

The Murine Local Lymph Node Assay: Daicel Chemical Industries, Ltd., Based on ATP Content (LLNA: DA)



Eleni Salicru, Ph.D.
ILS Inc., Contractor Supporting NICEATM

ICCVAM Workshop Series on Best Practices for Regulatory
Safety Testing: Assessing the Potential for Chemically Induced
Allergic Contact Dermatitis

January 20, 2011

William H. Natcher Conference Center
National Institutes of Health
Bethesda, MD

Overview of the LLNA: DA

- Developed by Daicel Chemical Industries, Ltd., as a nonradioactive LLNA^{1, 2}
- Measures ATP content in draining auricular lymph nodes as an estimate of cell number at the end of cell proliferation
 - ATP content is favorably correlated with living cell number
 - ATP content is assessed by a luciferin-luciferase assay to measure bioluminescence (relative luminescence units [RLU])



- ATP bioluminescence shown to have a strong correlation between thymidine uptake in a variety of cell lines³

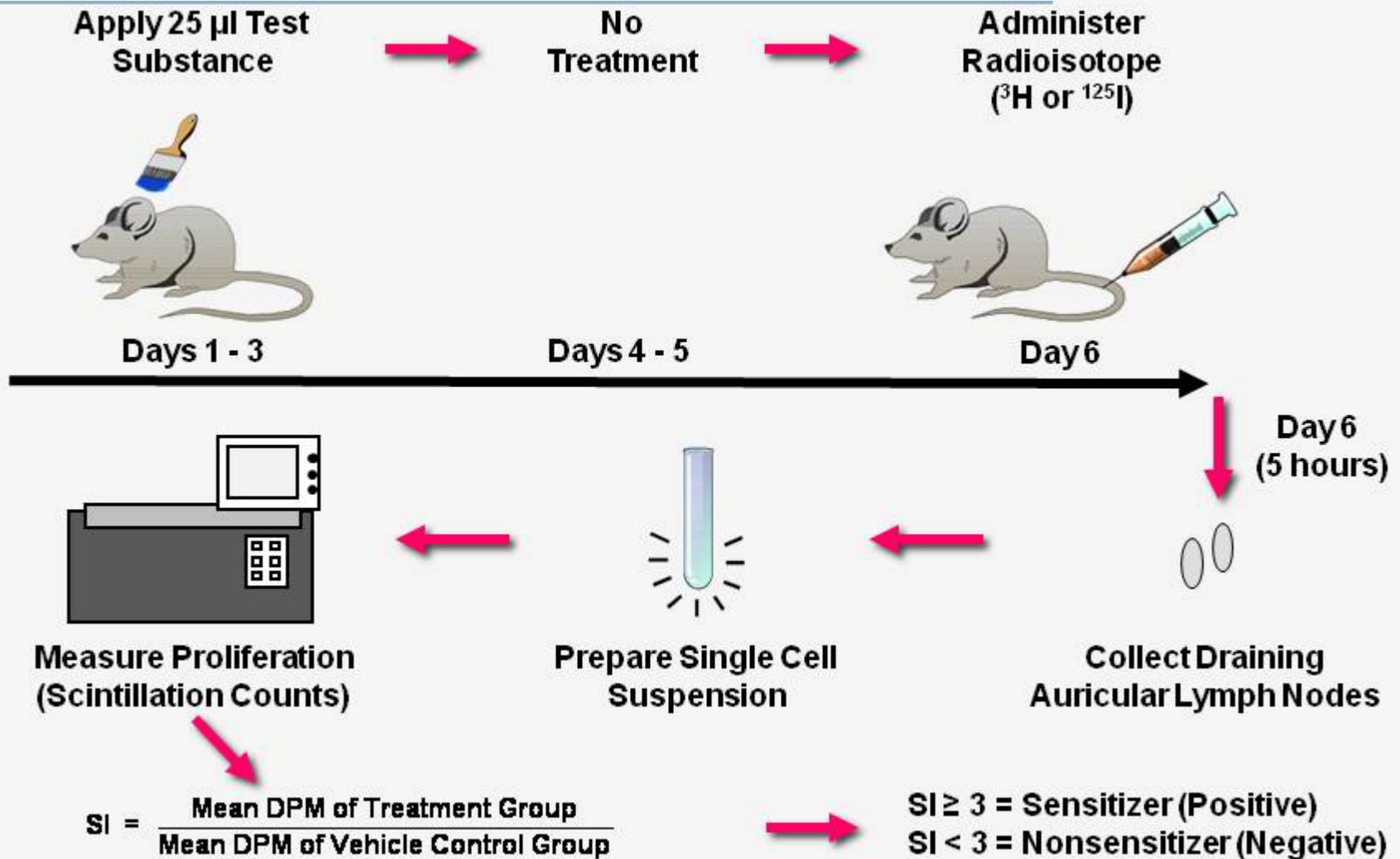
¹Yamashita et al. 2005. AATEX. 11:136-144.

