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

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Overview of the LLNA: DA

- Developed by Daicel Chemical Industries, Ltd., as a nonradioactive LLNA\textsuperscript{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])

\[
\text{ATP} + \text{Luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + P_{\text{Pi}} + \text{CO}_2 + \text{Light}
\]

- ATP bioluminescence shown to have a strong correlation between thymidine uptake in a variety of cell lines\textsuperscript{3}

\textsuperscript{1}Yamashita et al. 2005. AATEX. 11:136-144.
LLNA Test Method Protocol

Apply 25 μl Test Substance

No Treatment

Administer Radioisotope (³H or ¹²⁵I)

Days 1 - 3

Days 4 - 5

Day 6

Day 6 (5 hours)

Measure Proliferation (Scintillation Counts)

Prepare Single Cell Suspension

Collect Draining Auricular Lymph Nodes

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

\[ SI = \frac{\text{Mean DPM of Treatment Group}}{\text{Mean DPM of Vehicle Control Group}} \]

SI ≥ 3 = Sensitizer (Positive)
SI < 3 = Nonsensitizer (Negative)
LLNA: DA Test Method Protocol - 1

Pretreat: 1% SLS Solution ↓ 1 hour
Apply 25 μl Test Substance

Days 1 - 3

No Treatment

Days 4 - 6

Pretreat: 1% SLS Solution ↓ 1 hour
Apply 25 μl Test Substance

Day 7

Day 8 (24 – 30 hours)

Estimate Proliferation (ATP Content by Luciferin/Luciferase)

Prepare Single Cell Suspension

Collect Draining Auricular Lymph Nodes

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

SI = Mean RLU/Mouse for Treatment Group
Mean RLU/Mouse for Vehicle Control Group

SI ≥ 1.8 = Sensitizer (Positive)
SI < 1.8 = Nonsensitizer (Negative)
LLNA: DA Test Method Protocol - 2

- Technical points:

Procedure for preparation of cell suspension

1 mL PBS

PBS: Phosphate buffered saline
GIBCO PBS, pH 7.2; Invitrogen Co., Carlsbad, CA, USA

20 μL

1.98 mL PBS

Schematic courtesy of Dr. Kenji Idehara, Daicel Chemical Industries, Ltd.
LLNA: DA Test Method Protocol - 3

Technical points:

Procedure for measurement of ATP content

CheckLite™ 250 Plus kit
Kikkoman Co., Chiba, Japan

Luciferase solution

Lumitester™ C-100
Kikkoman Co., Chiba, Japan

100 μL
Extraction regent

20 s

100 μL

Relative light units (RLU)

Record

Preparation of the cell suspension

Extraction of ATP

Measurement of ATP (RLU)

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

- Measurement of RLU is conducted by assembly line.

- Experimenter 1: Excise the lymph node
  - ATP content gradually decreases with time after excision. The assay time for each individual animal should be similar, and within approximately 30 min.

- Experimenter 2: Measure the weight

- Experimenter 3: Prepare the cell suspension

- Experimenter 4: Measure the ATP (RLU)

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

- **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

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

<table>
<thead>
<tr>
<th>Traditional LLNA Sensitizers (n = 32)</th>
<th>Traditional LLNA Nonsensitizers (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abietic acid</td>
<td>Diethyl maleate</td>
</tr>
<tr>
<td>3-Aminophenol</td>
<td>Ethyl acrylate</td>
</tr>
<tr>
<td>Benzalkonium chloride</td>
<td>Ethylene glycol</td>
</tr>
<tr>
<td>p-Benzquinone</td>
<td>dimethacrylate</td>
</tr>
<tr>
<td>Butyl glycidyl ether</td>
<td>Eugenol</td>
</tr>
<tr>
<td>5-Chloro-2-methyl-4-isothiazolin-3-one</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Cinnamic alcohol</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>Cinnamic aldehyde</td>
<td>Hexyl cinnamic aldehyde</td>
</tr>
<tr>
<td>Citral</td>
<td>Hydroxycitronellal</td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>Imidazolidinyl urea</td>
</tr>
<tr>
<td>2,4-Dinitrochlorobenzene</td>
<td>Isoeugenol</td>
</tr>
</tbody>
</table>

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.
### LLNA: DA vs. LLNA (n = 44)

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

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
  
  - 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

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

Individual animal data from a two-phased interlaboratory validation study\textsuperscript{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 \(\geq 1.8\)) in 1/10 laboratories
    - Two LLNA sensitizers tested negative in the LLNA: DA (SI < 1.8) in 1 to 4 laboratories

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
  - 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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