The Murine Local Lymph Node Assay: 5-Bromo-2-deoxyuridine Enzyme-linked Immunosorbent Assay (LLNA: BrdU-ELISA)

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Bethesda, MD
Overview of the LLNA: BrdU-ELISA

- Developed by Takeyoshi et al. as a nonradioactive LLNA\(^1\)
- Measures BrdU incorporation in draining auricular lymph nodes as a measure of lymph node cell proliferation
  - BrdU is a thymidine analog, incorporated into proliferating cell DNA in a manner similar to radioisotope incorporation in the LLNA
  - ELISA detects peroxidase-labeled BrdU antibody
    - Colorimetric reaction measured with a plate reader

LLNA Test Method Protocol

Abbreviations: DPM = disintegrations per minute; SI = stimulation index
Abbreviations: BrdU = 5-bromo-2-deoxyuridine; ELISA = enzyme-linked immunosorbent assay; SI = stimulation index
LLNA: BrdU-ELISA Test Method Protocol - 2

- Lymphocyte suspension
  - Crush lymph nodes
  - Pass through #70 nylon mesh
  - Suspend lymph node cells in 15 mL physiological saline
  - (return to webpage to view Cell Suspension video clip)

Video courtesy of Dr. Takeyoshi – CERI, Japan
Sample Processing
- 100 μl cell suspension added to flat-bottom microplate wells
- Centrifuge (300 x g, 10 minutes)
- Remove supernatants and dry
- *(return to webpage to view Supernatant Removal video clip)*
LLNA: BrdU-ELISA Test Method Protocol – ICCVAM Recommendations - 1

- SI ≥ 1.6 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: BrdU-ELISA
- 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: BrdU-ELISA
- 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: BrdU-ELISA protocol specifications
Advantages of Using the LLNA: BrdU-ELISA

- 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
  - 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: BrdU-ELISA

- 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
47 total substances; 43 with comparative traditional LLNA data

Intralaboratory data
- Individual animal data for 12 substances tested 2 to 6 times in one laboratory1-7

Interlaboratory data
- Individual animal data from Phase II of the Japanese Society for Alternative Animal Experiments (JSAAE) validation study8
  • 10 coded substances tested in three to seven laboratories
    ■ 4/10 substances not previously tested in the LLNA: BrdU-ELISA

5Takeyoshi et al. 2007. 6th World Congress Presentation; 6Takeyoshi et al. 2008. J Appl Toxicol 28:530-534;
NICEATM-ICCVAM Evaluation of LLNA: BrdU-ELISA – Validation Database - 2

- **Test method reference substances (n = 43)**
  - Includes 16/18 required (and 2/4 optional) LLNA performance standards reference substances

<table>
<thead>
<tr>
<th>Traditional LLNA Sensitizers (n = 32)</th>
<th>Traditional LLNA Nonsensitizers (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Aminophenol</td>
<td>Diethyl phthalate</td>
</tr>
<tr>
<td>Aniline</td>
<td>Dimethyl isophthalate</td>
</tr>
<tr>
<td>Benzoquinone</td>
<td>Glycerol</td>
</tr>
<tr>
<td>4-Chloroaniline</td>
<td>Hexane</td>
</tr>
<tr>
<td>5-Chloro-2-methyl-4-isothiazolin-3-one</td>
<td>2-Hydroxypropyl methacrylate</td>
</tr>
<tr>
<td>Cinnamic alcohol</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>Cinnamic aldehyde</td>
<td>Lactic acid</td>
</tr>
<tr>
<td>trans-Cinnamic aldehyde</td>
<td>Methyl salicylate</td>
</tr>
<tr>
<td>Citral</td>
<td>Propylene glycol</td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>Cyclamen aldehyde</td>
<td>Sulfanilamide</td>
</tr>
<tr>
<td>Diethyl maleate</td>
<td></td>
</tr>
<tr>
<td>2,4-Dinitrochlorobenzene</td>
<td></td>
</tr>
<tr>
<td>Diphenylcyclopropenone</td>
<td></td>
</tr>
<tr>
<td>Ethyl acrylate</td>
<td></td>
</tr>
<tr>
<td>Ethylene glycol dimethacrylate</td>
<td></td>
</tr>
<tr>
<td>Eugenol</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td></td>
</tr>
<tr>
<td>Hexyl cinnamic aldehyde</td>
<td></td>
</tr>
<tr>
<td>Hydroxycitronellal</td>
<td></td>
</tr>
<tr>
<td>Imidazolidinyl urea</td>
<td></td>
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<tr>
<td>Isoeugenol</td>
<td></td>
</tr>
<tr>
<td>Isopropyl myristate</td>
<td></td>
</tr>
<tr>
<td>Linalool</td>
<td></td>
</tr>
<tr>
<td>2-Mercaptobenzothiazole</td>
<td></td>
</tr>
<tr>
<td>4-Methylaminophenol sulfate</td>
<td></td>
</tr>
<tr>
<td>Nickel sulfate</td>
<td></td>
</tr>
<tr>
<td>Phenyl benzoate</td>
<td></td>
</tr>
<tr>
<td>4-Phenylenediamine</td>
<td></td>
</tr>
<tr>
<td>Sodium lauryl sulfate</td>
<td></td>
</tr>
<tr>
<td>Trimellitic anhydride</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: n = number of substances.
Bold type = LLNA performance standards reference substance.
## NICEATM-ICCVAM Evaluation of LLNA: BrdU-ELISA – Test Method Accuracy - 1

