NICEATM

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

ICCVAM

Interagency Coordinating Committee on the Validation of Alternative Methods

The Reduced Murine Local Lymph Node Assay (rLLNA) – Validation Status and Appropriate Use

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National Toxicology Program

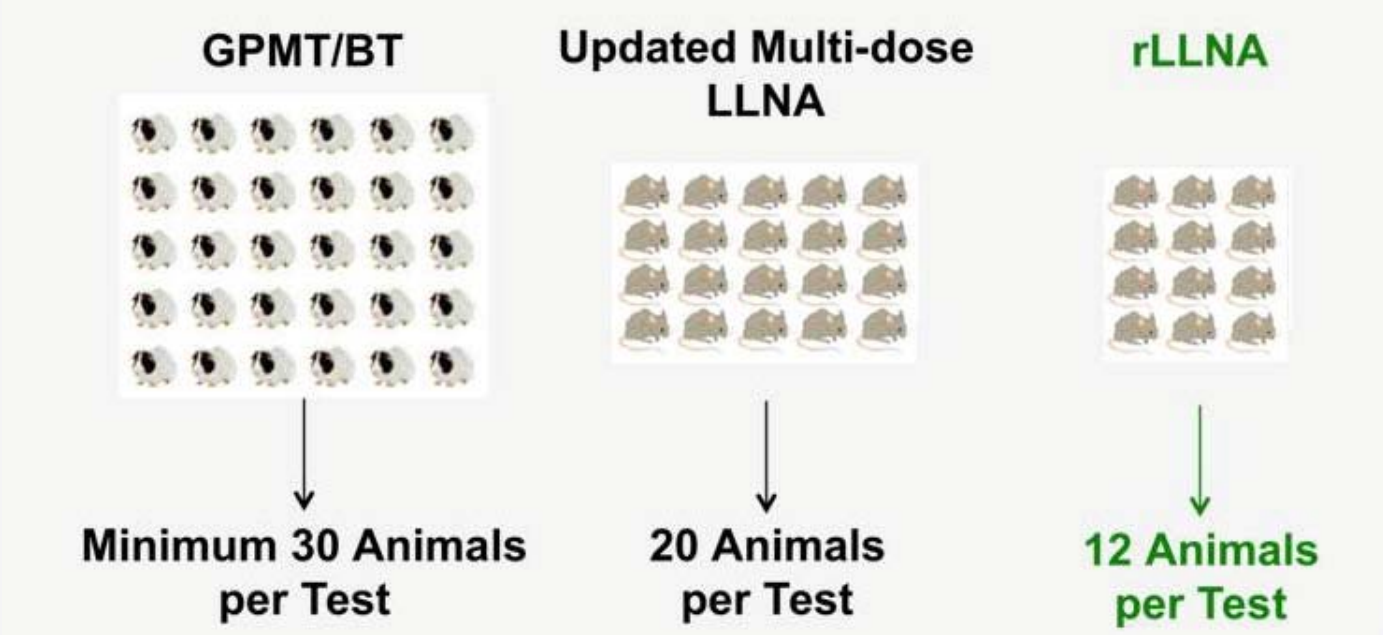
NIEHS

National Institutes of Health

Department of Health & Human Services

Overview of the rLLNA - 1

- Modification of multi-dose murine local lymph node assay (LLNA) that uses fewer animals to assess the allergic contact dermatitis (ACD) hazard potential of chemicals and products
  - For each test substance, rLLNA tests only the highest dose vs. at least three doses for LLNA
  - rLLNA reduces animal number by 40% for each test vs. multi-dose LLNA



