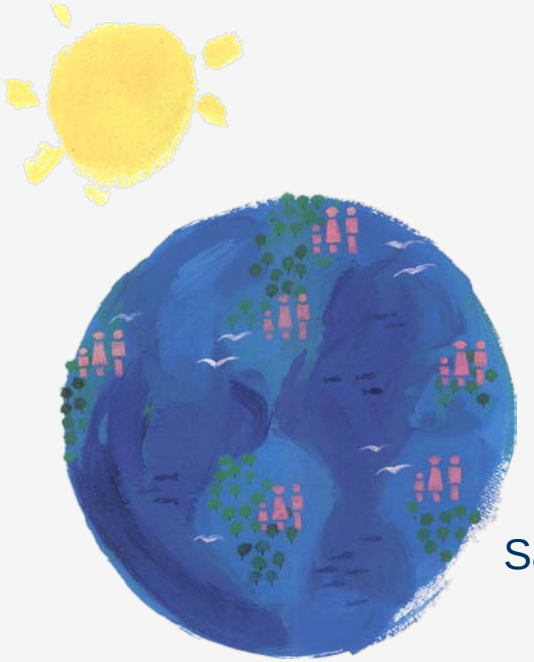
NICEATM

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)

Judy Strickland, Ph.D., DABT
ILS Inc., Contractor Supporting NICEATM

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

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

National Toxicology Program

NIEHS

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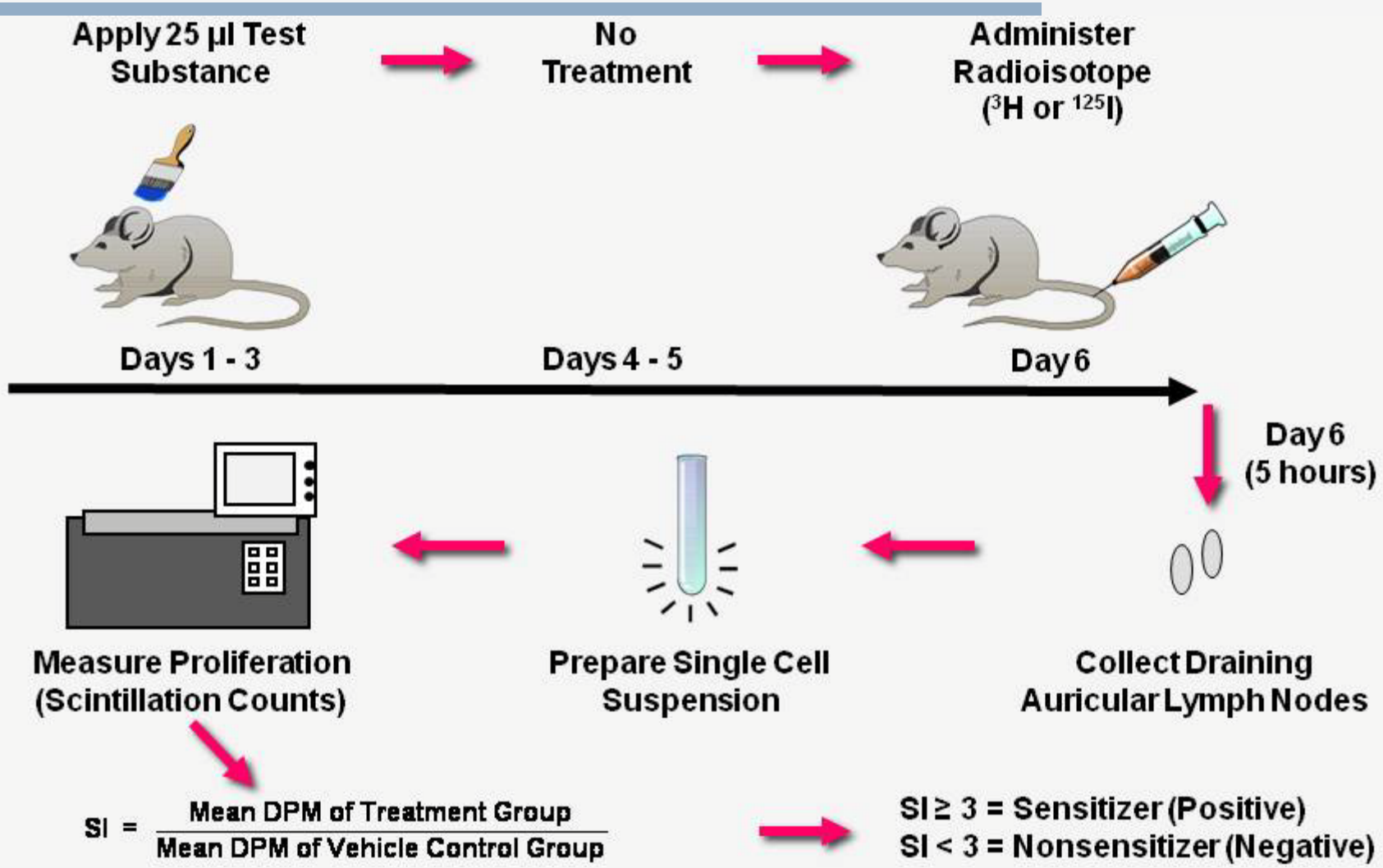
Department of Health & Human Services

