NICEATM — ***National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods***

ICCVAM — ***Interagency Coordinating Committee on the Validation of Alternative Methods***

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

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

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National Institutes of Health
Bethesda, MD

National Toxicology Program

NIEHS

National Institutes of Health

Department of Health & Human Services

Overview of the LLNA: DA

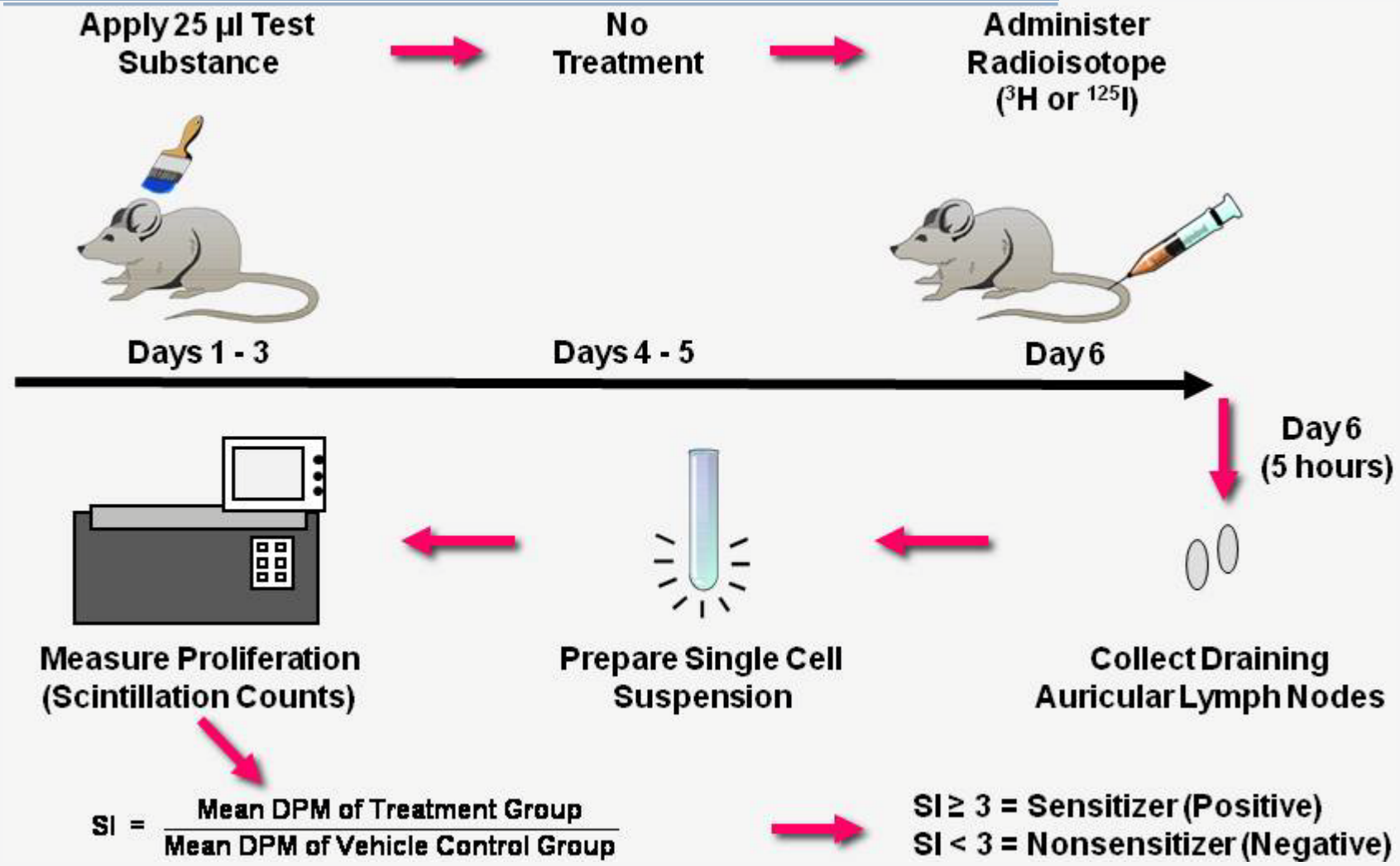
- Developed by Daicel Chemical Industries, Ltd., as a nonradioactive LLNA[1, 2]
- Measures ATP content in draining auricular lymph nodes as an estimate of cell number at the end of cell proliferation
  - ATP content is favorably correlated with living cell number
  - ATP content is assessed by a luciferin-luciferase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

