Two Validation Studies on a Non-RI Modification of the Murine LLNA Using ATP Measurement

ECVAM Workshop

Alternative Endpoints for the Local Lymph Node Assay

Sep 26, 2007

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Outline

- Background
- First study
- Second study
- Summary of the 2 studies

Before the JaCVAM was founded

- Japanese Society of Alternatives to Animal Experiments (JSAAE)
  - Promoted validation studies for evaluating alternatives
    - Evaluation Committee
    - Validation Committee

Announcement seeking participant laboratories

- Nomination of 19 laboratories.
- Problem: Shortage of materials!
  - It was impossible to arrange the experimental animals and carry out ATP measurements during the same study period.

Participant experimental laboratories

- Two studies
  - 1st study: 10 laboratories
  - 2nd study: 9 laboratories

  (Finally, only 7 laboratories participated)

Overall plan for these studies

- Main aim of the 2 studies: **Evaluation of inter-laboratory reproducibility** using masked chemicals.
- The 1st study will precede the 2nd study.
- Any problem detected in the 1st study will be investigated in the 2nd study.

Organization and roles

- Roles played by researchers in the 2 studies
  - Study manager
  - Chemical selector
  - Chemical & material distributor
  - Staff for technology transfer
  - Validation committee members
  - Representative of each experimental facility
  - Biostatistician

Face-to-face meetings

Feb 6, 2006: 1st meeting

Mar 27, 2006: 2nd meeting

Aug 21, 2006: 3rd meeting

Nov 27, 2006: 4th meeting

Mar 16, 2007: 5th meeting

Steps to avoid extra variation

- Prepare a study protocol and an experimental protocol
- **Employ technology transfer** and **preliminary tests**
- Use web tools
- Format the data file

Interpretation of results as positive or negative

- Interpretation was based on stimulation index (SI) values.
- Positive: SI ≥ 3

  Negative: otherwise

Confidence interval (CI) for the SI values

- CI for the SI values was calculated using the following formula:

$$\exp\left(\ln(SI) \pm 1.96\sqrt{\left(\mathrm{Var}(\ln SI)\right)}\right)$$

Where,

$$\mathrm{Var}(\ln SI) \cong \frac{SE(Y)^2}{\mathrm{Mean}(Y)^2} + \frac{SE(X)^2}{\mathrm{Mean}(X)^2}$$

Confidence interval for the SI values

- When the lower limit of the CI is greater than 1, it indicates statistical significance.

- We conducted **to show the CIs for the SI values**, but no statistical tests were conducted as a part of these studies.

First study

First study

Purposes

- Evaluation of the **reliability** of LLNA-DA
- Evaluation of the **relevance** of LLNA-DA

First study

Selected chemicals and their allocation

Chemical	Vehicle*	LLNA	GPMT/ BT**	Laboratory***									
				1	2	3	4	5	6	7	8	9	10
A: 2,4-Dinitrochlorobenzene	AOO	+	+	□	□	□	□	○	△	□	□	△	○
B: Hexylcinnamic aldehyde	AOO	+	+	○	○	△	△	△	□	△	○	○	△
C: 3-Aminophenol	AOO	+	+nonstd	□		○					□		
D: Glutaraldehyde	ACE	+		△	△			□					
E: Cobalt chloride	DMSO	+	+				○		○		△		
F: Isoeugenol	AOO	+	+				□	○				△	
G: Formaldehyde	ACE	+	+	△	△			□					
H: Dimethyl isophthalate	AOO	−	−	□		□				□			
I: Isopropanol	AOO	−	−	○	○	△	△	△	□	△	△	○	△
J: Nickel sulfate	DMSO	−	+				○		○		○		
K: Abietic acid	AOO	+	+		□				△	○			
L: Methyl salicylate	AOO	−	−			○				○			○

*: ACE, acetone; AOO, acetone-olive oil; and DMSO, dimethyl sulfoxide
**: +nonstd, non-standard guinea pig maximization tests
***: Allocated pairs for the experiment in each laboratory:
○, 1st experiment; △, 2nd experiment; and □, 3rd experiment