²Idehara et al. 2008. J Pharmacol Toxicol Methods. 58:1-10.

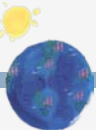
³Crouch et al. 1993. J Immunol Methods. 160:81-88.



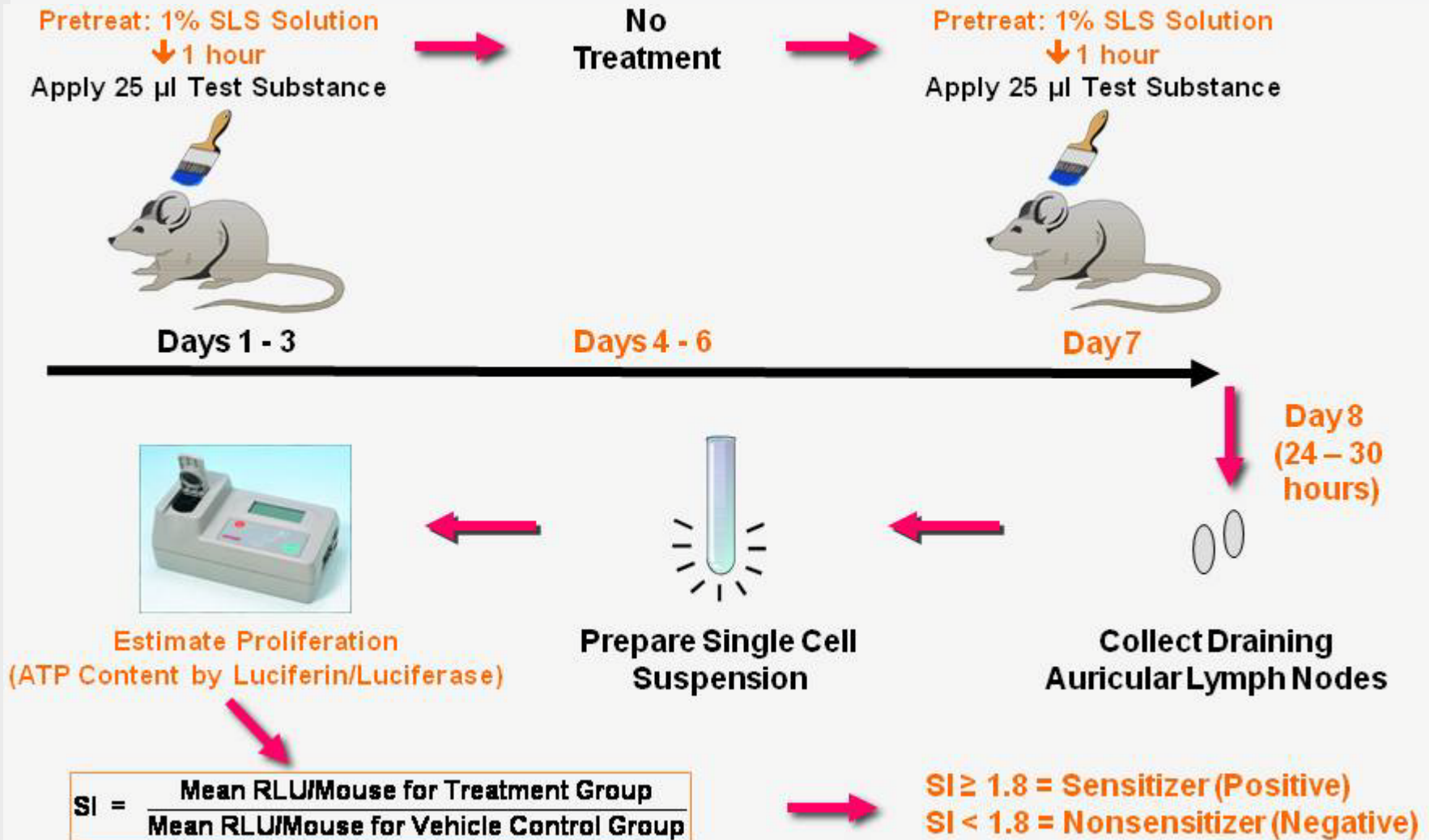
LLNA Test Method Protocol



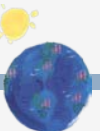
Abbreviations: DPM = disintegrations per minute; SI = stimulation index