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

    - ATP bioluminescence shown to have a strong correlation between thymidine uptake in a variety of cell lines[3]

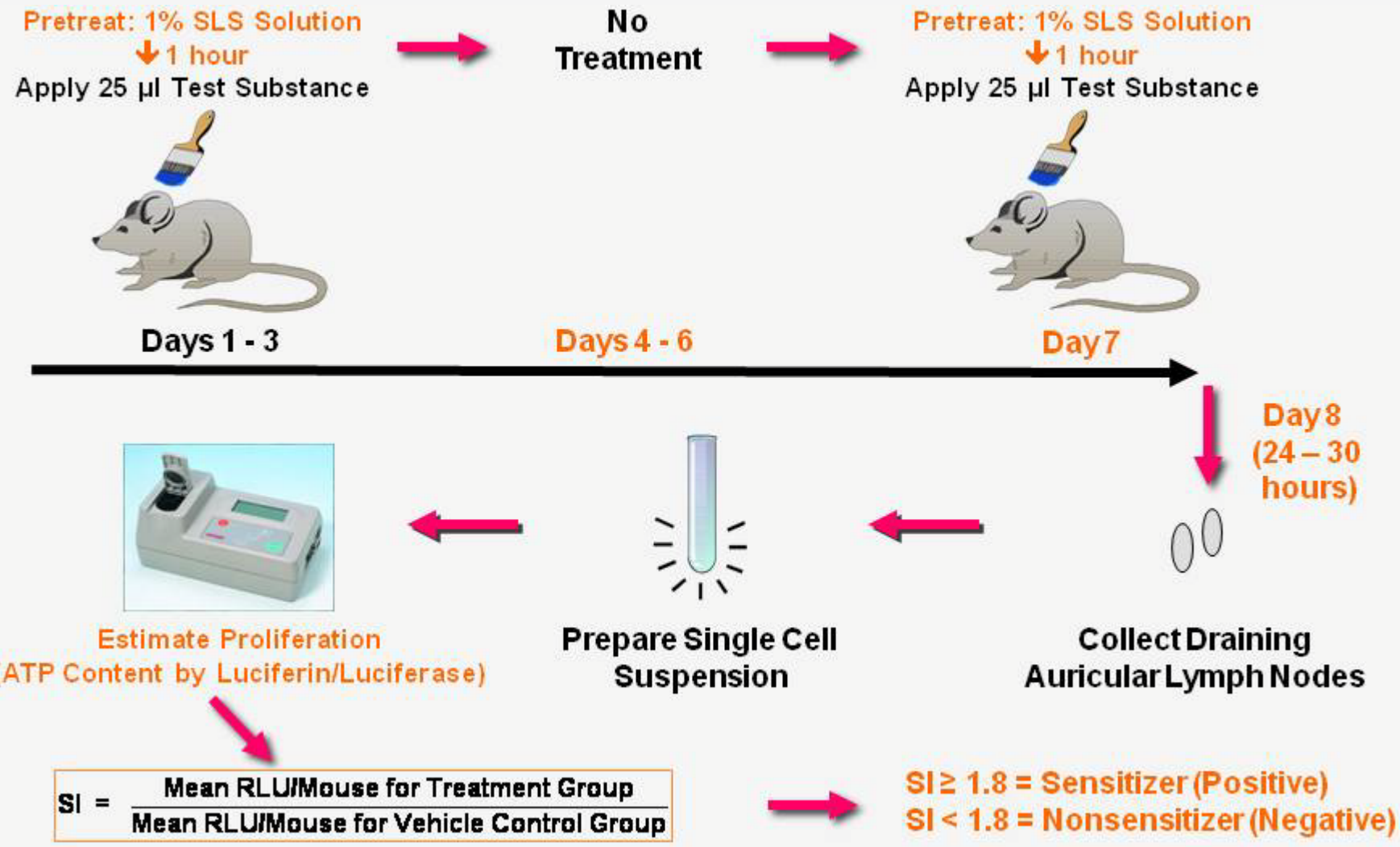
[1]Yamashita et al. 2005. AATEX. 11:136-144.
[2]Idehara et al. 2008. J Pharmacol Toxicol Methods. 58:1-10.
[3]Crouch et al. 1993. J Immunol Methods. 160:81-88.