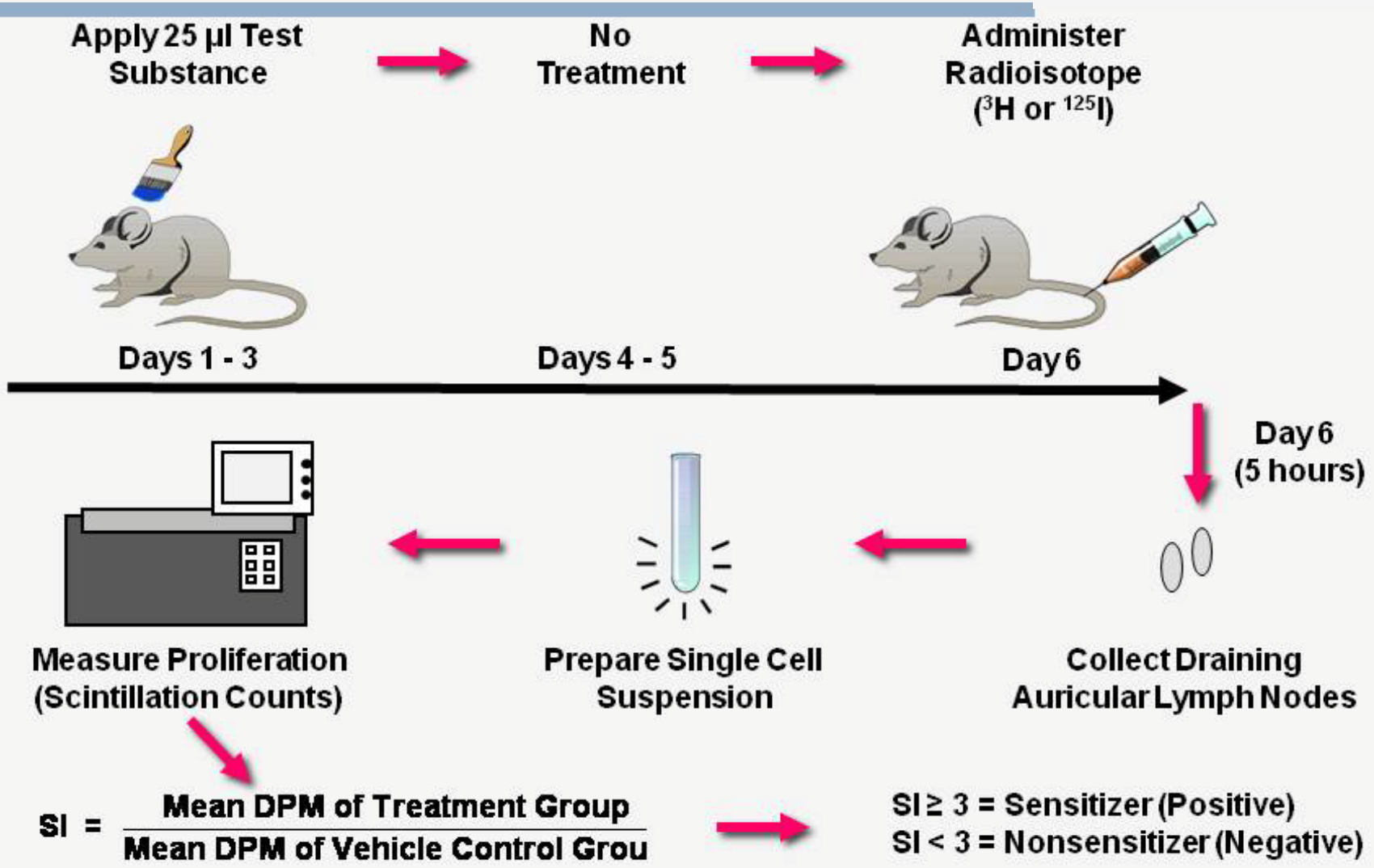
Abbreviations: GPMT/BT = guinea pig maximization test/Buehler test

Overview of rLLNA - 2

- The rLLNA (radioactive and nonradioactive LLNA) should be used for most testing since 80% of chemical products are nonsensitizers in standardized tests[1]

