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Japan

DRAFT

Characterization and current status of the LLNA-DA method: a non-RI modified LLNA based on ATP content

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What is LLNA-DA?

《A non-RI modified method》

LLNA : Measure ^{3}H-TdR incorporation
to determine the endpoint of cell proliferation

LLNA-DA : Measure ATP content in the lymph node
to determine the cell number at the end of cell proliferation

ATP: Adenosine triphosphate
Principal energy source for all living organisms.
ATP content is known to correlate with living cell number.
Bioluminescence is measured by luciferin-luciferase assay
to determine the ATP content.

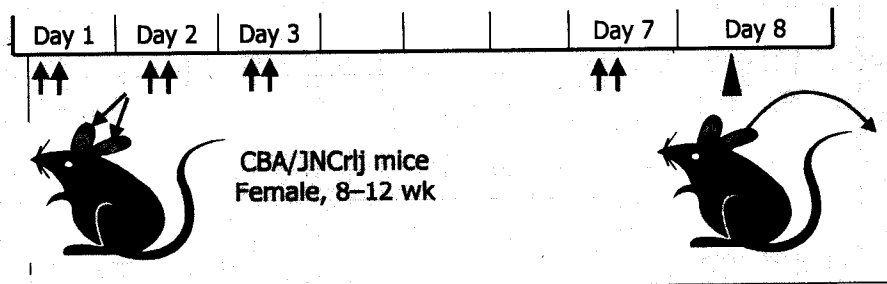
$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{Light}$$

LLNA-DA: LLNA modified by Daicel based on ATP content

Agenda

- Characterization of LLNA-DA
  - Method
  - Results of 31 well-known chemicals
  - Performance of LLNA-DA
- Inter-laboratory validation studies
  - Summary of results

Protocol of the LLNA-DA method



Days 1, 2, and 3, and Day 7

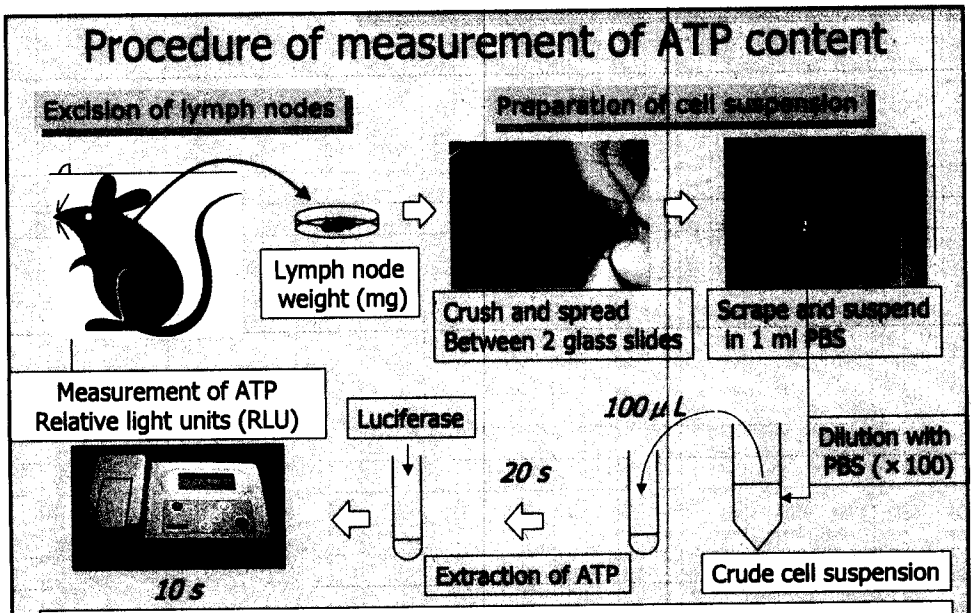
- Application of chemicals or vehicle control: 25 μL on the dorsum of both ears
- Pretreatment with 1% SLS solution: 1 h before each application

Day 8
(24–30 h after the last application)

Excision of auricular lymph nodes

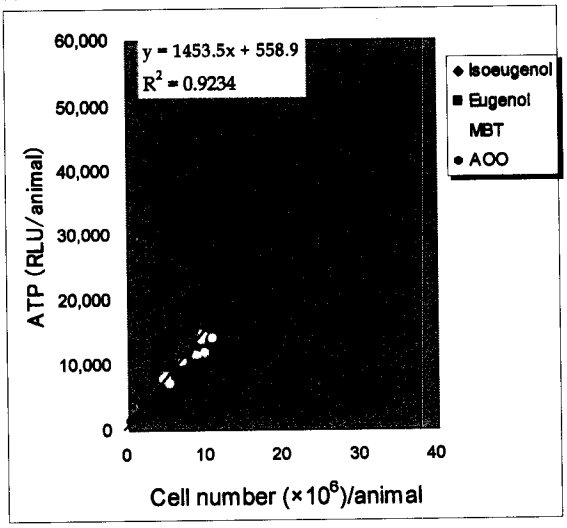
Measurement of ATP content by luciferin-luciferase assay

Procedure of measurement of ATP content



Several measurement kits are easily available.
Measuring the ATP is very easy and rapid.
Measurements should be performed immediately after lymph node excision.