LLNA: DA Test Method Protocol - 1

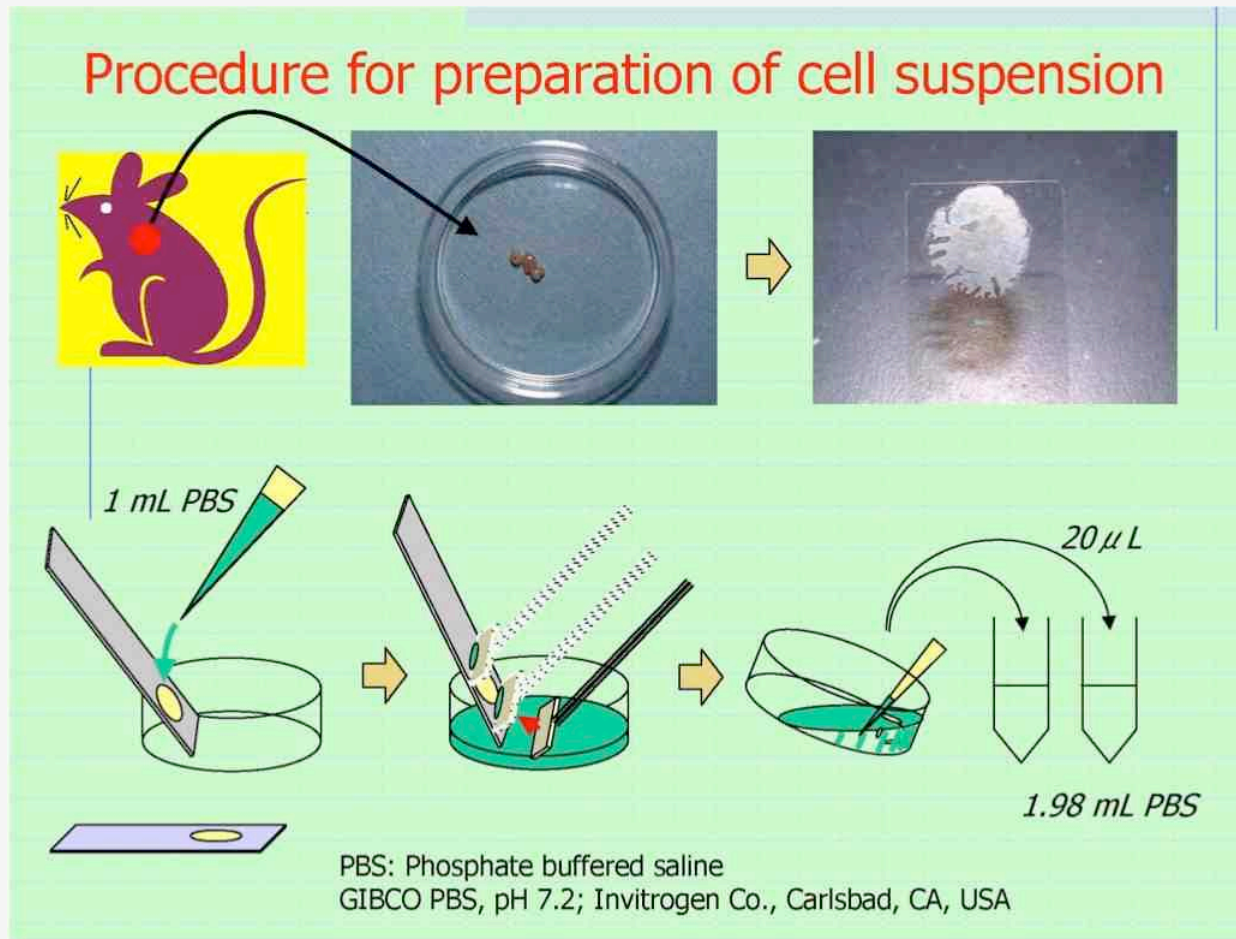


Abbreviations: RLU = relative luminescence units; SI = stimulation index; SLS = sodium lauryl sulfate