First study

Dose for chemicals

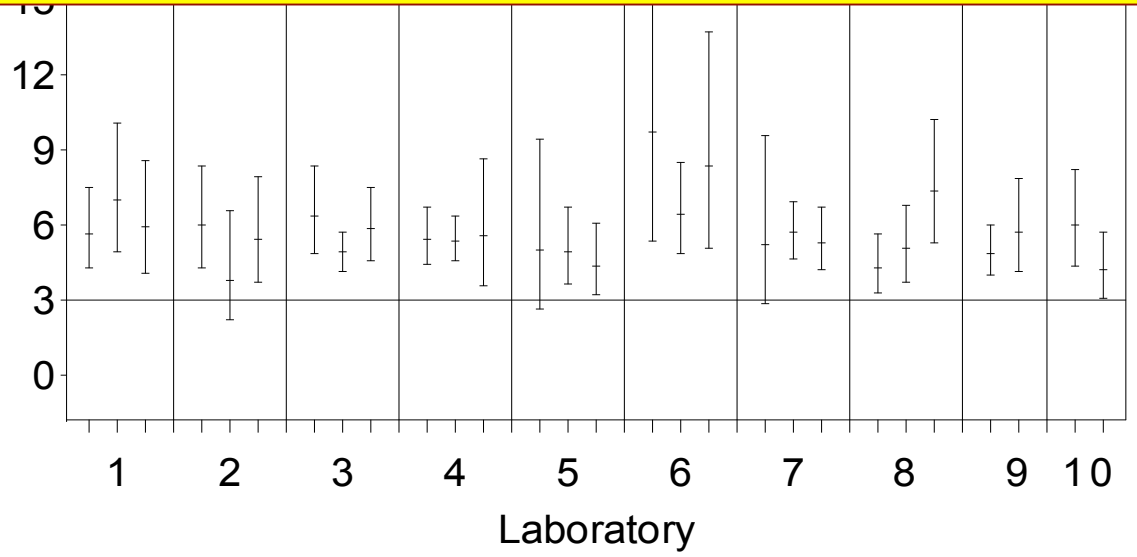
Chemical	Vehicle	Low	Middle	High
A: 2,4-Dinitrochlorobenzene	AOO	0.03%	0.10%	0.30%
B: Hexylcinnamic aldehyde	AOO	5%	10%	25%
C: 3-Aminophenol	AOO	1%	3%	10%
D: Glutaraldehyde	ACE	0.05%	0.15%	0.50%
E: Cobalt chloride	DMSO	0.30%	1.00%	3.00%
F: Isoeugenol	AOO	1%	3%	10%
G: Formaldehyde	ACE	0.5%	1.5%	5.0%
H: Dimethyl isophthalate	AOO	5%	10%	25%
I: Isopropanol	AOO	10%	25%	50%
J: Nickel sulfate	DMSO	1%	3%	10%
K: Abietic acid	AOO	5%	10%	25%
L: Methyl salicylate	AOO	5%	10%	25%

First study

Assay sensitivity

Positive control

- SI values were greater than 3 for all the experiments conducted in all the laboratories

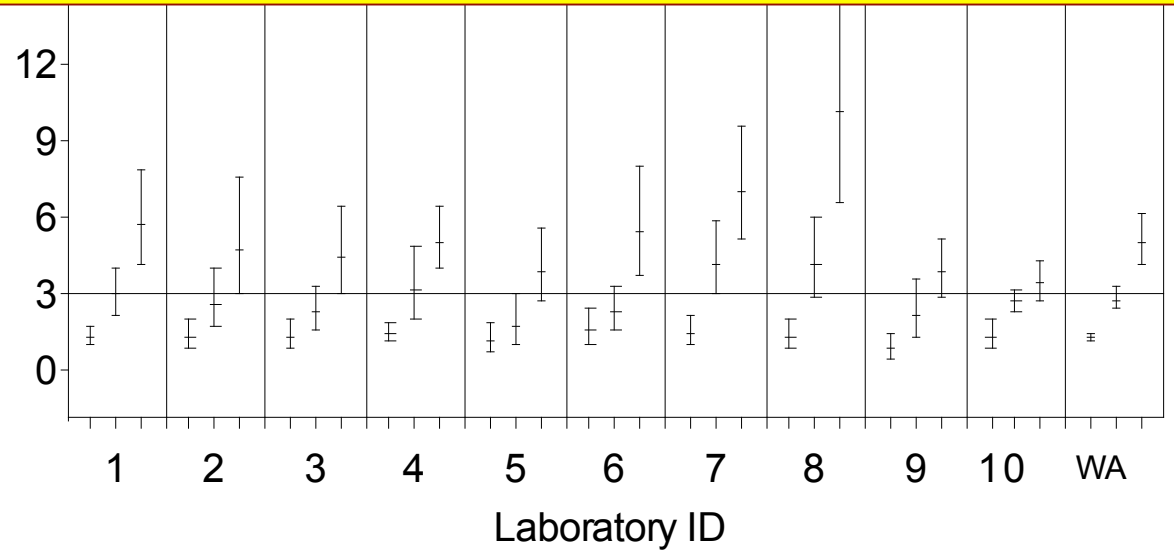


First study

Dose-response relationships of SI values

B: Hexylcinnamic aldehyde

- SI values were greater than 3 for the high-dose groups at all the laboratories.