### LLNA: BrdU-ELISA vs. LLNA (n = 43)

<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>≥2.0</td>
<td>43</td>
<td>95%</td>
<td>94%</td>
<td>100%</td>
<td>0%</td>
<td>6%</td>
<td>100%</td>
<td>85%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(41/43)</td>
<td>(30/32)</td>
<td>(11/11)</td>
<td>(0/11)</td>
<td>(2/32)</td>
<td>(30/30)</td>
<td>(11/13)</td>
</tr>
<tr>
<td>≥1.8</td>
<td>43</td>
<td>91%</td>
<td>94%</td>
<td>82%</td>
<td>18%</td>
<td>6%</td>
<td>94%</td>
<td>82%</td>
</tr>
<tr>
<td>≥1.6</td>
<td>43</td>
<td>95%</td>
<td>100%</td>
<td>82%</td>
<td>18%</td>
<td>0%</td>
<td>94%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(41/43)</td>
<td>(32/32)</td>
<td>(9/11)</td>
<td>(2/11)</td>
<td>(0/32)</td>
<td>(32/34)</td>
<td>(9/9)</td>
</tr>
<tr>
<td>≥1.4</td>
<td>43</td>
<td>93%</td>
<td>100%</td>
<td>73%</td>
<td>27%</td>
<td>0%</td>
<td>91%</td>
<td>100%</td>
</tr>
</tbody>
</table>

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

![Graph showing the relationship between Stimulation Index (SI) and accuracy, false positives, and false negatives.]
NICEATM-ICCVAM Evaluation of LLNA: BrdU-ELISA – Intralaboratory Reproducibility

- Individual animal data for 12 substances tested (2-6 times) in one laboratory\(^1\)-\(^7\)
  - 10/12 substances were LLNA sensitizers
    - Repeat positive LLNA: BrdU-ELISA results (SI ≥ 1.6) for 8/10 LLNA sensitizers
    - One positive LLNA: BrdU-ELISA result (SI ≥ 1.6) and one negative LLNA: BrdU-ELISA result (SI < 1.6) for 2/10 LLNA sensitizers
  - 2/12 substances were LLNA nonsensitizers
    - Repeat negative LLNA: BrdU-ELISA results (SI < 1.6) for 1/2 LLNA nonsensitizers
    - Repeat positive LLNA: BrdU-ELISA results (SI ≥ 1.6) for 1/2 LLNA nonsensitizers

NICEATM-ICCVAM Evaluation of LLNA: BrdU-ELISA – Interlaboratory Reproducibility

- Individual animal data from Phase II of the JSAAE validation study
  - 10 coded substances tested in three to seven laboratories
    - 7/10 substances were LLNA sensitizers
    - 3/10 substances were LLNA nonsensitizers
  - Concordant results for 9/10 substances among all the laboratories tested
    - One LLNA nonsensitizer with concordant positive LLNA: BrdU-ELISA results (SI ≥ 1.6)
  - Discordant results for 1/10 substances among 2/7 laboratories tested
    - Substance was an LLNA nonsensitizer
    - LLNA: BrdU-ELISA results were positive (SI ≥ 1.6) in two laboratories and negative (SI < 1.6) in 5 laboratories

ICCVAM Test Method Recommendations for LLNA: BrdU-ELISA – Usefulness and Limitations

**Usefulness**

- Can be used to identify potential skin sensitizers or nonsensitizers
  - Use SI ≥ 1.6 to identify potential sensitizers
  - Produced no false negatives, relative to traditional LLNA

**Limitations**

- Borderline weak positives (1.6 < SI < 1.9) 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

- Same limitations as those associated with LLNA applicability domain except for nickel
LLNA: BrdU-ELISA International Acceptance

- OECD TG 442B Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA
  - Adopted July 22, 2010
  - Based on ICCVAM-recommended LLNA: BrdU-ELISA 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
See poster at this workshop (Room C1/C2):

**ICCVAM Evaluation and International Acceptance of the Nonradioactive LLNA: BrdU-ELISA Test Method**

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<th>Masahiro Takeyoshi, Ph.D.</th>
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<td>Japanese Center for the Validation of Alternative Methods</td>
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<td>Ministry of Health, Labour and Welfare</td>
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- NICEATM Staff