LLNA: DA Test Method Protocol - 2

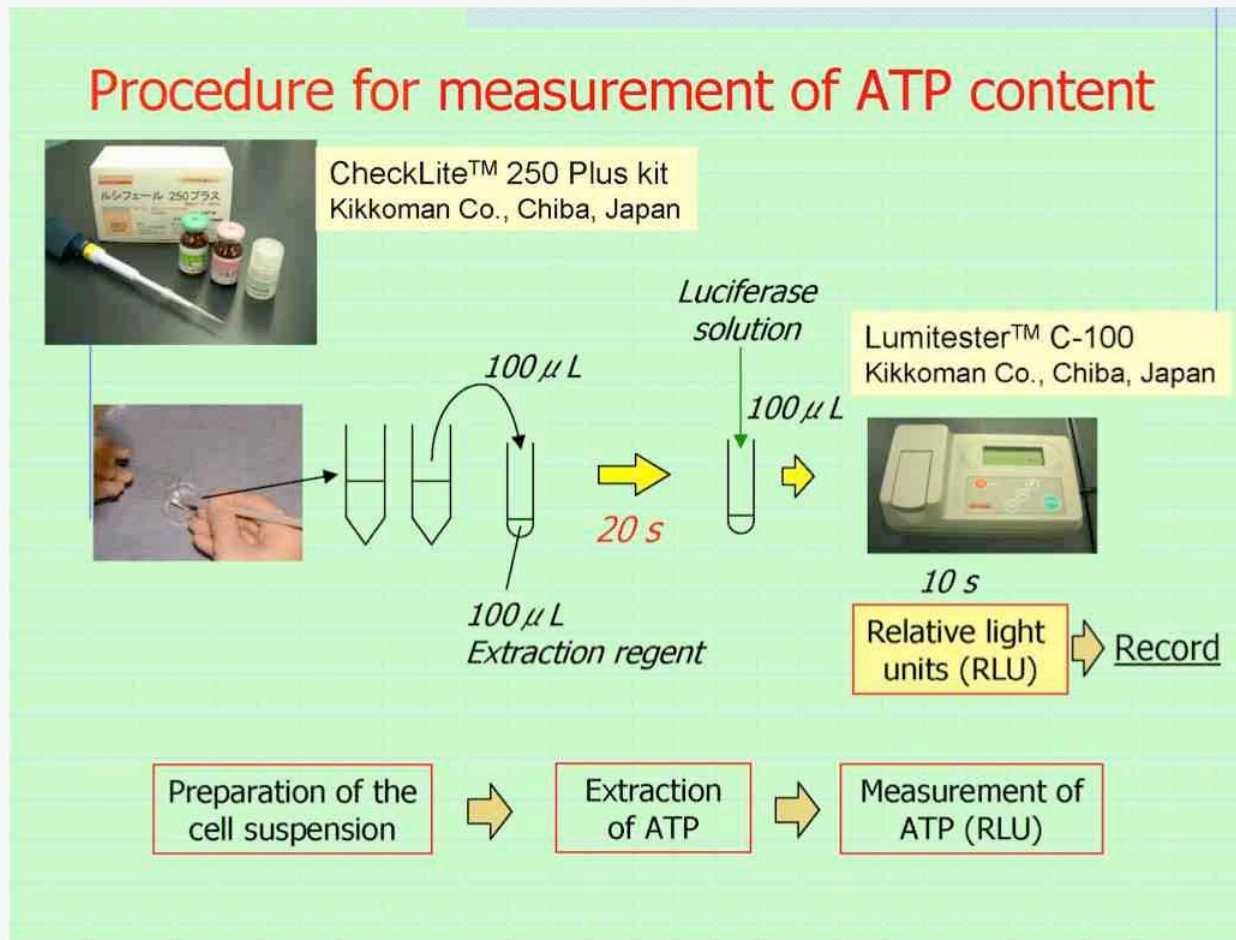
- Technical points:



Schematic courtesy of Dr. Kenji Idehara, Daicel Chemical Industries, Ltd.