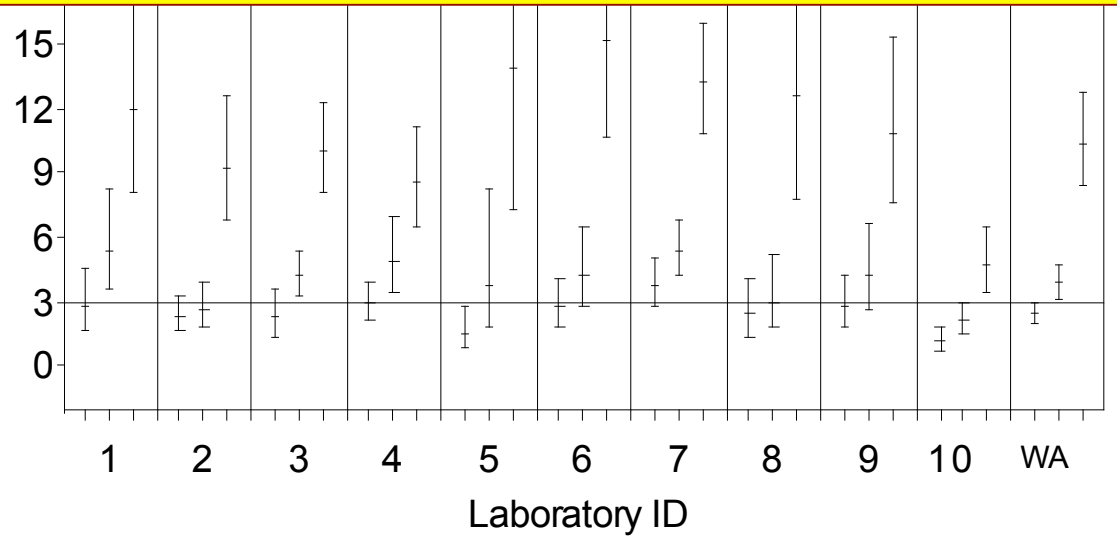


First study

Dose-response relationships of SI values

A: 2,4-Dinitro chlorobenzene

- SI values were greater than 3 for the high-dose groups at all the laboratories.

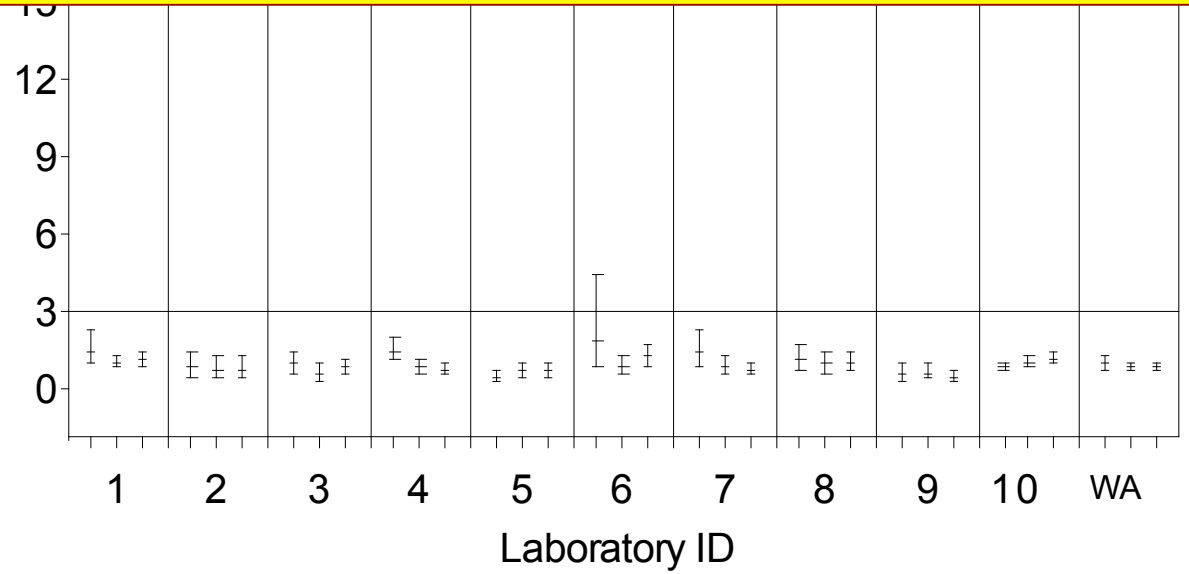


First study

Dose-response relationships of SI values

I: Isopropanol

- SI values were less than 3 for all the dose groups at all the laboratories.



