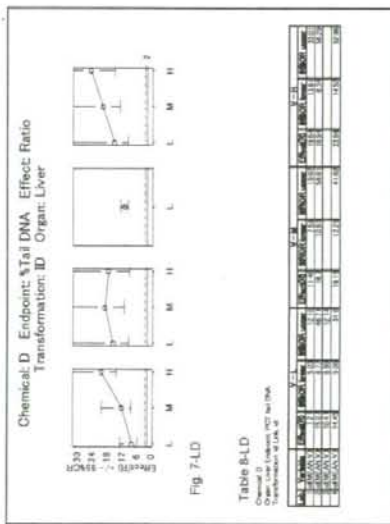
\* Data acceptance criteria may be revised based on the 3<sup>rd</sup> phase validation results

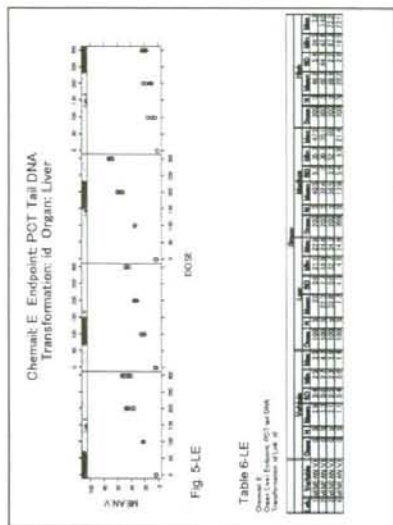
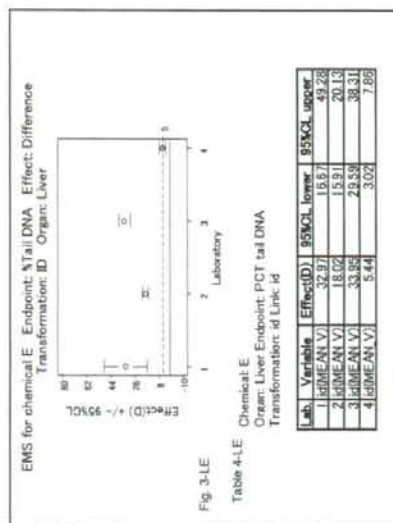
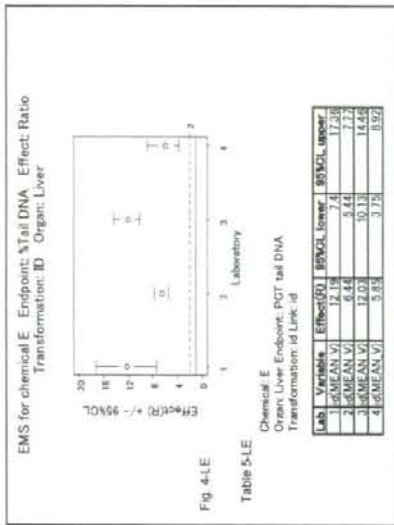
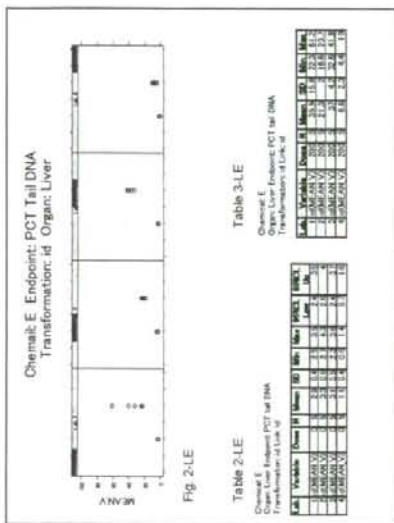












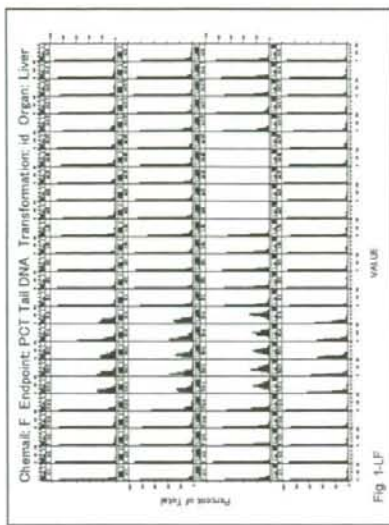
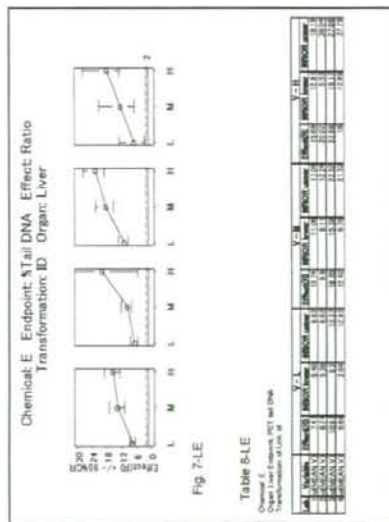
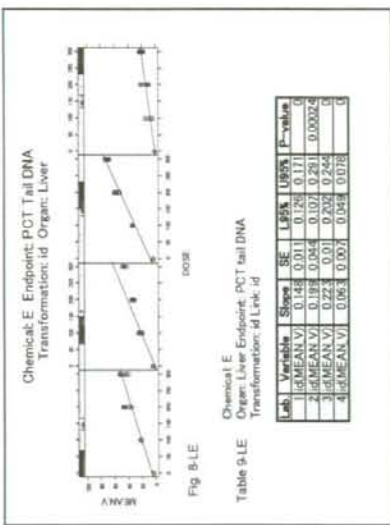
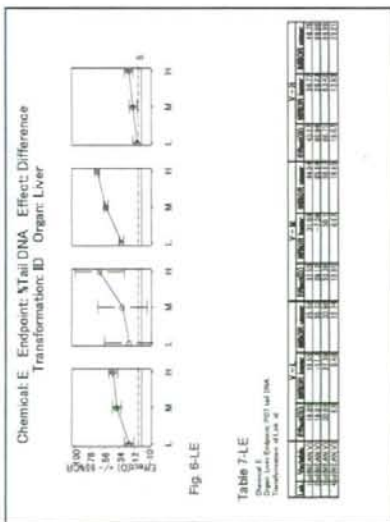


Table 1-LF