LLNA: DA Test Method Protocol - 3

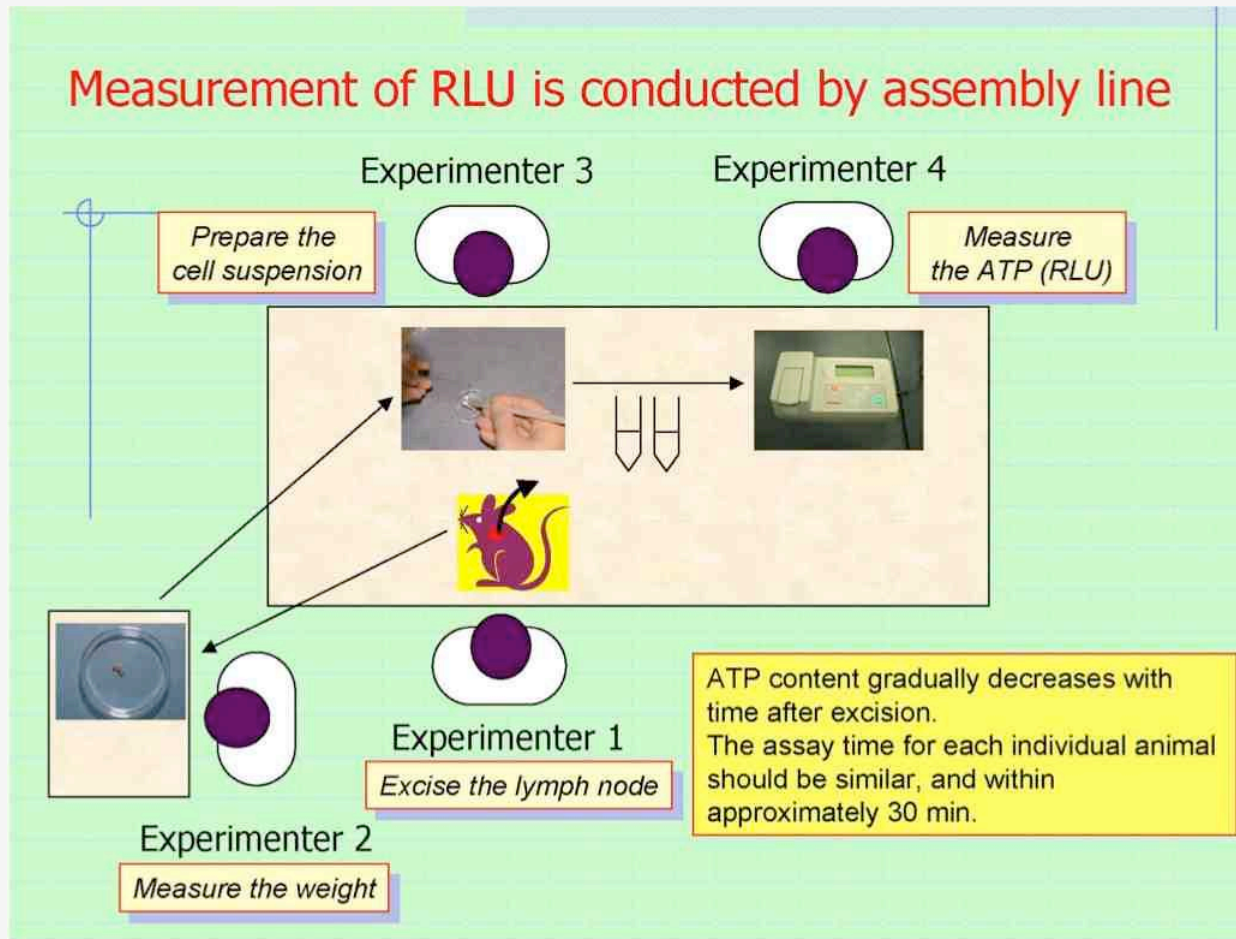
- Technical points:



Schematic courtesy of Dr. Kenji Idehara, Daicel Chemical Industries, Ltd.

LLNA: DA Test Method Protocol - 4

- Technical points:



Schematic courtesy of Dr. Kenji Idehara, Daicel Chemical Industries, Ltd.

LLNA: DA Test Method Protocol – ICCVAM Recommendations - 1

- $SI \geq 1.8$ decision criterion
- Minimum of four animals per group
- Individual animal data
 - Allows for statistical analysis for detection of outliers and comparison to vehicle control group
- Concurrent vehicle control
 - Used as the baseline to determine any increase in lymphocyte proliferation of treated animals
- Concurrent positive control
 - Demonstrates that the assay as conducted is capable of producing a positive response
 - Required by U.S. agencies
 - Absence of a concurrent positive control could result in a requirement to repeat negative results



LLNA: DA Test Method Protocol – ICCVAM

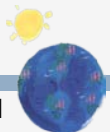
Recommendations - 2

- Dose selection with adequate scientific rationale
 - Select 3 consecutive doses from an appropriate concentration series (100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc.)
 - Consider existing toxicological information (acute toxicity, dermal irritation)
 - Consider structural and physicochemical information on test material or structurally related test material
 - In absence of such existing information a prescreen test may be necessary
- Reduced LLNA: DA
 - Should be used routinely to determine the allergic contact dermatitis (ACD) hazard potential of chemicals and products
 - If existing information suggests a substance might have ACD hazard potential AND dose-response information is needed, consider testing in the multi-dose LLNA: DA
 - Reduces animal numbers by using only the high dose group
 - Maximum concentration that doesn't induce overt systemic toxicity and/or excessive local skin irritation
 - Adhere to all other LLNA: DA protocol specifications