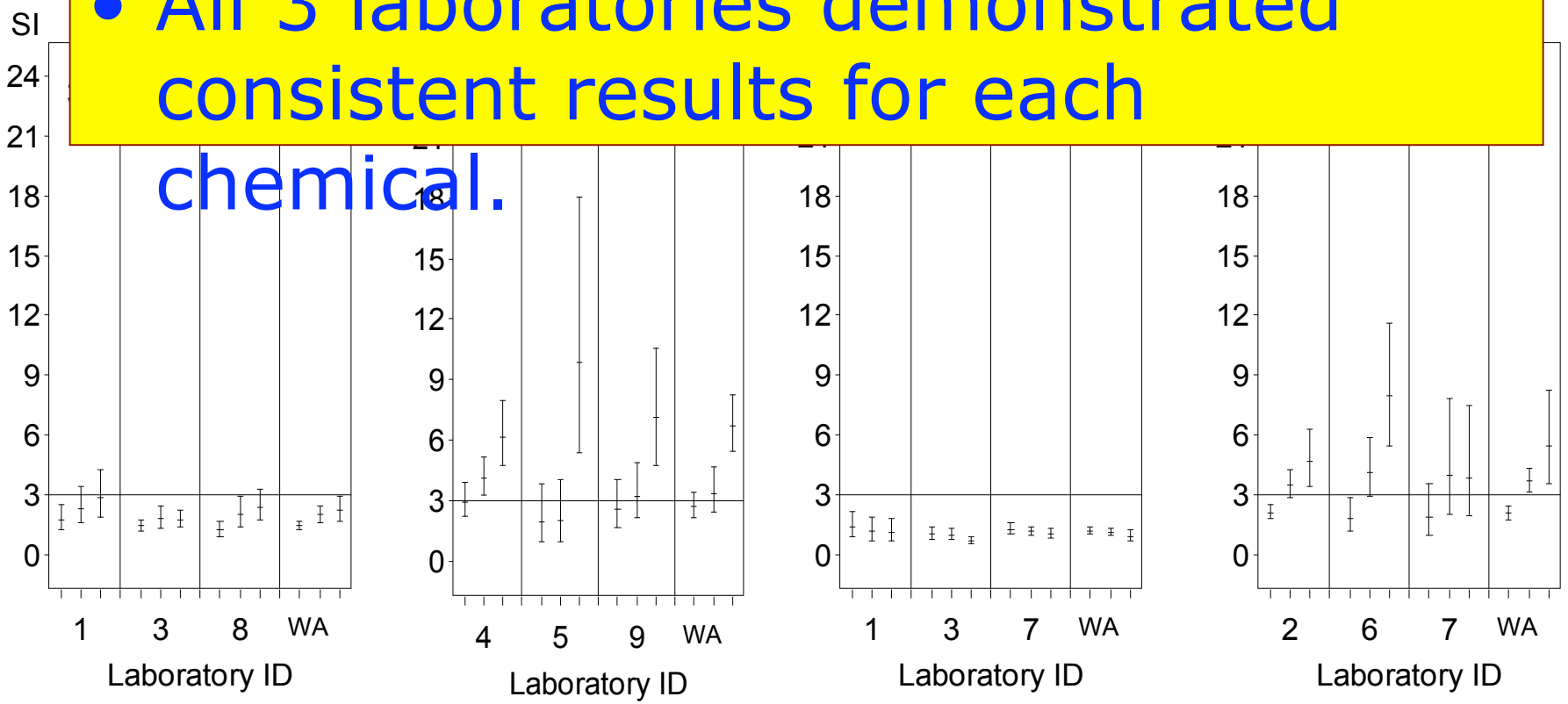
First study

Dose-response relationships of SI values

C: 3-Aminophenol, F: Isoeugenol,

H: Dimethyl isophthalate, K: Abietic acid

- All 3 laboratories demonstrated consistent results for each chemical.

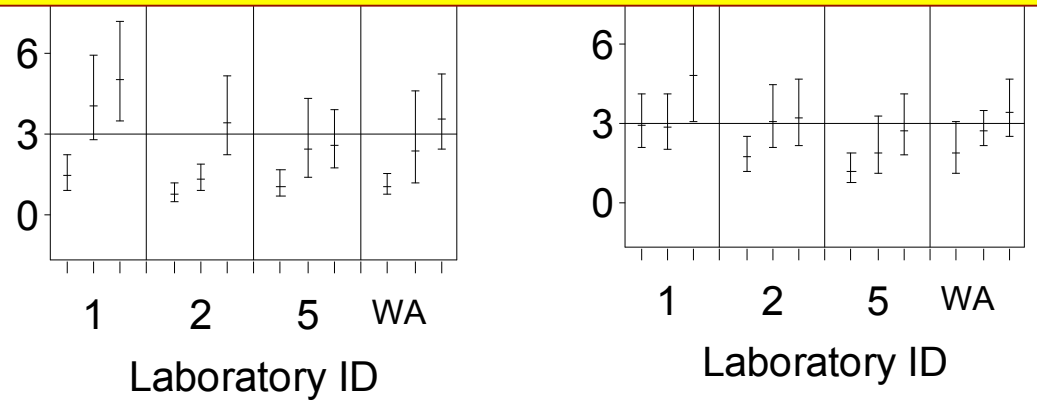


First study

Dose-response relationships of SI values

D: Glutaraldehyde, G: Formaldehyde

- Inconsistent results were observed among the 3 laboratories for each chemical.
- However, the variations were not large.