LLNA Test Method Protocol



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

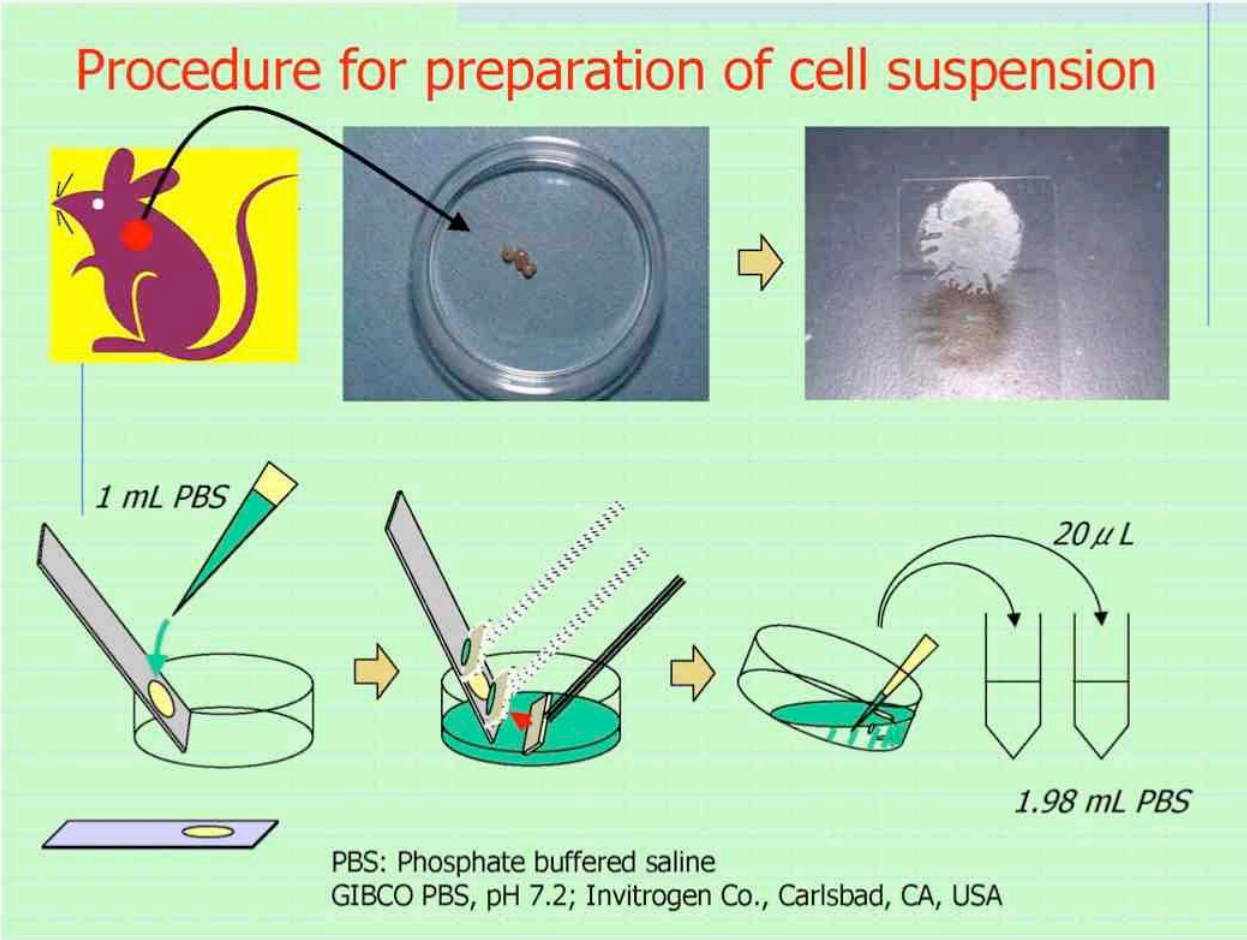
LLNA: DA Test Method Protocol - 1