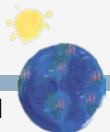
Advantages of Using the LLNA: DA

- Less hazardous, no radioisotopes
 - Makes LLNA available to laboratories where radioactivity is discouraged/prohibited
 - Refinement: avoids potential pain and distress associated with a positive response in guinea pig tests
 - Refinement: no injection of labeling agent required
 - Avoids costs associated with radioactive waste
- Reduction vs. guinea pig tests
 - Four mice per dose group
- Convenient and rapid assay
 - Commercially available reagent kits
- Training and time considerations are similar to LLNA



NICEATM-ICCVAM Evaluation of LLNA: DA

- Reviewed available data and information regarding the usefulness and limitations to assess the ACD hazard potential of chemicals and products
- Determined validation status
 - Accuracy: sensitivity and specificity
 - Reproducibility for identifying LLNA sensitizers and nonsensitizers
 - Scope of substances tested
 - Availability of a standardized test method protocol
- Independent international scientific peer review panel

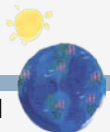


NICEATM-ICCVAM Evaluation of LLNA: DA – Validation Database - 1

- 46 total substances; 44 with comparative traditional LLNA data
- Intralaboratory data:
 - Individual animal data from Daicel Chemical Industries, Ltd. for 45 substances
 - Idehara et al. 2008¹: 31 substances
 - Idehara unpublished: 14 substances
- Interlaboratory data:
 - Individual animal data from a two-phased interlaboratory validation study for 14 substances²
 - One of the 14 substance not previously among the 45 substances from Daicel

¹Idehara et al. 2008. J Pharmacol Toxicol Methods. 58:1-10.

²Omori et al. 2008. J Pharmacol Toxicol Methods. 58:11-26.



NICEATM-ICCVAM Evaluation of LLNA: DA – Validation Database - 2

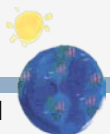
- Test Method Reference Substances (n = 44)
 - Includes 18/18 required (and 3/4 optional) LLNA performance standards reference substances

Traditional LLNA Sensitizers (n = 32)			Traditional LLNA Nonsensitizers (n = 12)
Abietic acid	Diethyl maleate	2-Mercaptobenzothiazole	1-Bromobutane
3-Aminophenol	Ethyl acrylate	Methyl methacrylate	Chlorobenzene
Benzalkonium chloride	Ethylene glycol dimethacrylate	Nickel (II) sulfate hexahydrate	Diethyl phthalate
p-Benzoquinone	Eugenol	Phenyl benzoate	Dimethyl isophthalate
Butyl glycidyl ether	Formaldehyde	p-Phenylenediamine	Hexane
5-Chloro-2-methyl-4-isothiazolin-3-one	Glutaraldehyde	Phthalic anhydride	Isopropanol
Cinnamic alcohol	Hexyl cinnamic aldehyde	Potassium dichromate	Lactic acid
Cinnamic aldehyde	Hydroxycitronellal	Propyl gallate	Methyl salicylate
Citral	Imidazolidinyl urea	Resorcinol	Nickel (II) chloride
Cobalt chloride	Isoeugenol	Sodium lauryl sulfate	Propylparaben
2,4-Dinitrochlorobenzene		Trimellitic anhydride	Salicylic acid
			Sulfanilamide

Abbreviations: n = number of substances.

Bold type = LLNA performance standards reference substance.

Benzocaine and toluene 2,4-diisocyanate have also been tested in the LLNA: DA but sufficient LLNA data for comparison were not available and are not included in the table.