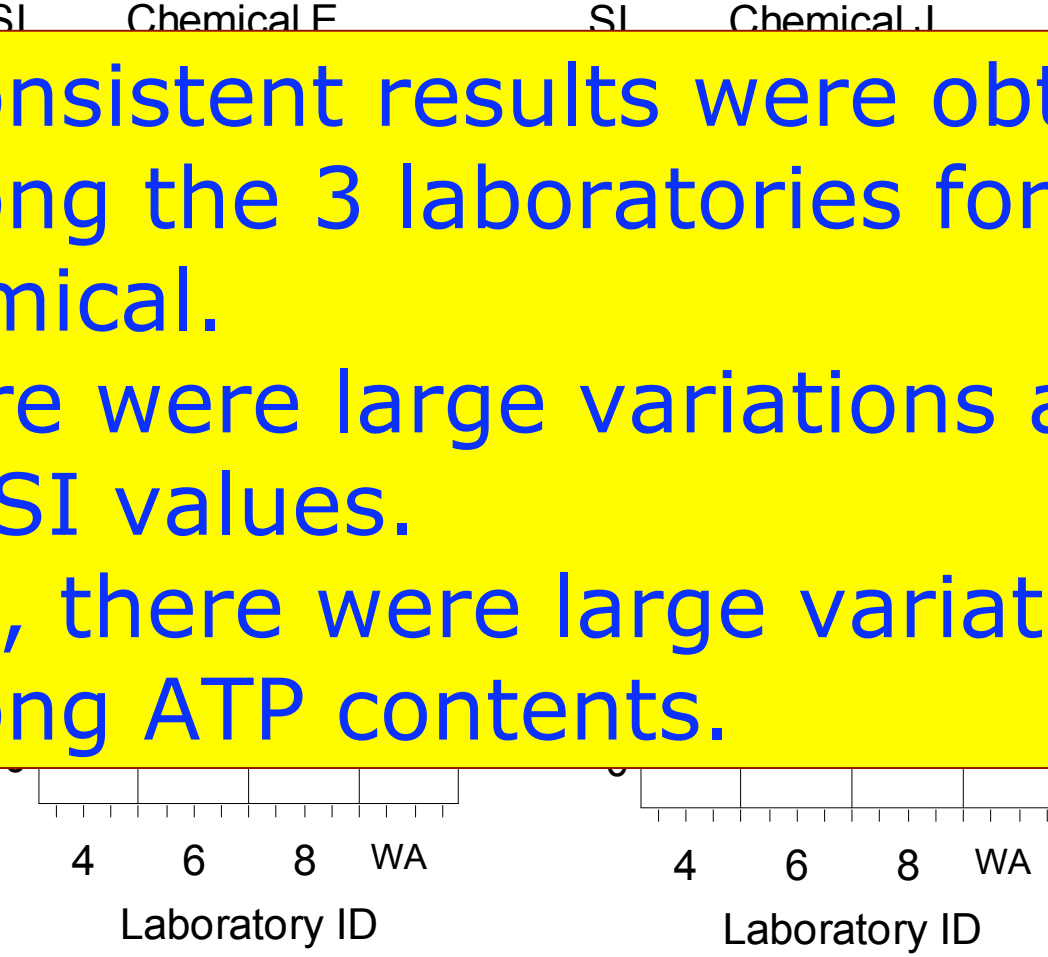


First study

Dose-response relationships of SI values

E: Cobalt chloride, J: Nickel sulfate

- Inconsistent results were obtained among the 3 laboratories for each chemical.
- There were large variations among the SI values.
- Also, there were large variations among ATP contents.



First study

Interpretation based on SI values

Chemical	LLNA	GPMT/BT	Laboratory									
			1	2	3	4	5	6	7	8	9	10
A: 2,4-Dinitrochlorobenzene	+	+	+	+	+	+	+	+	+	+	+	+
B: Hexylcinnamic aldehyde	+	+	+	+	+	+	+	+	+	+	+	+
C: 3-Aminophenol	+	+nonstd	−		−					−		
D: Glutaraldehyde	+		+	+			−					
E: Cobalt chloride	+	+				−		+		+		
F: Isoeugenol	+	+				+	+				+	
G: Formaldehyde	+	+	+	+			−					
H: Dimethyl isophthalate	−	−	−		−				−			
I: Isopropanol	−	−	−	−	−	−	−	−	−	−	−	−
J: Nickel sulfate	−	+				−		+		+		
K: Abietic acid	+	+		+				+	+			
L: Methyl salicylate	−	−			−				−			−

First study

Relevance

Statistical calculations were performed based on WA

- The performance of LLNA-DA was similar to that of LLNA.