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

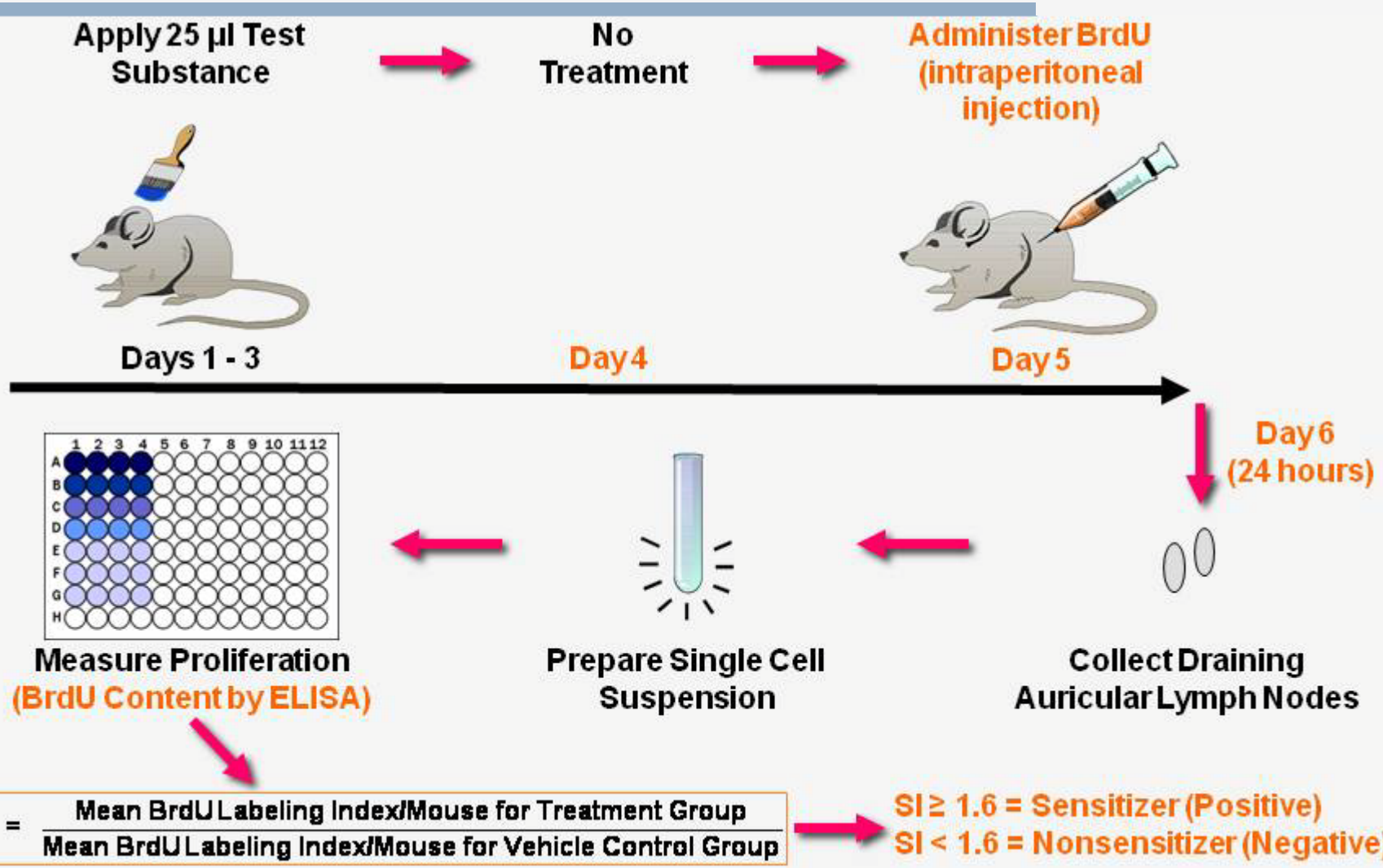
[1]Takeyoshi et al. 2001. Toxicol Lett. 119:203-8.