Correlation between of cell number and ATP



Wide dynamic range

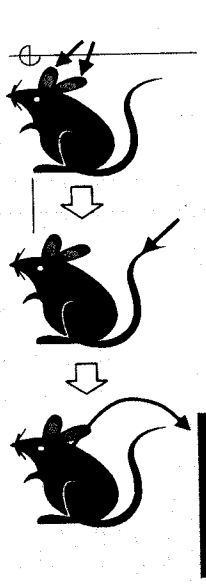
Detection range:
1,000~500,000 RLU

SI value: 1 to 20

RLU:relative light units

ATP content (RLU) is linearly related to the cell number

Difference between LLNA & LLNA-DA



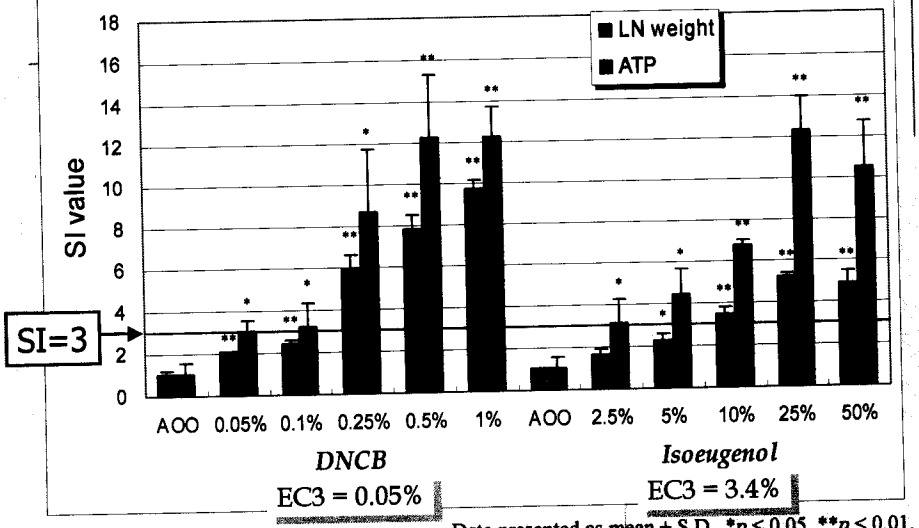
LLNA

LLNA-DA

Days 1, 2, and 3, and Day 7
Application
+pretreatment with
1% SLS solution

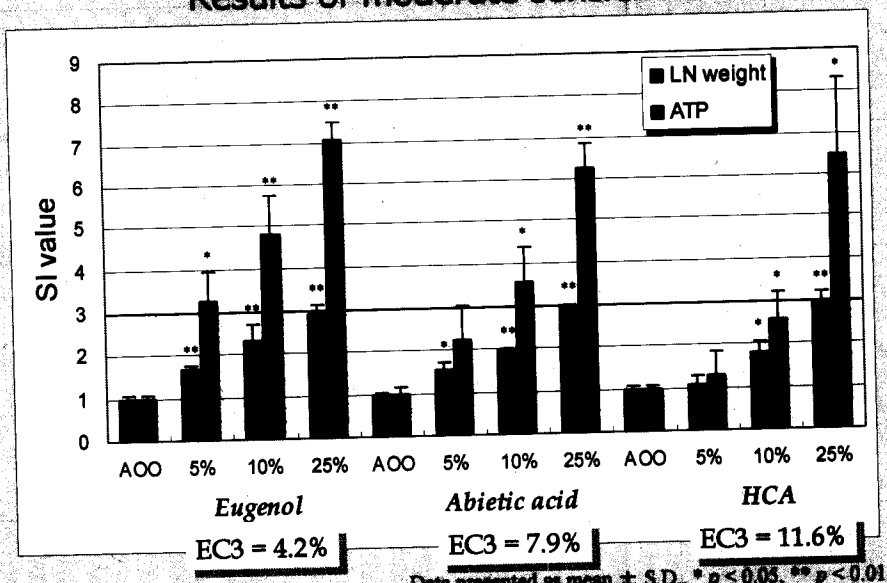
Day 8
Excision of auricular
lymph nodes
Measurement of ATP
(Index of cell number)

Results of extreme or strong sensitizers



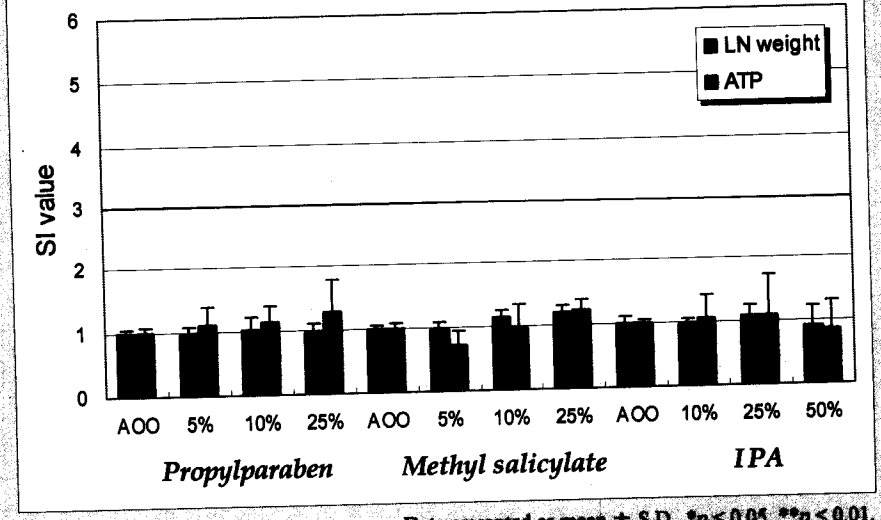
Data presented as mean ± S.D., $*p < 0.05$, $**p < 0.01$.
AOO: acetone/olive oil (4:1, v/v)
DNCB: 2,4-dinitrochlorobenzene

Results of moderate sensitizers



Data presented as mean ± S.D., * $p < 0.05$, ** $p < 0.01$
AOO: acetone/olive oil (4:1, v/v)
HCA: hexyl cinnamic aldehyde

Results of weak sensitizers and irritants



Data presented as mean ± S.D., *$p < 0.05$, **$p < 0.01$.
AOO: acetone/olive oil (4:1, v/v)
IPA: isopropyl alcohol