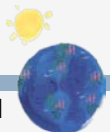


NICEATM-ICCVAM Evaluation of LLNA: DA – Test Method Accuracy - 1

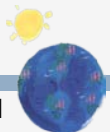
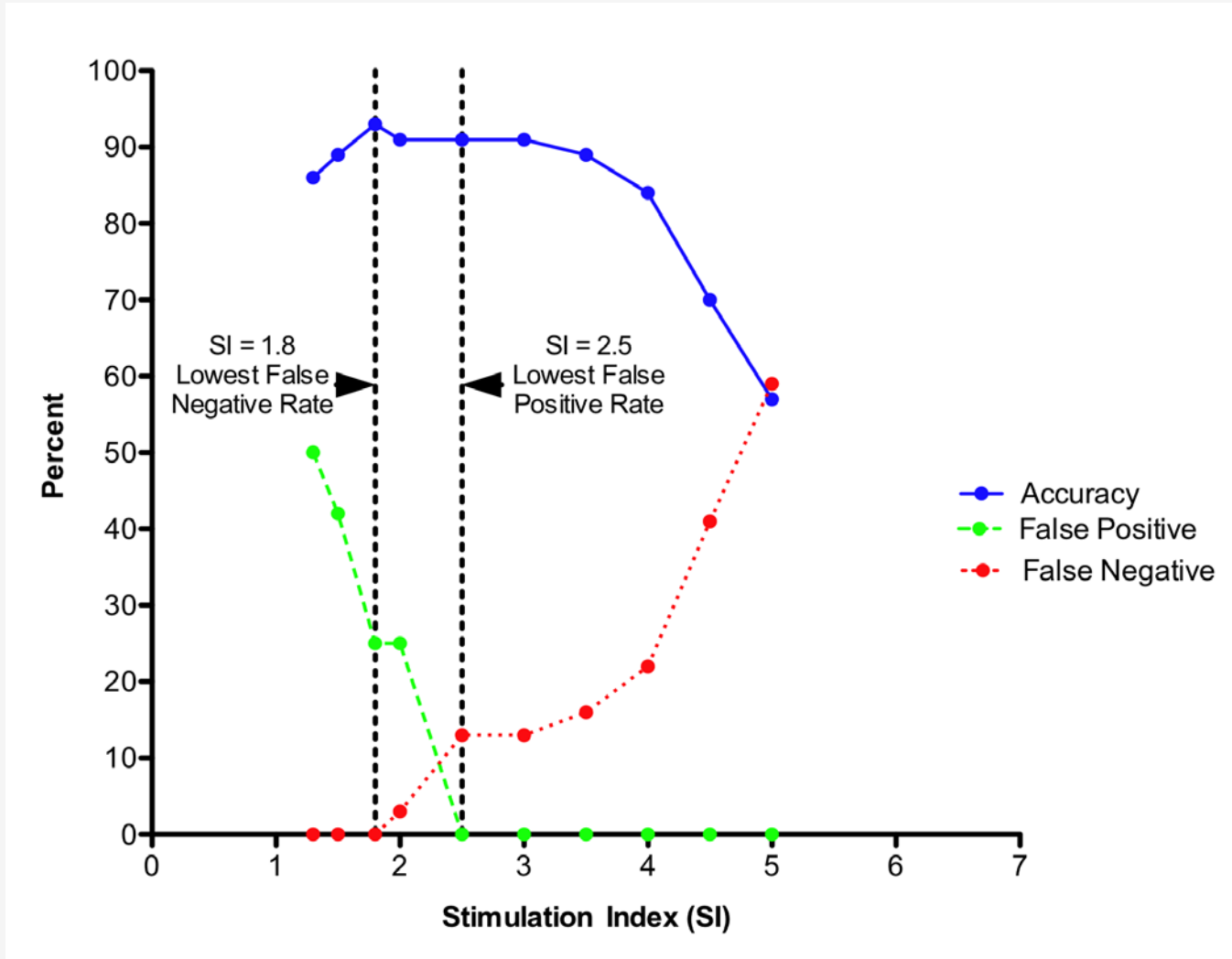
■ LLNA: DA vs. LLNA (n = 44)

SI Decision Criterion	n	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate	Positive Predictivity	Negative Predictivity
≥3.0	44	91% (40/44)	88% (28/32)	100% (12/12)	0% (0/12)	13% (4/32)	100% (28/28)	75% (12/16)
≥2.5	44	91% (40/44)	88% (28/32)	100% (12/12)	0% (0/12)	13% (4/32)	100% (28/28)	75% (12/16)
≥2.0	44	91% (40/44)	97% (31/32)	75% (9/12)	25% (3/12)	3% (1/32)	91% (31/34)	90% (9/10)
≥1.8	44	93% (41/44)	100% (32/32)	75% (9/12)	25% (3/12)	0% (0/32)	91% (32/35)	100% (9/9)

Abbreviations: n = number of substances; SI = stimulation index



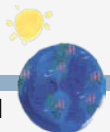
NICEATM-ICCVAM Evaluation of LLNA: DA - Test Method Accuracy - 2



NICEATM-ICCVAM Evaluation of LLNA: DA – Intralaboratory Reproducibility

- Individual animal data for two substances tested at varying concentrations, in three different experiments in one laboratory¹
 - Isoeugenol
 - Repeat positive results ($SI \geq 1.8$) obtained for all three experiments
 - Eugenol
 - Repeat positive results ($SI \geq 1.8$) obtained for all three experiments
 - Isoeugenol and eugenol are sensitizers in the LLNA

¹Idehara et al. 2008. J Pharmacol Toxicol Methods. 58:1-10.



