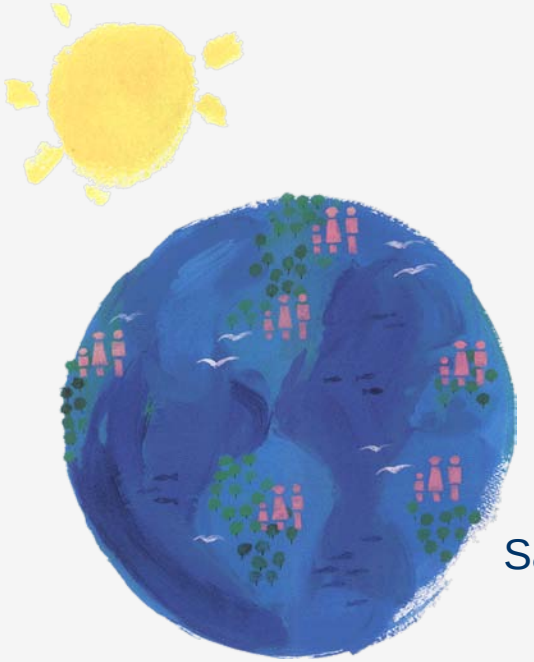
**NICEATM**

***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: 5-Bromo-2-deoxyuridine Enzyme-linked Immunosorbent Assay (LLNA: BrdU-ELISA)

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National Toxicology Program

NIEHS

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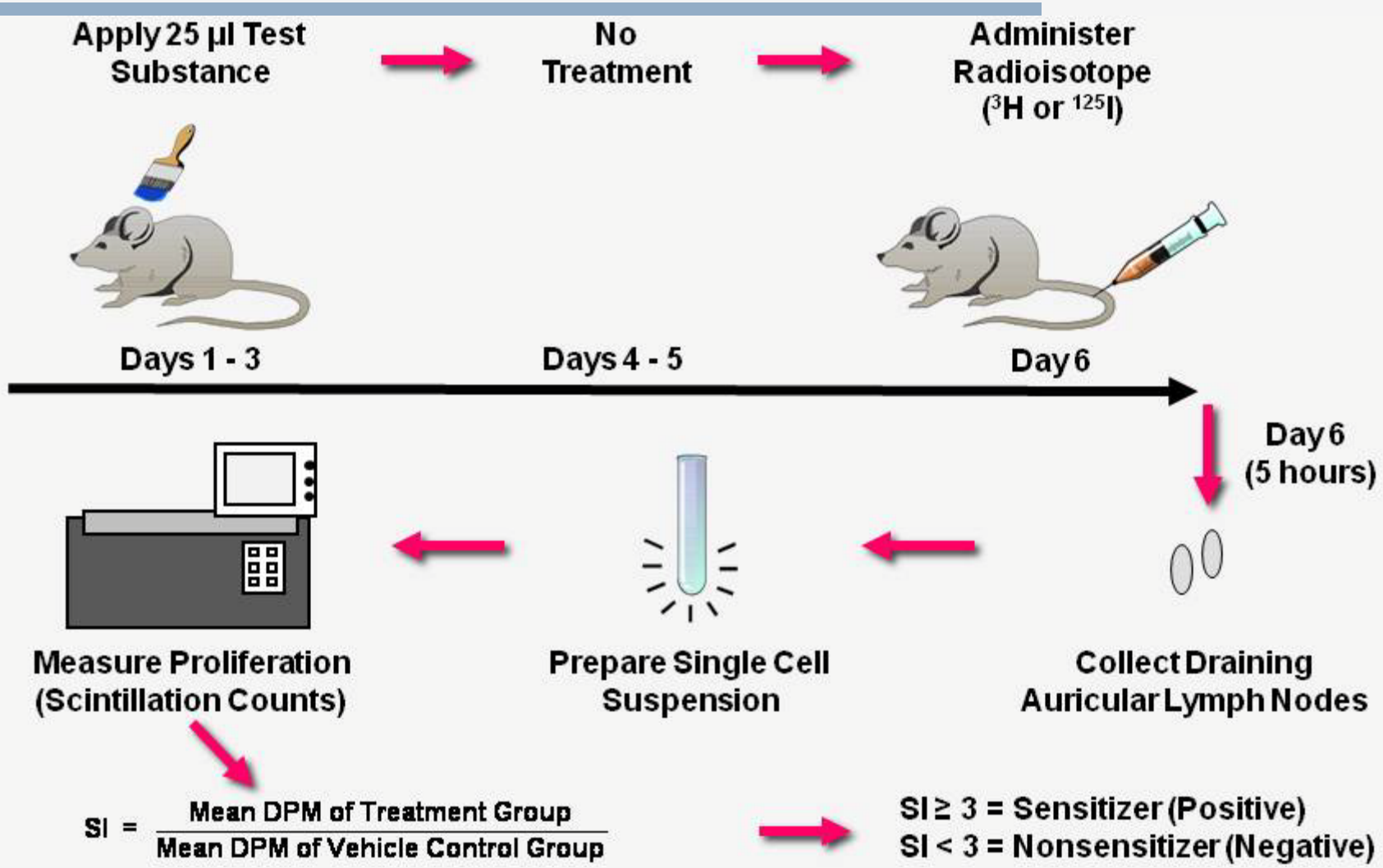
Department of Health & Human Services