Summary of results
31 well-known chemicals

GPMT: guinea pig maximization test
BA: Buehler assay
HMT: human maximization test
HPTA: human patch test allergen

Chemicals	LLNA	GPMT/BA	HMT/HPTA
2,4-Dinitrochlorobenzene	+	+	
p-Phenylenediamine	+	+	+
Toluene diisocyanate	+		
Glutaraldehyde	+		
$K_2Cr_2O_7$	+	+	+
Pthalic anhydride	+	+	
Trimellitic anhydride	+		
Formaldehyde	+	+	+
Cinnamic aldehyde	+	+	+
Isoeugenol	+	+	+
$CoCl_2$	+	+	+
Eugenol	+	+	+
Resorcinol	+	-	+
Benzocaine	+/-	+	+/-
Abietic acid	+	+	+
Hexyl cinnamic aldehyde	+	+	
Mercaptobenzothiazol	+	+	+
Citral	+	+	+
Hydroxycitronellal	+	+	+
Imidazolidinyl urea	+	+	+
SLS	+	-	-
$NiSO_4$	-	+	+
Benzalkonium chloride	-	-	+
Propyl paraben	-	-	+/-
Diethylphthalate	-		
1-Bromobutane	-		
Methysalicylate	-	-	-
Chlorobenzene	-	-	
Lactic acid	-	-	
Hexane	-		-
Isopropanol	-	-	

Performance of LLNA-DA (vs. LLNA)

		LLNA	
		Positive	**Negative**
LLNA-DA	**Positive**	DNCB *p*-Phenylenediamine Cinnamaldehyde Isoeugenol Eugenol Abietic acid Imidazolidinyl urea Trimellitic anhydride Phthalic anhydride Glutaraldehyde Formaldehyde Hydroxycitronellal $K_2Cr_2O_7$ $CoCl_2$ TDI HCA Citral Resorcinol SLS	Benzalkonium chloride
	Negative	Mercaptobenzothiazol	1-Bromobutane Diethyl phthalate Propylparaben Methyl salicylate Chlorobenzene $NiSO_4$ Hexane IPA Lactic acid

Comparison	No. of comparisons	Sensitivity	Specificity	Positive predictivity	Negative predictivity	Accuracy
LLNA-DA vs. LLNA	30	95% (19/20)	90% (9/10)	95% (19/20)	90% (9/10)	93% (28/30)

Performance of LLNA-DA (vs. GPMT/BA)

		GPMT/BA	
		Positive	**Negative**
LLNA-DA	**Positive**	2,4-Dinitrochlorobenzene *p*-Phenylenediamine Phthalic anhydride Formaldehyde Cinnamic aldehyde Isoeugenol Eugenol Abietic acid Hydroxycitronellal Imidazolidinyl urea Benzocaine [redacted] $K_2Cr_2O_7$ $CoCl_2$ HCA Citral	Resorcinol SLS Benzalkonium chloride [redacted]
	Negative	Mercaptobenzothiazol $NiSO_4$ [redacted]	Propylparaben Methyl salicylate Chlorobenzene Lactic acid IPA [redacted]

Comparison	No. of comparisons	Sensitivity	Specificity	Positive predictivity	Negative predictivity	Accuracy
LLNA-DA vs. GPMT/BA	25	**88%** (15/17)	**63%** (5/8)	**83%** (15/18)	**71%** (5/7)	**80%** (20/25)

GPMT: guinea pig maximization test
BA: Buehler assay

Performance of LLNA-DA (vs. HMT/HPTA)

		HMT/HPTA	
		Positive	**Negative**
LLNA-DA	**Positive**	*p*-Phenylenediamine Formaldehyde Cinnamic aldehyde Isoeugenol Eugenol Resorcinol Abietic acid Citral Hydroxycitronellal Imidazolidinyl urea Benzalkonium chloride [redacted] $K_2Cr_2O_7$ $CoCl_2$	SLS [redacted]
	Negative	Mercaptobenzothiazol $NiSO_4$ Propylparaben [redacted]	Methyl salicylate Hexane [redacted]

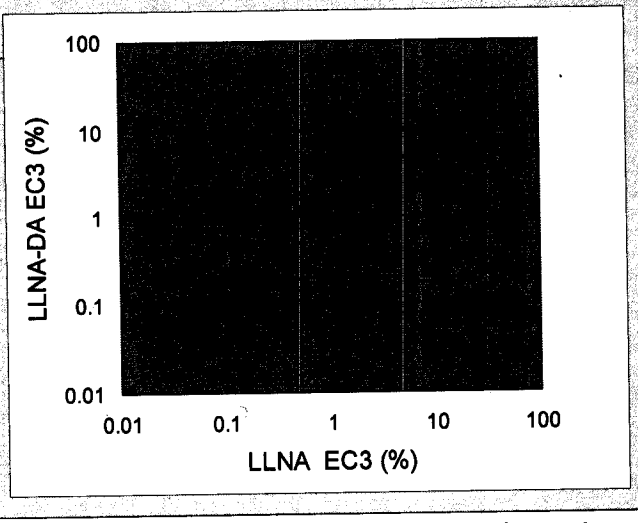
Comparison	No. of comparisons	Sensitivity	Specificity	Positive predictivity	Negative predictivity	Accuracy
LLNA-DA vs. HMT/HPTA	19	**81%** (13/16)	**67%** (2/3)	**93%** (13/14)	**40%** (2/5)	**79%** (15/19)