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

LLNA: DA Test Method Protocol - 2

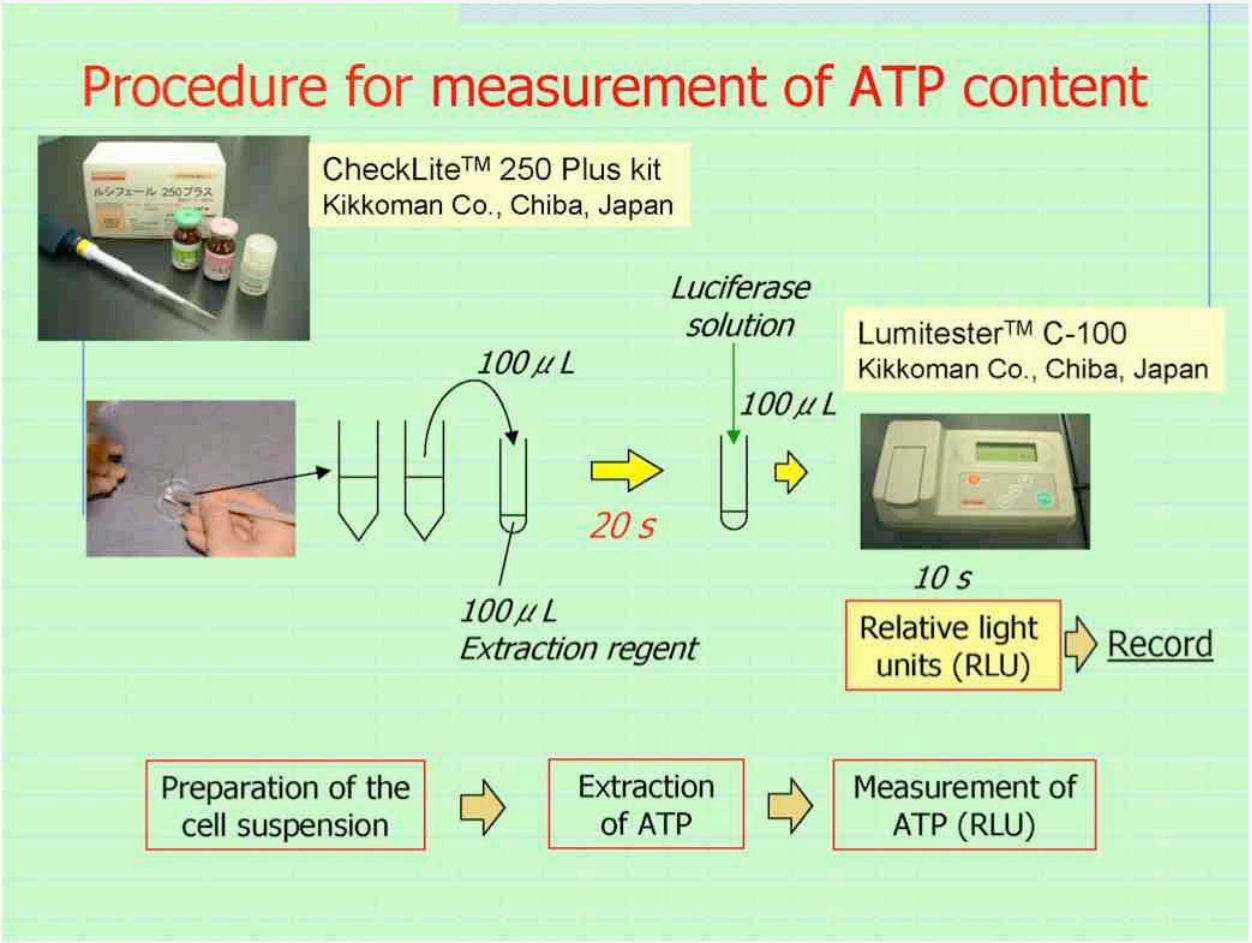
- Technical points:



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

LLNA: DA Test Method Protocol - 3

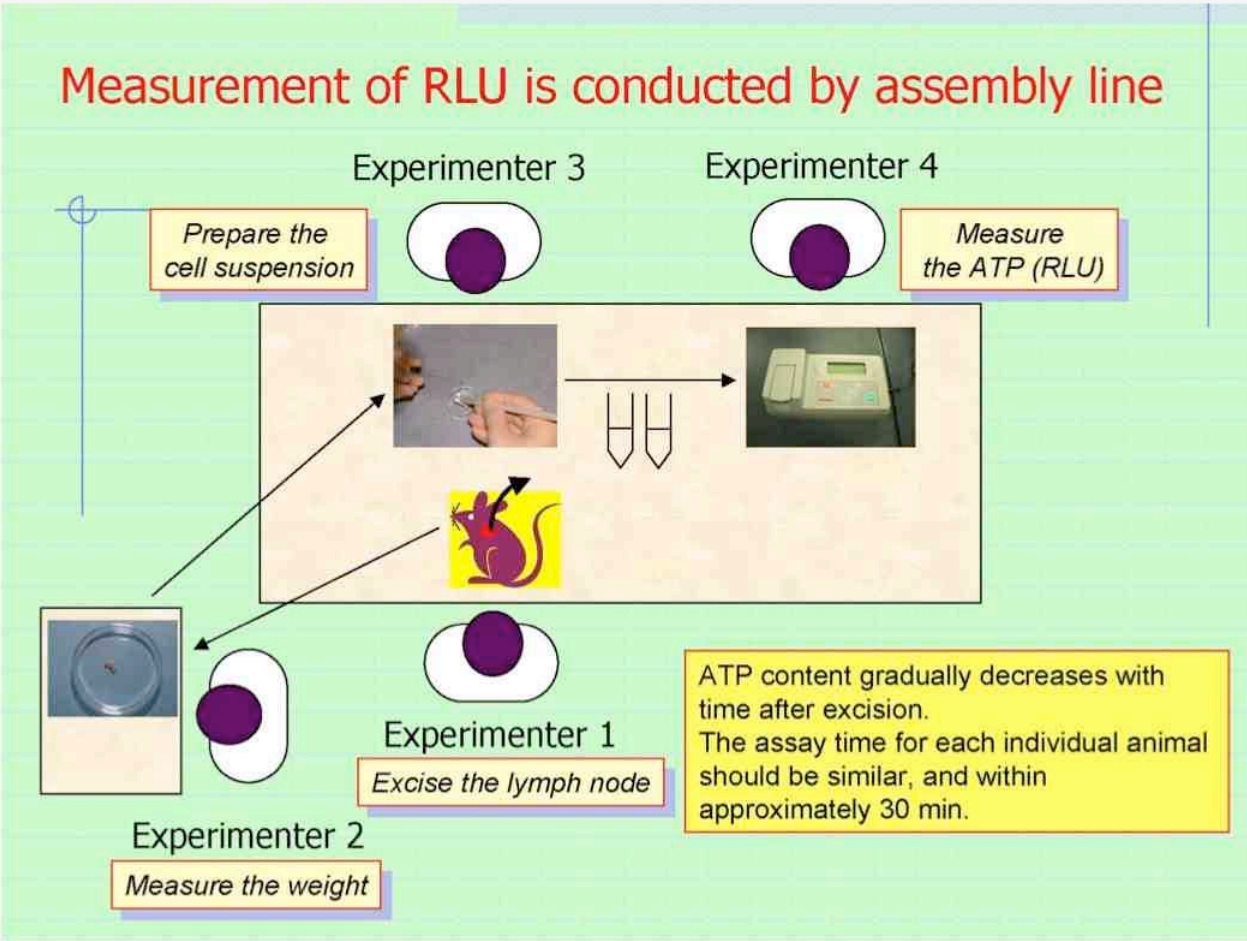
- Technical points:



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 ≥ 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. 2008[1]: 31 substances
    - Idehara unpublished: 14 substances
- Interlaboratory data:
  - Individual animal data from a two-phased interlaboratory validation study for 14 substances[2]
    - One of the 14 substance not previously among the 45 substances from Daicel