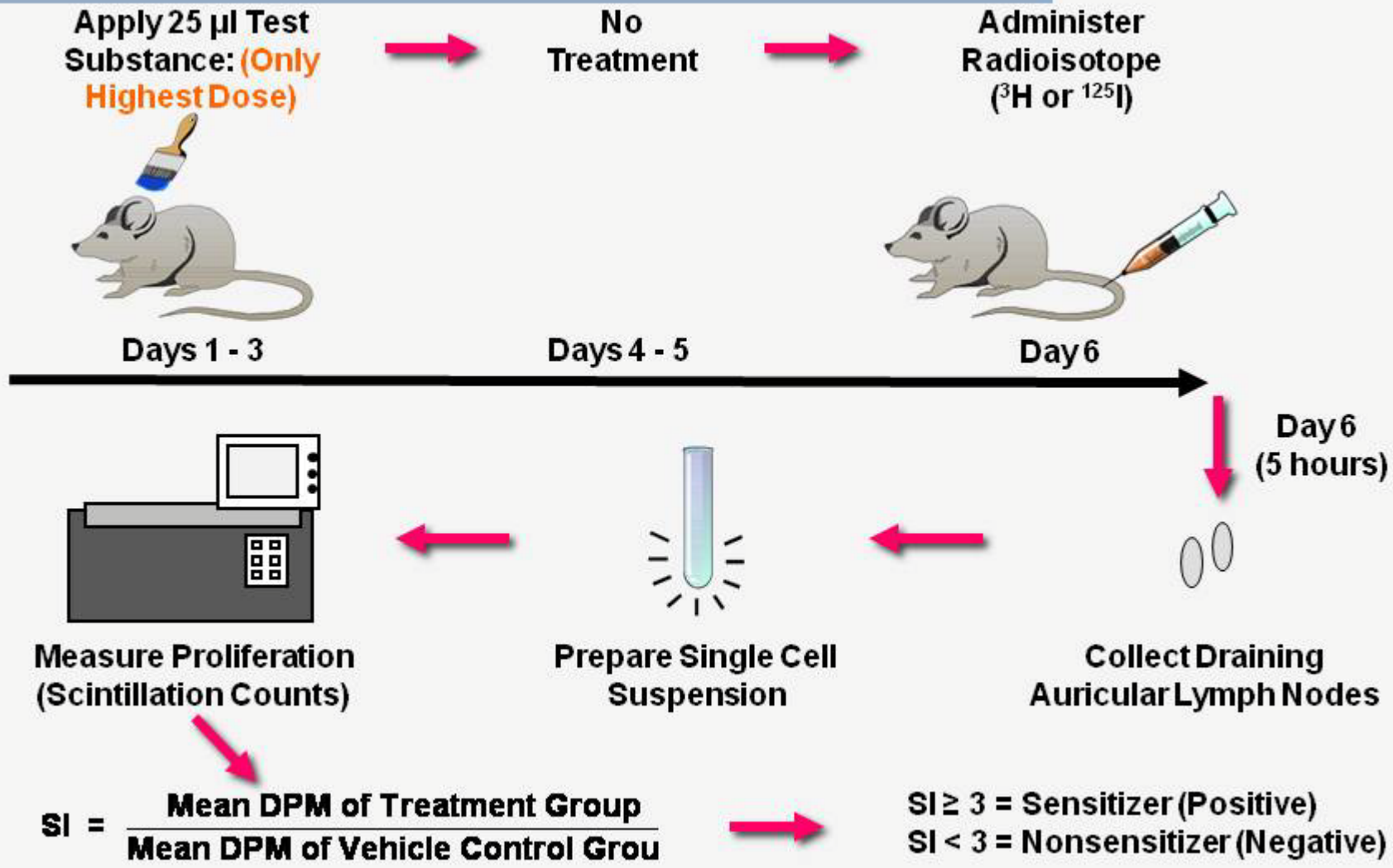
Safford et al. 2008. Reg Tox Pharm 51:195-200.

LLNA Test Method Protocol



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

rLLNA Test Method Protocol - 1



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

rLLNA Test Method Protocol - 2

- Criteria for selecting highest dose is the same as for multi-dose LLNA:
  - Maximum concentration that does not induce overt systemic toxicity and/or excessive local skin irritation
- Identify existing information to aid in selecting the appropriate maximum dose
  - Acute toxicity data
  - Dermal irritation data
  - Dose data from LLNA tests for structurally related substance(s)
- In absence of existing information, a prescreen test may be necessary
  - Identical experimental conditions except for:
    - Omission of lymph node cell proliferation assessment
    - Fewer animals per dose group

rLLNA Test Method Protocol – ICCVAM Recommendations - 1

- Use of a reduced procedure by testing only the highest dose is applicable to the radioactive LLNA and the nonradioactive LLNA
  - Radioactive rLLNA (stimulation index [SI] ≥ 3.0 decision criterion)
  - Nonradioactive reduced LLNA: BrdU-ELISA (SI ≥ 1.6 decision criterion)
  - Nonradioactive reduced LLNA: DA (SI ≥ 1.8 decision criterion)

rLLNA Test Method Protocol – ICCVAM Recommendations - 2

- 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

NICEATM-ICCVAM Evaluation of rLLNA

- 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 rLLNA – Validation Database - 1