HMT: human maximization test
HPTA: human patch test allergen

Summary of EC3 value

Chemical name	LLNA-DA EC3%	LLNA EC3%
2,4-Dinitrochlorobenzene	0.05	0.03~0.09
p-Phenylenediamine	0.35	0.06~0.2
Toluene diisocyanate	0.05	0.11
Glutaraldehyde	0.10	0.10~0.20
$K_2Cr_2O_7$	0.14	0.14
Trimellitic anhydride	0.20	0.22
Formaldehyde	1.16	0.4~0.7
Cinnamic aldehyde	2.98	1.7~3.1
Isoeugenol	2.46, 2.28, 3.40	1.3~1.8
$CoCl_2$	3.27	0.82
Eugenol	4.23, 5.09, 5.59	13
Resorcinol	6.44	6.3
Benzocaine	6.57	+/-
Abietic acid	7.90	11.0~14.7
Hexyl cinnamic aldehyde	11.6	4.0~11.9
Citral	15.6	13
Hydroxycitronellal	13.7	20~23
Imidazolidinyl urea	18.8	23.9

Correlation of EC3 (LLNA vs. LLNA-DA)



EC3 values of LLNA-DA and original LLNA are almost in agreement

Reproducibility of EC3 values based on ATP content

Isoeugenol

Concentration (%)	SI value (ATP) ± S.D.		
	Exp. 1	Exp. 2	Exp. 3
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54		1.22 ± 0.13
1	2.28 ± 0.60		2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98
5	3.39 ± 0.69	4.39 ± 1.25	
10	5.68 ± 1.19	6.77 ± 0.23	
EC3	**3.40%**	**2.28%**	**2.46%**

2.71% ± 0.60% CV: 22%

Eugenol

Concentration (%)	SI value (ATP) ± S.D.		
	Exp. 1	Exp. 2	Exp. 3
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	**5.09%**	**5.59%**	**4.23%**

4.97% ± 0.69% CV: 14%

Mini-summary

- We developed a modified LLNA method with a non-RI endpoint (LLNA-DA)
- In LLNA-DA, we measure the ATP content as the endpoint.
  ~Luciferin-luciferase reaction~
- Simple operation to determine the ATP content and availability of a wide dynamic range
- Performance of LLNA-DA is similar to that of original LLNA.
- EC3 of LLNA-DA is almost equal to that of LLNA.

Inter-laboratory validation study

Objectives: To evaluate the reliability and relevance of LLNA-DA

T. Omori (Kyoto University): Study manager
H. Kojima (JaCVAM): Chemical selector, Chemical and material distributor
T. Sozu (Osaka University): Biostatistician
I. Yoshimura (Tokyo University of Science)

Experimental laboratories

National Institute of Health Sciences
Taisho Pharmaceutical Co., Ltd.
Otsuka Pharmaceutical Co., Ltd.
Food and Drug Safety Center
Sumitomo Chemical Co., Ltd.
Meiji Seika Kaisha, Ltd.
Fuji Film Co., Ltd.
Biosafety Research Center, Food, Drugs, and Pesticides
Chemicals Evaluation and Research Institute
Daicel Chemical Industries, Ltd.
Ishihara Sangyo Kaisha, Ltd.
Pias Corporation
Drug Safety Testing Center Co., Ltd.
TOAEIYO Ltd.
Nippon Shinyaku Co., Ltd.
Hoyu Co., Ltd.
Santen Pharmaceutical Co. Ltd.
Nakano Seiyaku Co., Ltd.
Institute of Environmental Toxicology

Progression of the validation study

Feb 2006	Established a committee for this study Prepared SOP for this study	
Mar	Technology transfer Pre-study Meeting Study	1st round by 10 facilities Blinded test of 12 chemicals
Aug	Meeting	
Oct	Technology transfer Pre-study	2nd round by 7 facilities
Nov	Meeting	Blinded test of 5 chemicals, including 3 metal salts/DMSO
Dec	Study	
Feb 2007		
Mar	Meeting	

Poster session in WC6

August 24, 2007 Room1-B

P2-2082 **First inter-laboratory validation study on LLNA-DA**

Y. Ikarashi (National Institute of Health Sciences) and co-workers

P2-2083 **Second inter-laboratory validation study on LLNA-DA**

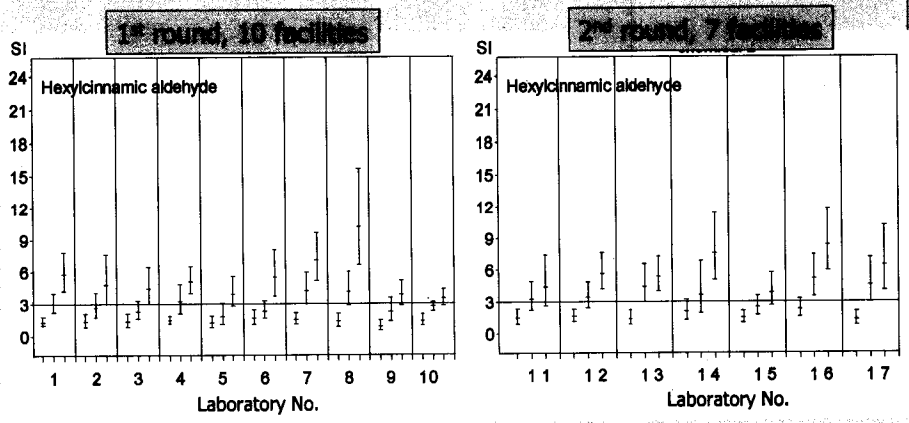
Y. Kanazawa (Food and Drug Safety Center) and co-workers

P2-2101 **Validation studies on LLNA:**

Importance of study management

T. Omiri (Kyoto University) and co-workers

Results of HCA in the validation studies



5%, 10%, and 25% hexylcinnamaldehyde (HCA) /AOO

All 17 laboratories judged HCA as positive.
Small variances were observed in the SI values at the same concentration.