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

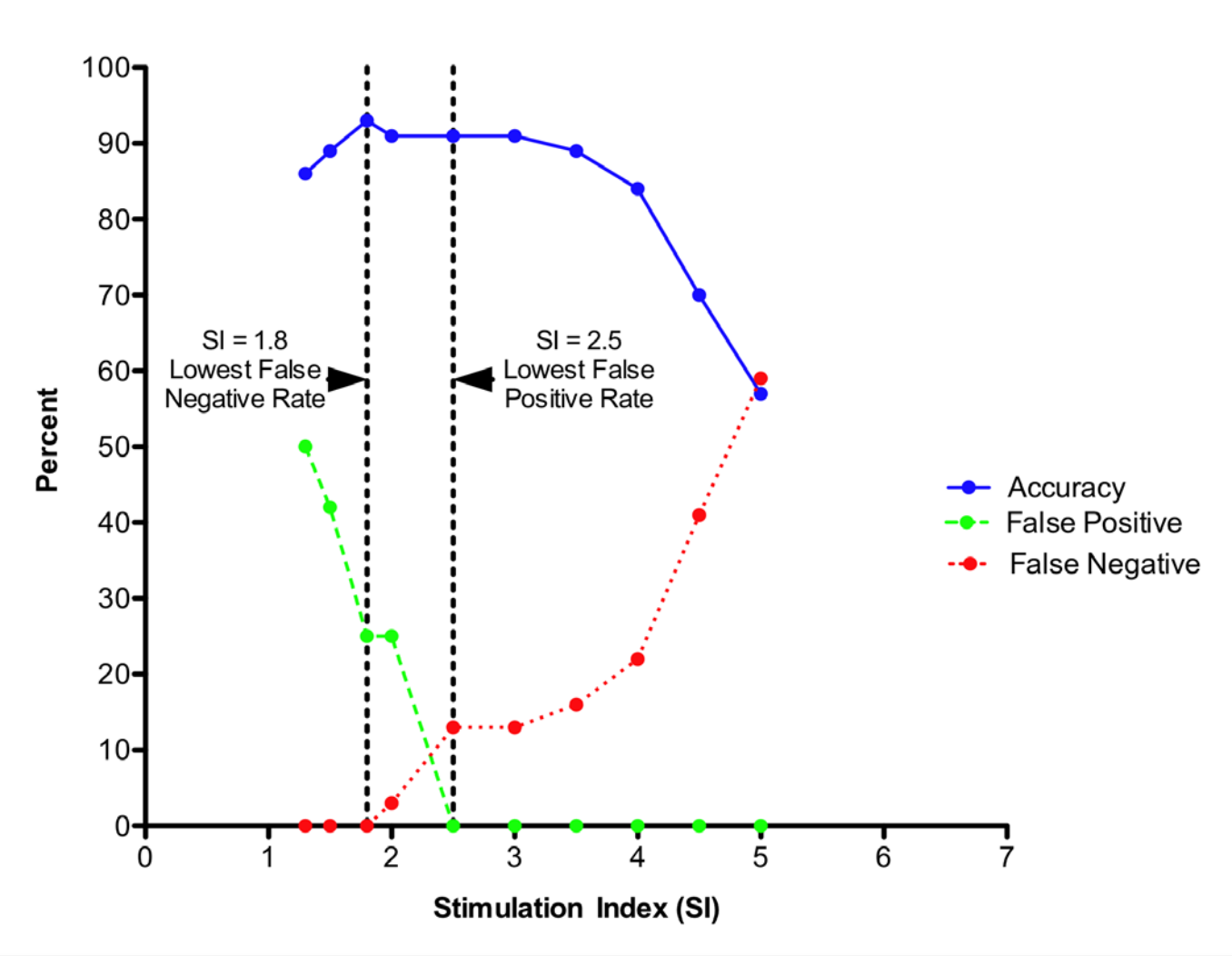
NICEATM-ICCVAM Evaluation of LLNA: DA – Test Method Accuracy - 1

- 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 laboratory[1]
  - Isoeugenol
    - Repeat positive results (SI ≥ 1.8) obtained for all three experiments
  - Eugenol
    - Repeat positive results (SI ≥ 1.8) obtained for all three experiments
  - Isoeugenol and eugenol are sensitizers in the LLNA

[1]Idehara 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 study[1]
  - 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

[1]Omori 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 study[1] (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 ≥ 1.8) in 1/10 laboratories
      - Two LLNA sensitizers tested negative in the LLNA: DA (SI < 1.8) in 1 to 4 laboratories

[1]Omori 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 ≥ 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 Matheson[1], A Jacobs[2], P Brown[2], R Ward[3], E Margosches[3], E Salicru[4], D Allen[4], F Stack[4], W Stokes[5]

  [1]U.S. CPSC, Bethesda, MD; [2]U.S. FDA, Silver Spring, MD; [3]U.S. EPA, Washington, DC; [4]ILS, Inc., Contractor Supporting NICEATM, RTP, NC; [5]NICEATM/NTP/NIEHS/NIH/DHHS, RTP, NC

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- NICEATM Staff