# 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

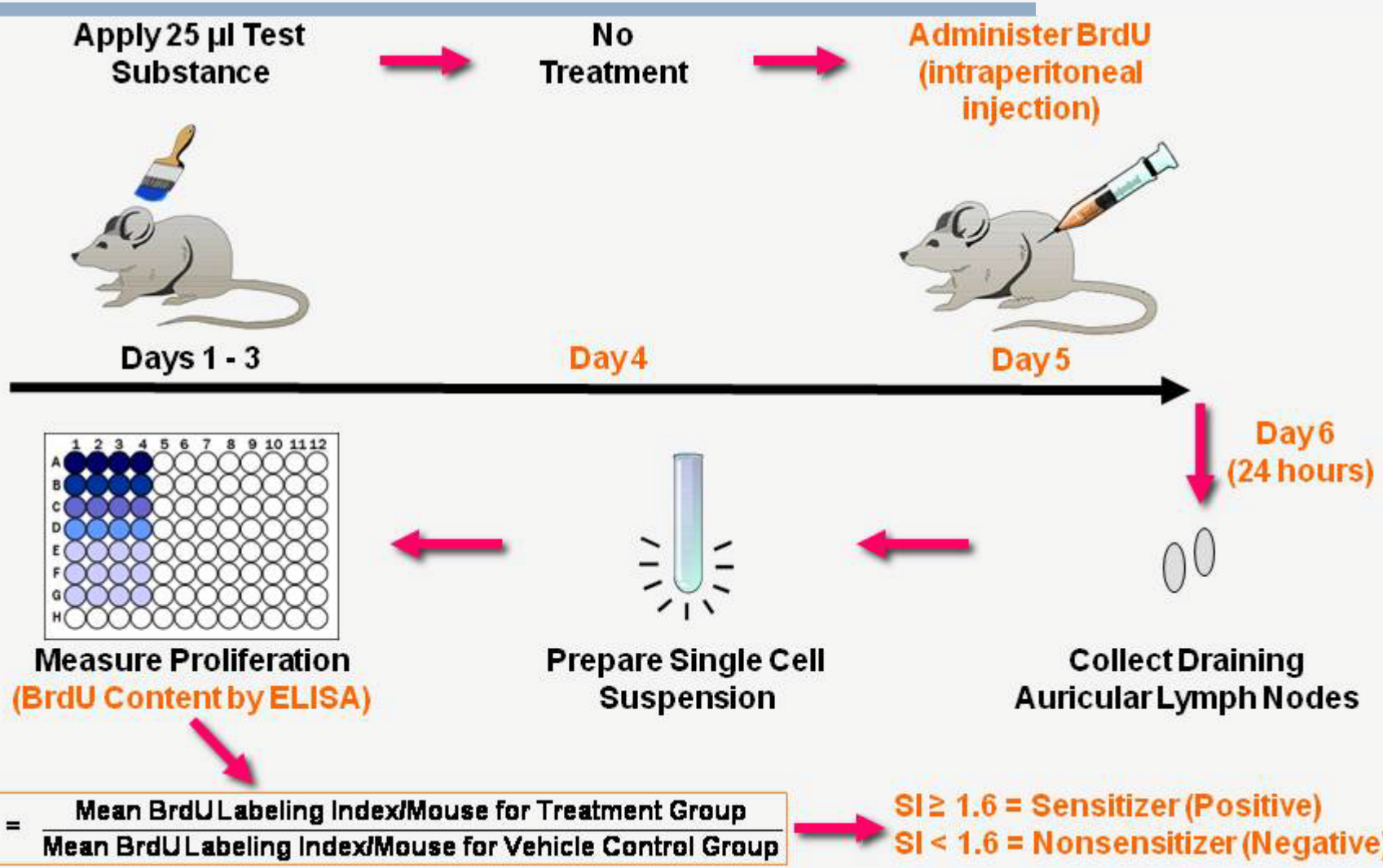
[1]Takeyoshi et al. 2001. Toxicol Lett. 119:203-8.