Performance of LLNA-DA in the first-round study

Comparison	n	Sensitivity	Specificity	Positive predictivity	Negative predictivity	Accuracy
LLNA-DA vs. GPMT/BA	11	87.5% (7/8)	100% (3/3)	100% (7/7)	75% (3/4)	90.9% (10/11)
LLNA-DA vs. LLNA	12	87.5% (7/8)	75.0% (3/4)	88% (7/8)	75% (3/4)	83.3% (10/12)
LLNA vs. GPMT/BA	11	87.5% (7/8)	100% (3/3)	100% (7/7)	75% (3/4)	90.9% (10/11)

GPMT: guinea pig maximization test
BA: Buehler assay

The sensitivity, specificity, and accuracy of LLNA-DA vs. GPMT/BA are similar to those of LLNA vs. GPMT/BA.

Conclusion

We have developed LLNA-DA—a non-RI LLNA method—in which ATP content is used as the endpoint.

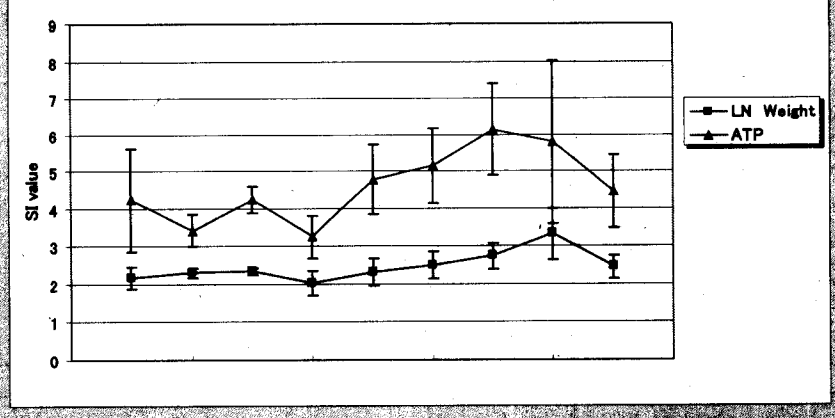
We tested 31 well-known chemicals and confirmed the performance of LLNA-DA.

Two rounds of inter-laboratory validation studies were conducted at 17 facilities.

The results of these studies are acceptable as a catch-up validation study, at least with regard to the 14 examined chemicals.

Thank you for your attention

Reproducibility of SI value (10% [illegible])



4.99% ± 1.35% CV: 27%

[illegible]

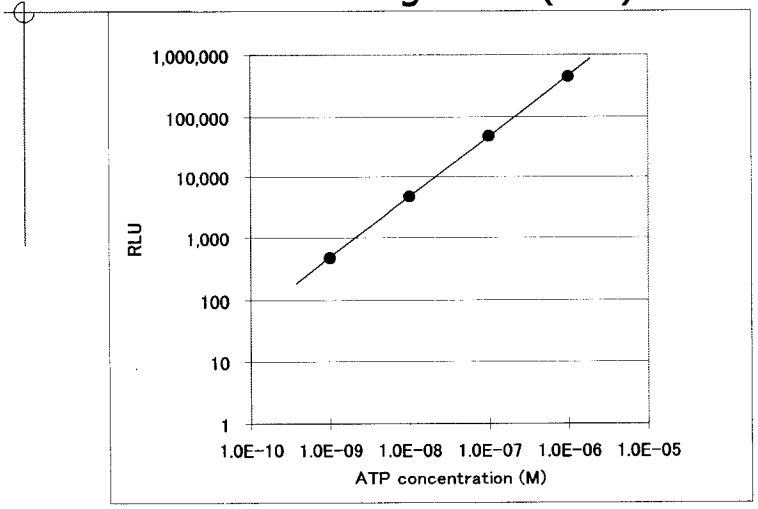
Performance of LLNA-DA against LLNA and other [illegible]

Comparison	No. of comparisons	Sensitivity	Specificity	Positive predictivity	Negative predictivity
LLNA-DA vs. LLNA	30	95% (19/20)	90% (9/10)	95% (19/20)	90% (9/10)
LLNA-DA vs. GPMT/BA	25	88% (15/17)	63% (5/8)	83% (15/18)	71% (5/7)
LLNA-DA vs. HMT/HPTA	19	81% (15/17)	67% (9/10)	93% (19/20)	40% (9/10)
*LLNA-DA vs. GPMT/BA	97	91% (62/28)	83% (24/29)	93% (62/67)	80% (24/30)
*LLNA-DA vs. HMT/HPTA	74	72% (49/68)	67% (4/6)	96% (49/51)	17% (4/23)

*[illegible]

Reference information

Correlation of ATP concentration with relative light unit (RLU)

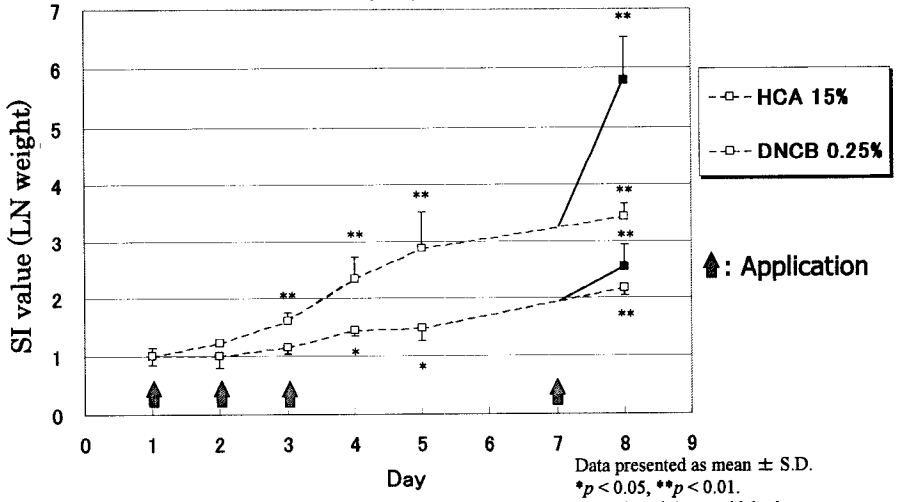


Correlation of ATP concentration with relative light unit (RLU)