LLNA-DA vs GPMT/BT	11	87.5% (7/8)	100% (3/3)	90.9% (10/11)
LLNA-DA vs LLNA	12	87.5% (7/8)	75.0% (3/4)	83.3% (10/12)
LLNA vs GPMT/BT	11	87.5% (7/8)	100% (3/3)	90.9% (10/11)

First study

Summary of the first study

- **Acceptable inter-laboratory reproducibility was obtained** for 10 of the 12 chemicals.
- There were **large variations** for E (**cobalt chloride**) and J (**nickel sulfate**), which were **metallic salts** dissolved in **dimethyl sulfoxide (DMSO)**.
- **Performance was similar to that of LLNA.**

Second study

Second study

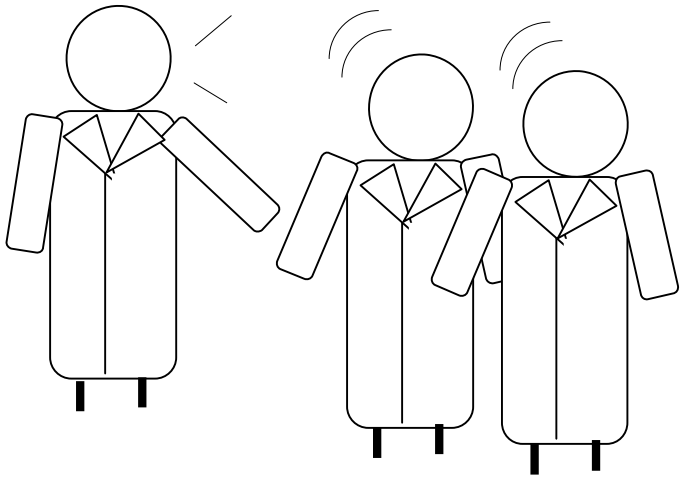
Purposes

- Development of a method to evaluate transferability
- Evaluation of the **reliability** of LLNA-DA for **metallic salts dissolved in DMSO**

Second study

Technology transfer

- In the seminar, the operation of **DMSO application** was included.



Second study

Selected chemicals and their allocation

Chemical	Vehicle*	LLNA	GPMT/ BT	Laboratory**						
				11	12	13	14	15	16	17
B: Hexylcinnamic aldehyde	AOO	+	+	○	○	○	○	○	○	○
J: Nickel sulfate	DMSO	−	+	□	△		△		△	
M: Lactic acid	DMSO	−	−	△		△		△	△	
E: Cobalt chloride	DMSO	+	+	□		△	△			△
N: Potassium dichromate	DMSO	+	+	△	△			△		△

*: ACE, acetone; AOO, acetone-olive oil; and DMSO, dimethyl sulfoxide
**: Allocated pairs for the experiment in each laboratory:
○, 1st experiment; △, 2nd experiment; and □, 3rd experiment

Second study

Dose for chemicals

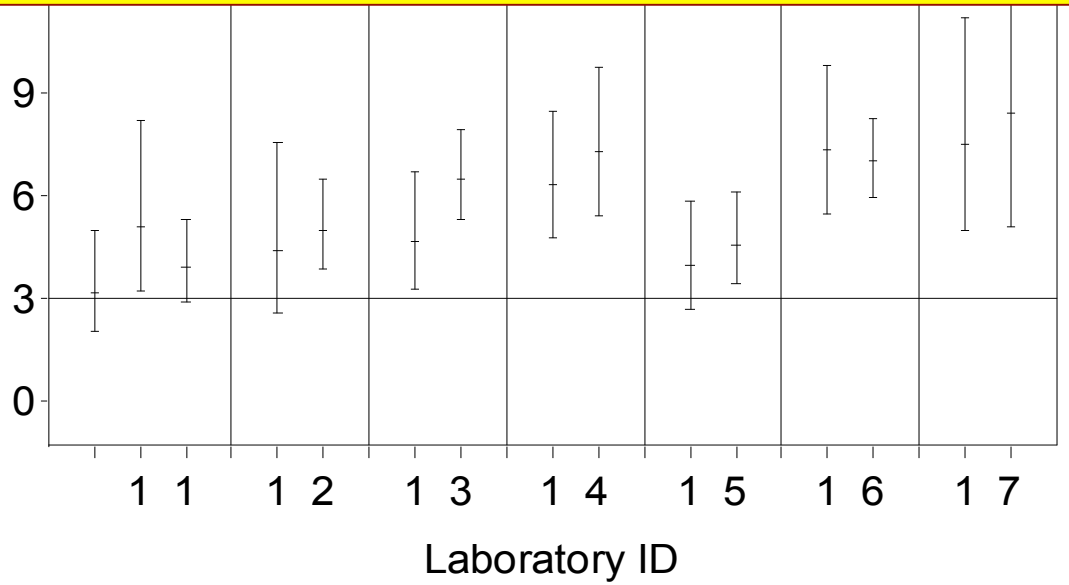
Chemical	Vehicle	Low	Middle	High
B: Hexylcinnamic aldehyde	AOO	5%	10%	25%
E: Cobalt chloride	DMSO	1%	3%	5%
J: Nickel sulfate	DMSO	1%	3%	10%
M: Lactic acid	DMSO	5%	10%	25%
N: Potassium dichromate	DMSO	0.1%	0.3%	1.0%

Second study

Assay sensitivity

Positive control

- SI values for all the experiments conducted in all the laboratories were greater than 3.