# LLNA Test Method Protocol




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

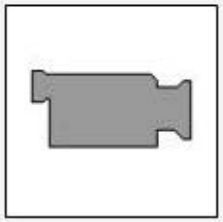
# LLNA: BrdU-ELISA Test Method Protocol - 1




Abbreviations: BrdU = 5-bromo-2-deoxyuridine; ELISA = enzyme-linked immunosorbent assay; SI = stimulation index

# LLNA: BrdU-ELISA Test Method Protocol - 2

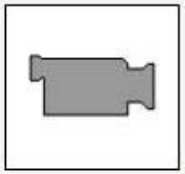
- Lymphocyte suspension
  - Crush lymph nodes
  - Pass through #70 nylon mesh
  - Suspend lymph node cells in 15 mL physiological saline
  - *(return to webpage to view Cell Suspension video clip)*



Video courtesy of Dr. Takeyoshi – CERI, Japan

# LLNA: BrdU-ELISA Test Method Protocol - 3

- 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)*



Video courtesy of Dr. Takeyoshi – CERI, Japan

# 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

# LLNA: BrdU-ELISA 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: 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

# NICEATM-ICCVAM Evaluation of LLNA: BrdU-ELISA – Validation Database - 1

- 47 total substances; 43 with comparative traditional LLNA data
- Intralaboratory data
  - Individual animal data for 12 substances tested 2 to 6 times in one laboratory[1-7]
- Interlaboratory data
  - Individual animal data from Phase II of the Japanese Society for Alternative Animal Experiments (JSAAE) validation study[8]
    - 10 coded substances tested in three to seven laboratories
      - 4/10 substances not previously tested in the LLNA: BrdU-ELISA

[1]Takeyoshi et al. 2003. Toxicology. 191:259-263; [2]Takeyoshi et al. 2004. Exp Anim 53:171-173; [3]Takeyoshi et al. 2005. J Appl Toxicol 25:129-134; [4]Takeyoshi et al. 2006. J Appl Toxicol 26:5-9; [5]Takeyoshi et al. 2007. 6th World Congress Presentation; [6]Takeyoshi et al. 2008. J Appl Toxicol 28:530-534; [7]Takeyoshi et al. unpublished data; [8]Kojima et al. 2011. J Appl Toxicol 31: 63-74

# 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

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

Abbreviations: n = number of substances.
Bold type = LLNA performance standards reference substance.