Reference information

Effect of fourth application on Day 7

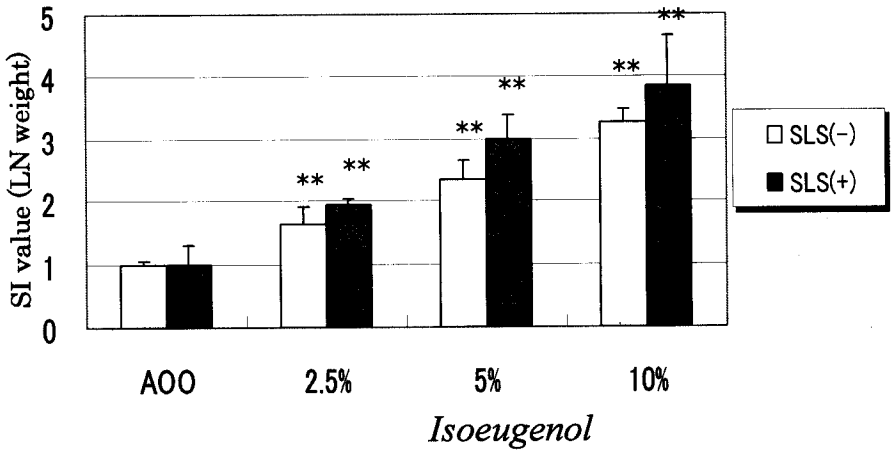
—Variation in lymph node weight—



Data presented as mean ± S.D.
*$p < 0.05$, **$p < 0.01$.
HCA: hexylcinnamaldehyde
DNCB: 2,4-dinitrochlorobenzene

Reference information

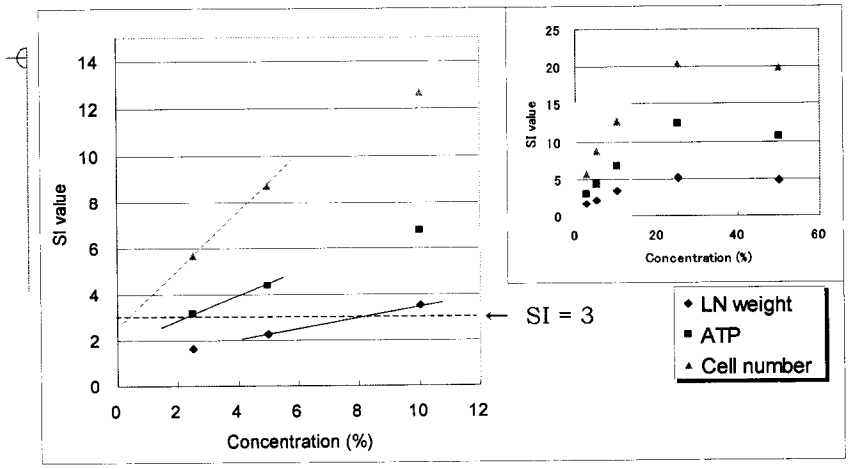
Effect of pretreatment with 1% SLS solution



Data presented as mean ± S.D.
*$p < 0.05$, **$p < 0.01$.

Reference information

Variation in EC3 by difference of endpoints for isoeugenol



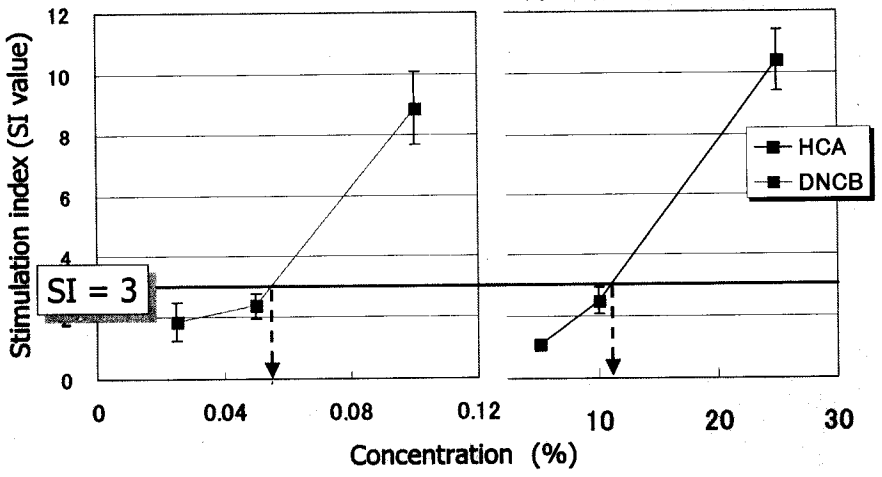
EC3 = 8.02%　(LN weight)
EC3 = 2.28%　(ATP)
EC3 = 0.31%　(Cell number)

EC3 = 1.3～3.3%
(Original LLNA)

Reference information

Potency estimation in LLNA by EC3 value

Gerberick, G. F., *et al.*, *Methods* (1999) 19, 48–55.