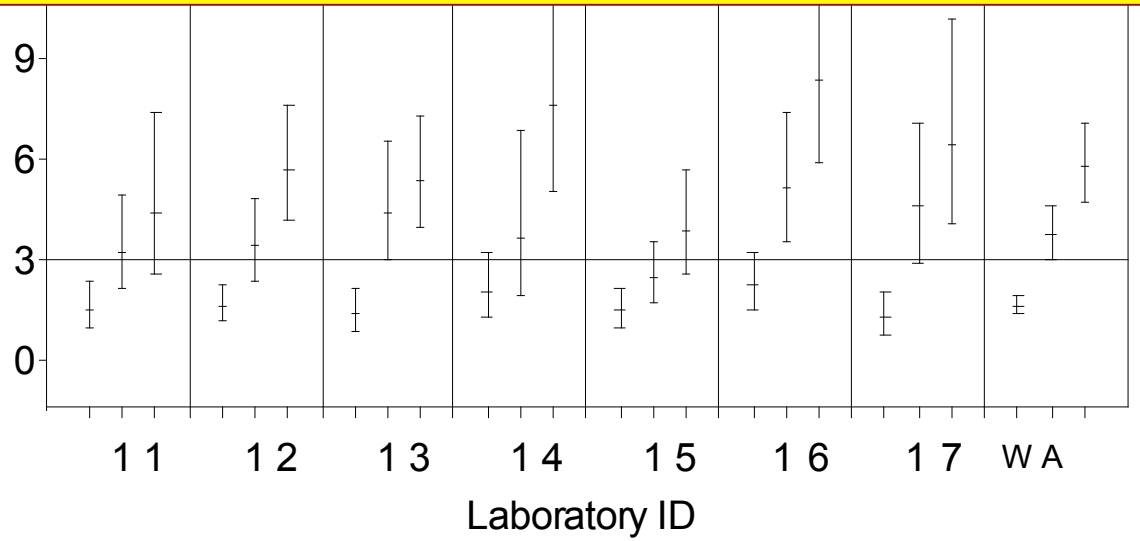


Second study

Dose-response relationships of SI values

B: Hexylcinnamic aldehyde

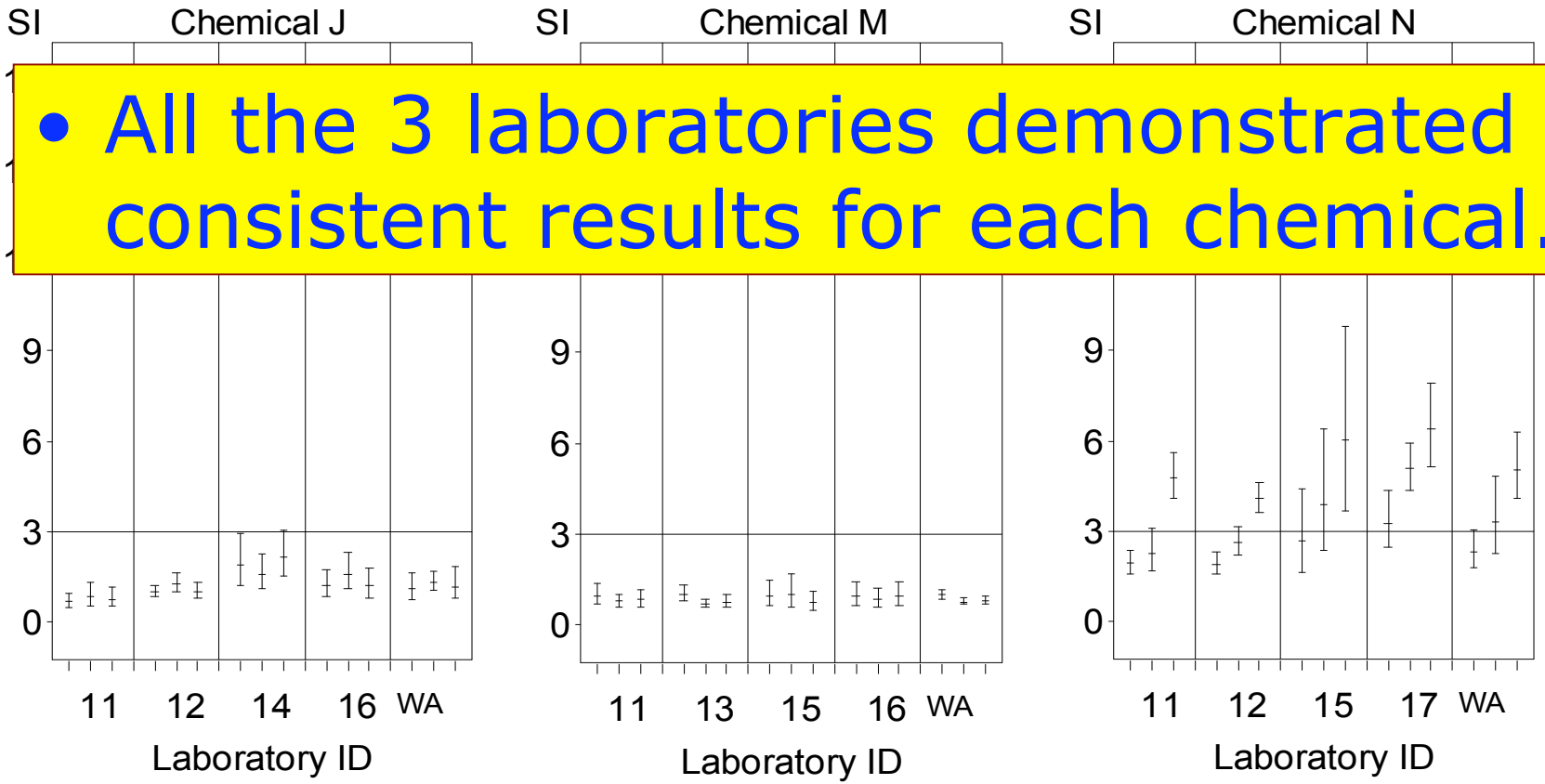
- SI values were greater than 3 for high-dose groups at all the laboratories.