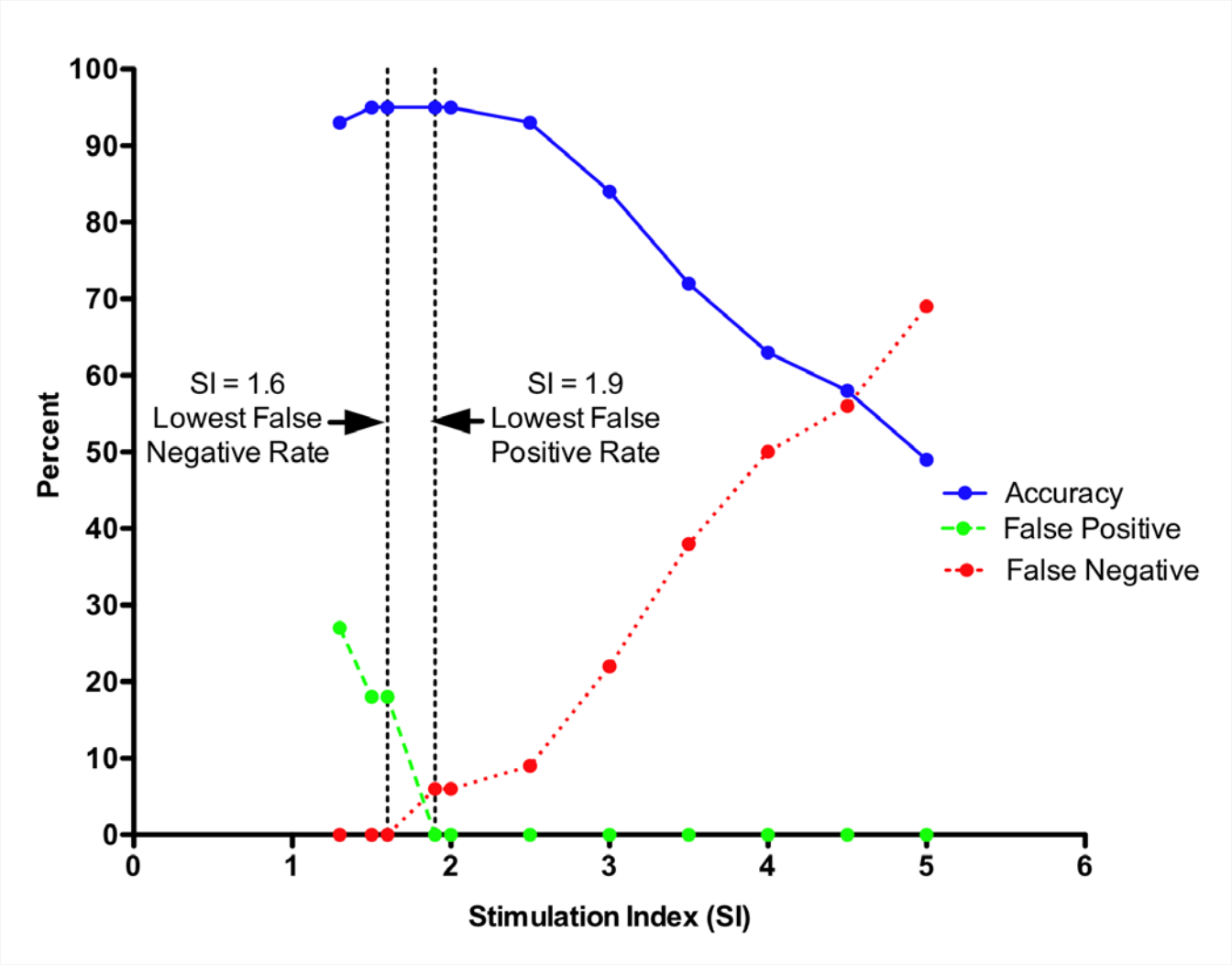
# NICEATM-ICCVAM Evaluation of LLNA: BrdU-ELISA – Test Method Accuracy - 1

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

| SI Decision Criterion | n | Accuracy | Sensitivity | Specificity | False Positive Rate | False Negative Rate | Positive Predictivity | Negative Predictivity |
|---|---|---|---|---|---|---|---|---|
| ≥2.0 | 43 | 95% (41/43) | 94% (30/32) | 100% (11/11) | 0% (0/11) | 6% (2/32) | 100% (30/30) | 85% (11/13) |
| ≥1.8 | 43 | 91% (39/43) | 94% (30/32) | 82% (9/11) | 18% (2/11) | 6% (2/32) | 94% (30/32) | 82% (9/11) |
| **≥1.6** | **43** | **95% (41/43)** | **100% (32/32)** | **82% (9/11)** | **18% (2/11)** | **0% (0/32)** | **94% (32/34)** | **100% (9/9)** |
| ≥1.4 | 43 | 93% (40/43) | 100% (32/32) | 73% (8/11) | 27% (3/11) | 0% (0/32) | 91% (32/35) | 100% (8/8) |

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

# NICEATM-ICCVAM Evaluation of LLNA: BrdU-ELISA – Test Method Accuracy - 2



# 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

[1]Takeyoshi et al. 2003. Toxicology. 191:259-263; [2]Takeyoshi et al. 2004. Exp Anim 53:171-173;
[3]Takeyoshi et al. 2005. J Appl Toxicol 25:129-134; [4]Takeyoshi et al. 2006. J Appl Toxicol 26:5-9;
[5]Takeyoshi et al. 2007. 6th World Congress Presentation; [6]Takeyoshi et al. 2008. J Appl Toxicol 28:530-534;
[7]Takeyoshi et al. unpublished data.

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

- Individual animal data from Phase II of the JSAAE validation study[1]
  - 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

[1]Kojima et al. 2011. J Appl Toxicol 31: 63-74

# 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
  - Available at http://www.oecd-ilibrary.org/environment/test-no-442b-skin-sensitization_9789264090996-en
  - 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

# Poster Available for Viewing

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

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

  A Jacobs[1], J Matheson[2], M Wind[2], V Malshet[1], J Toy[1], J Strickland[3], D Allen[3], T Burns[3], F Stack[3], W Stokes[4]

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

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| | |
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