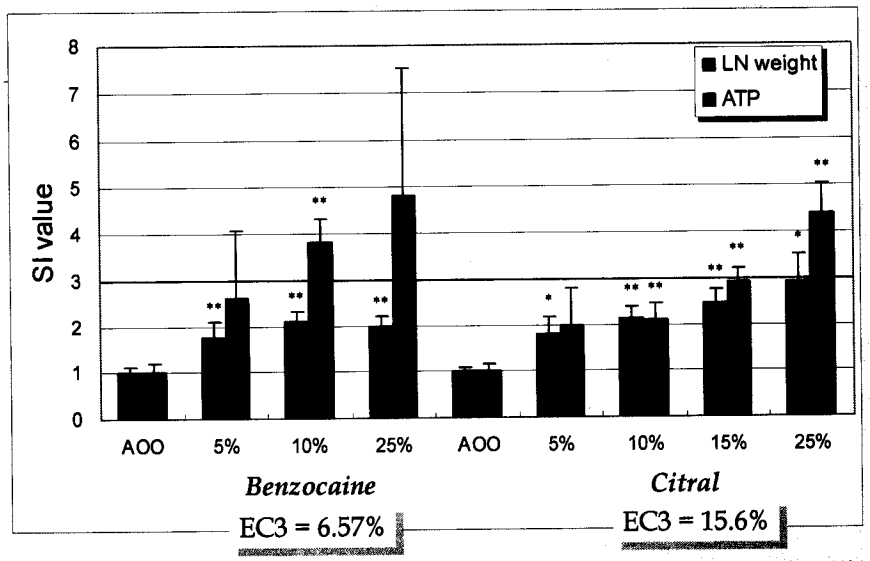


EC3 = 0.05%

EC3 = 11%

Reference information

Results of moderate and weak sensitizers



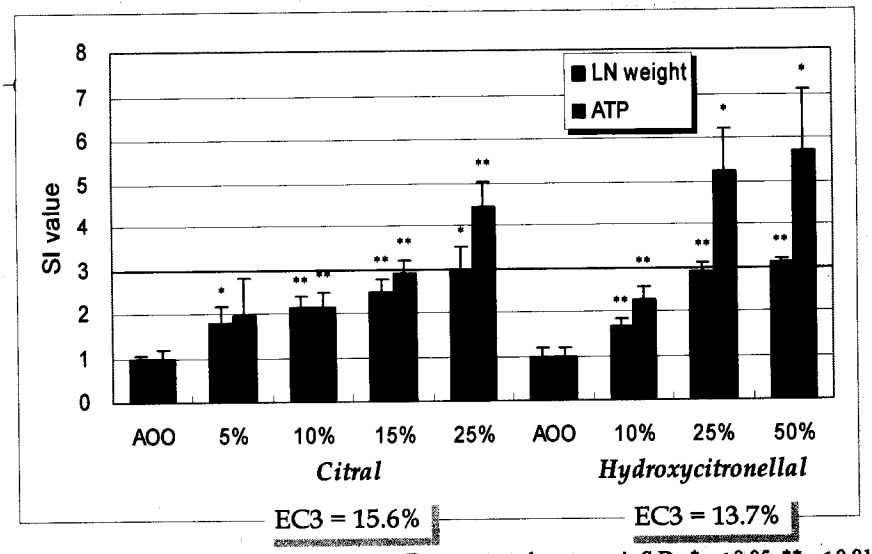
EC3 = 6.57%

EC3 = 15.6%

Data presented as mean ± S.D., $*p < 0.05$, $**p < 0.01$.
AOO: acetone/olive oil (4:1, v/v)

Reference information

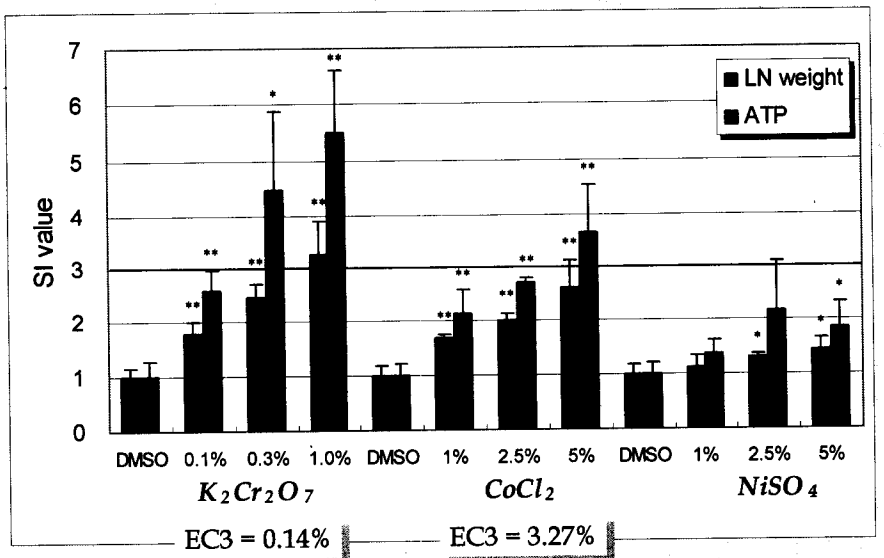
Results of weak sensitizers



Data presented as mean ± S.D., $*p < 0.05$, $**p < 0.01$.
AOO: acetone/olive oil (4:1, v/v)