NICEATM-ICCVAM Evaluation of LLNA: DA – Interlaboratory Reproducibility - 1

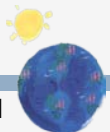
- Individual animal data from a two-phased interlaboratory validation study¹
 - First phase: 10 laboratories, 12 coded substances
 - 3 substances tested in all 10 laboratories
 - 9 substances randomly assigned to subsets of 3/10 laboratories
 - Each substance tested once in each laboratory at 3 doses
 - Second phase: 7 laboratories, 5 coded substances (2/5 substances unique to second phase)
 - 1 substance tested in all 7 laboratories
 - 4 substances randomly assigned to subsets of 4/7 laboratories
 - Each substance tested once in each laboratory at 3 doses

¹Omori et al. 2008. J Pharmacol Toxicol Methods. 58:11-26.

NICEATM-ICCVAM Evaluation of LLNA: DA – Interlaboratory Reproducibility - 2

- Individual animal data from a two-phased interlaboratory validation study¹ (continued)
 - Combined phases: 17 laboratories, 14 different coded substances
 - 10 LLNA sensitizers and 4 LLNA nonsensitizers
 - Concordant results for 11/14 substances among all the laboratories tested and same result as LLNA
 - Discordant results for 3/14 substances among some of the laboratories tested
 - One LLNA nonsensitizer tested positive in the LLNA: DA ($SI \geq 1.8$) in 1/10 laboratories
 - Two LLNA sensitizers tested negative in the LLNA: DA ($SI < 1.8$) in 1 to 4 laboratories

¹Omori et al. 2008. J Pharmacol Toxicol Methods. 58:11-26.



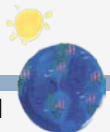
ICCVAM Test Method Recommendations for LLNA: DA – Usefulness and Limitations

Usefulness

- Can be used to identify potential skin sensitizers or nonsensitizers
 - Use $SI \geq 1.8$ to identify potential sensitizers
 - Produced no false negatives, relative to traditional LLNA

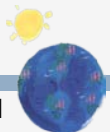
Limitations

- Borderline weak positives ($1.8 < SI < 2.5$) have a slight potential to be false positives
 - If not consistent with predicted ACD hazard potential, consider all other available information
 - Nature of dose response
 - Evidence of systemic toxicity and/or excessive local skin irritation
 - Where appropriate, statistical significance
 - Structural relationship to known skin sensitizers
- Should consider if test material might be a potent ATP inhibitor or ATP degrading enzyme



LLNA: DA International Acceptance

- OECD TG 442A Skin Sensitization: Local Lymph Node Assay: DA
 - Adopted July 22, 2010
 - Available at http://www.oecd-ilibrary.org/environment/test-no-442a-skin-sensitization_9789264090972-en
 - Based on ICCVAM-recommended LLNA: DA protocol
 - Expected to result in broader use of LLNA tests, which will further reduce and refine animal use for ACD hazard assessments on a global basis, while ensuring human safety



Poster Available for Viewing

- See poster at this workshop (Room C1/C2):

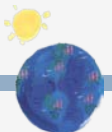
ICCVAM Evaluation and International Acceptance of the Nonradioactive LLNA: DA Test Method

J Matheson¹, A Jacobs², P Brown², R Ward³, E Margosches³, E Salicru⁴, D Allen⁴, F Stack⁴, W Stokes⁵

¹U.S. CPSC, Bethesda, MD; ²U.S. FDA, Silver Spring, MD;

³U.S. EPA, Washington, DC; ⁴ILS, Inc., Contractor Supporting NICEATM, RTP, NC;

⁵NICEATM/NTP/NIEHS/NIH/DHHS, RTP, NC



Acknowledgements

- ICCVAM and NICEATM gratefully acknowledge the following individuals and institutions for submitting data to NICEATM for the LLNA: DA evaluation

Kenji Idehara, Ph.D.

Daicel Chemical Industries, Ltd.
Hyogo, Japan

Takashi Omori, Ph.D.

Kyoto University School of Public
Health
Kyoto, Japan



Additional Acknowledgements

- ICCVAM
- ICCVAM Interagency Immunotoxicity Working Group
- ICCVAM Independent Scientific Peer Review Panel
- NICEATM Staff