LLNA Test Method Protocol



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

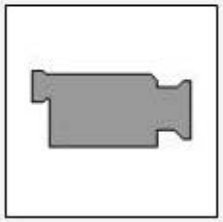
LLNA: BrdU-ELISA Test Method Protocol - 1



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

LLNA: BrdU-ELISA Test Method Protocol - 2

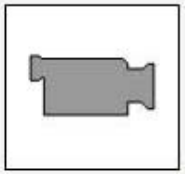
- Lymphocyte suspension
  - Crush lymph nodes
  - Pass through #70 nylon mesh
  - Suspend lymph node cells in 15 mL physiological saline
  - *(return to webpage to view Cell Suspension video clip)*



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.

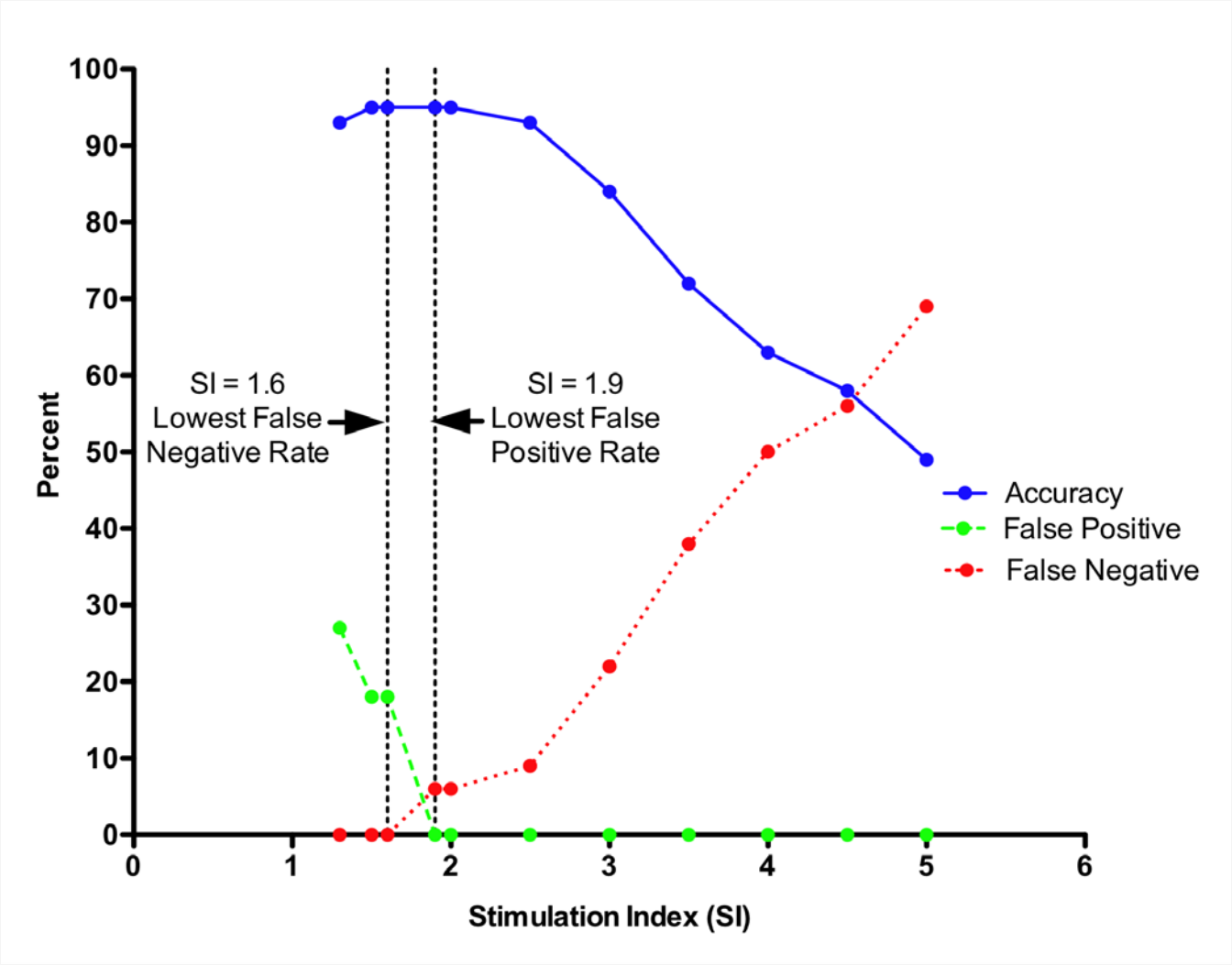
NICEATM-ICCVAM Evaluation of LLNA: BrdU-ELISA – Test Method Accuracy - 1

- 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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