Second study

Dose-response relationships of SI values

J: Nickel sulfate, M: Lactic acid,
N: Potassium dichromate



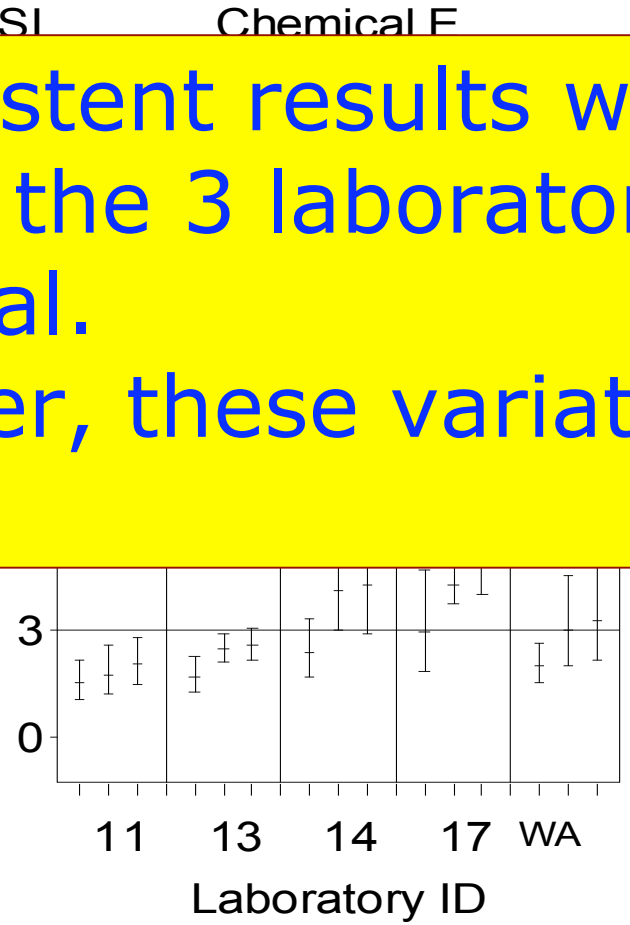
- All the 3 laboratories demonstrated consistent results for each chemical.

Second study

Dose-response relationships of SI values

E: Cobalt chloride

- Inconsistent results were obtained among the 3 laboratories for each chemical.
- However, these variations were not large.



Second study

Interpretation based on SI values

Chemical	LLNA	GPMT/BT	Laboratory						
			11	12	13	14	15	16	17
B: Hexylcinnamic aldehyde	+	+	+	+	+	+	+	+	+
E: Cobalt chloride	+	+	−		−	+			+
J: Nickel sulfate	−	+	−	−		−		−	
M: Lactic acid	−	−	−		−		−	−	
N: Potassium dichromate	+	+	+	+			+		+

Second study

Summary of the second study

- **Acceptable inter-laboratory reproducibility was obtained** for 5 chemicals.
- LLNA-DA can be used for testing **metallic salts** with **DMSO** as the vehicle.

Summary of the 2 studies and other information

Some factors responsible for the small variation

- All the laboratories used **the same experimental protocol.**
- All the laboratories used **the same luminometer** (Lumitester C-100, Kikkoman Co., Tokyo).

- All the laboratories used **the same dose of** each masked chemical.

Good Laboratory Practice (GLP) compliance

- We were not able to conduct these studies under the full compliance of GLP.
- However, all the laboratories were GLP laboratories.
- Formats for recording individual experiments were prepared and the formatted records of all the experiments were collected.

Number of tested chemicals

- Only 14 chemicals were tested.
- However, to date, **approximately 40 chemicals** have been tested and examined for relevance by Daicel Ltd.

Conclusions

- A total of **17 laboratories** tested the validity of the assay by using **14 chemicals**.
- **Small inter-laboratory variation** and **good relevance** were obtained.

Conclusions

- These results provide evidence that **the performance of LLNA-DA is similar to that of LLNA.**