Reference information

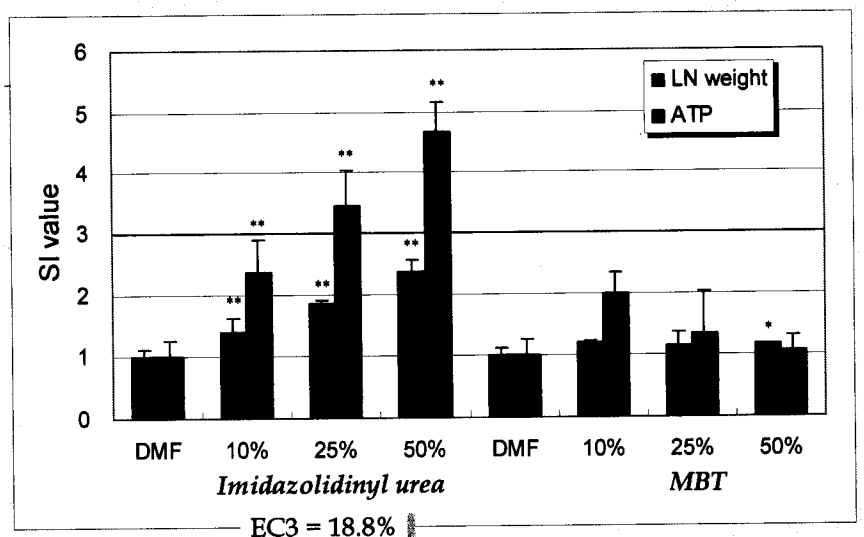
Results of metal salts



Data presented as mean ± S.D., $*p < 0.05$, $**p < 0.01$.
DMSO: dimethylsulfoxide

Reference information

Using DMF as vehicle

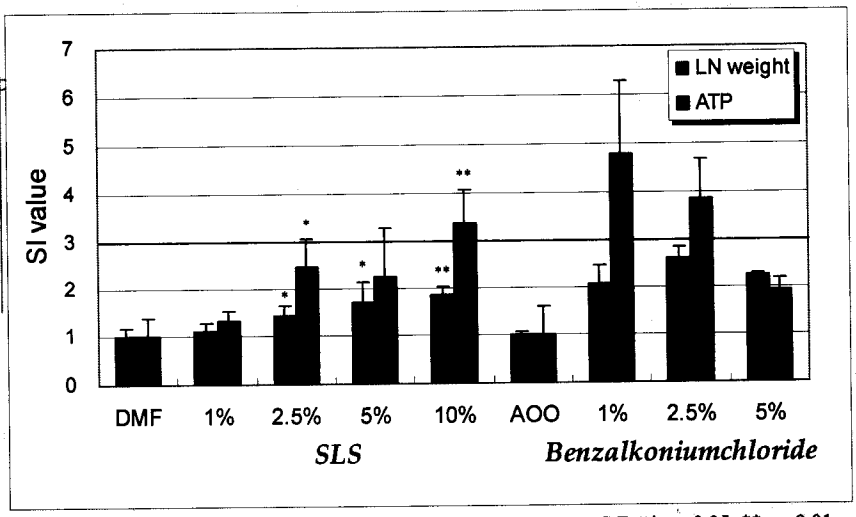


EC3 = 18.8%

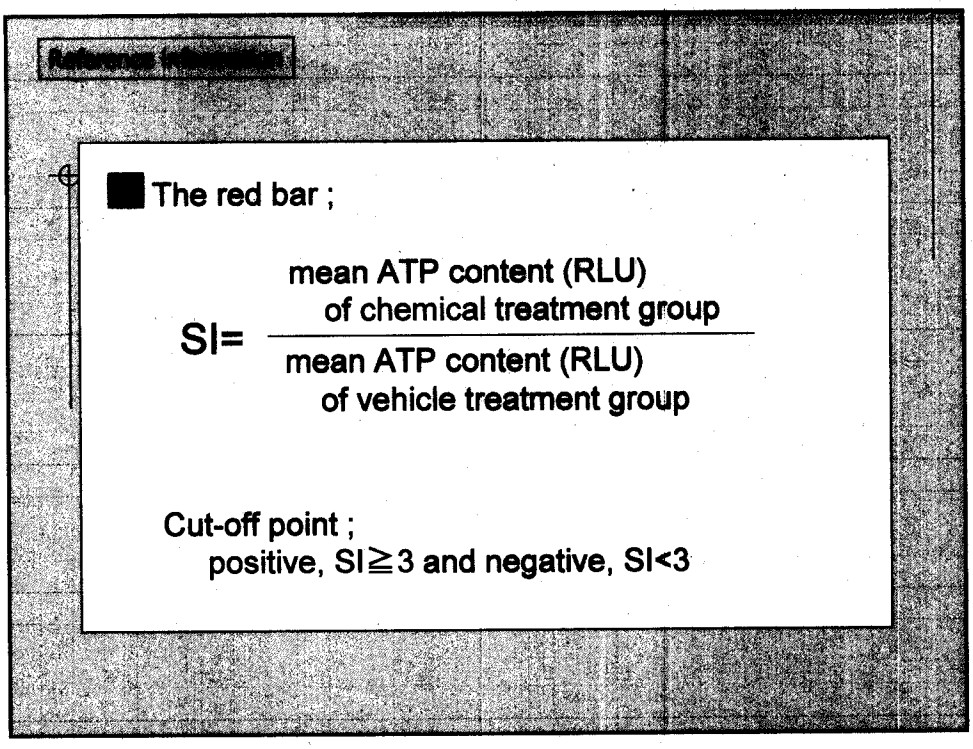
Data presented as mean ± S.D., $*p < 0.05$, $**p < 0.01$.
DMF ; *N, N* –Dimethylformamide
MBT ; 2-Mercaptobenzothiazol

Reference information

False positive substances



Data presented as mean ± S.D., $*p < 0.05$, $**p < 0.01$.
DMF ; *N, N* -Dimethylformamide
AOO: acetone/olive oil (4:1, v/v)
SLS: Sodium lauryl sulfate


The red bar ;
SI= mean ATP content (RLU) of chemical treatment group / mean ATP content (RLU) of vehicle treatment group
Cut-off point ;
positive, SI≧3 and negative, SI<3