- 471 traditional LLNA studies
  - Published reports and unpublished data in response to May 17, 2007 Federal Register (FR)[1]
  - 318 sensitizers (SI ≥ 3)
  - 153 nonsensitizers (SI < 3)
  - Studies for substances tested more than once in the same vehicle were combined to yield an overall skin-sensitization classification
    - 465 studies with unique substance/vehicle combinations[2]
    - 315 sensitizers (SI ≥ 3)
    - 150 nonsensitizers (SI < 3)
    - 211 substances in the original ICCVAM LLNA evaluation[3]

[1] 72 FR 27815 available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf
[2] 457 unique substances but some substances tested in more than 1 vehicle; each substance-vehicle combination considered separately (n = 465).
[3] ICCVAM. 1999. The Murine Local Lymph Node Assay: A Test Method for Assessing The Allergic Contact Dermatitis Potential of Chemical/Compounds. NIH Publication No. 99-4494. Research Triangle Park, NC: National Toxicology Program.

NICEATM-ICCVAM Evaluation of rLLNA – Validation Database - 2

Data Source	Number of Studies	Primary Data Source and Substance Selection Rationale
Gerberick et al. (2005)	210	Historical data: Substances of varying skin sensitization potential
M.J. Olson/GlaxoSmithKline	124	Pharmaceuticals, pharmaceutical intermediates
Basketter, Gerberick, and Kimber	31	Historical data: Substances of varying skin sensitization potential
K. Skirda/CESIO (TNO Report V7217)	18	CESIO data in paper: “Limitations of the LLNA as preferred test for skin sensitisation: concerns about false positive and false negative test results”
Lalko and Api (2006)	17	Essential oils, commonly used in perfumery (contain significant known skin sensitizers)
H.W. Vohr/BGIA	16	Epoxy resin components
Ryan et al. (2002)	15	Water-soluble haptens and known skin sensitizers to assess usefulness of a novel vehicle
D. Germolec/NIEHS	15	Substances evaluated by the National Toxicology Program
E. Debruyne/Bayer CropScience SA	10	Pesticide types and formulations
P. Ungeheur/EFfCI	9	Unsaturated chemicals
P. Botham/ECPA	6	Pesticides evaluated in the LLNA with a novel vehicle
Total	**471[1]**	

Abbreviations: BGIA = German Institute for Occupational Safety and Health; CESIO = European Committee on Organic Surfactants and their Intermediates; ECPA = European Crop Protection Association; EFfCI = European Federation for Cosmetic Ingredients; NIEHS = National Institute of Environmental Health Sciences: TNO = Netherlands Organisation for Applied Research.

[1] The total number of studies does not take into account the fact that some substances were tested more than once.

NICEATM-ICCVAM Evaluation of rLLNA – Validation Database - 3

Chemical Class	No. Kimber et al. 2006	No. Additional	Chemical Class	No. Kimber et al. 2006	No. Additional	Chemical Class	No. Kimber et al. 2006	No. Additional
Alcohols	9	4	Hydrocarbons Acyclic	2	1	Nitriles	1	1
Aldehydes	21	4	Hydrocarbons Cyclic	14	7	Nitro Compounds	2	0
Amides	4	0	Hydrocarbons Halogenated	27	1	Nitroso Compounds	3	0
Amidines	1	0	Hydrocarbons Other	7	8	Onium Compounds	1	0
Amines	14	7	Imines	0	1	Pharmaceutical chemicals	0	125
Anhydrides	1	0	Inorganic Chemicals	0	2	Phenols	18	2
Carbohydrates	3	2	Isocyanates	1	0	Polycyclic Compounds	5	3
Carboxylic Acids	29	15	Ketones	5	0	Quinones	1	1
Esters	3	0	Lactones	2	2	Sulfur Compounds	20	2
Ethers	14	2	Lipids	7	14	Urea	3	0
Formulations	0	10	Inorganic Chemicals	0	2	Unknown	28	42
Heterocyclic Compounds	18	4	Macromolecular Substances	0	5			

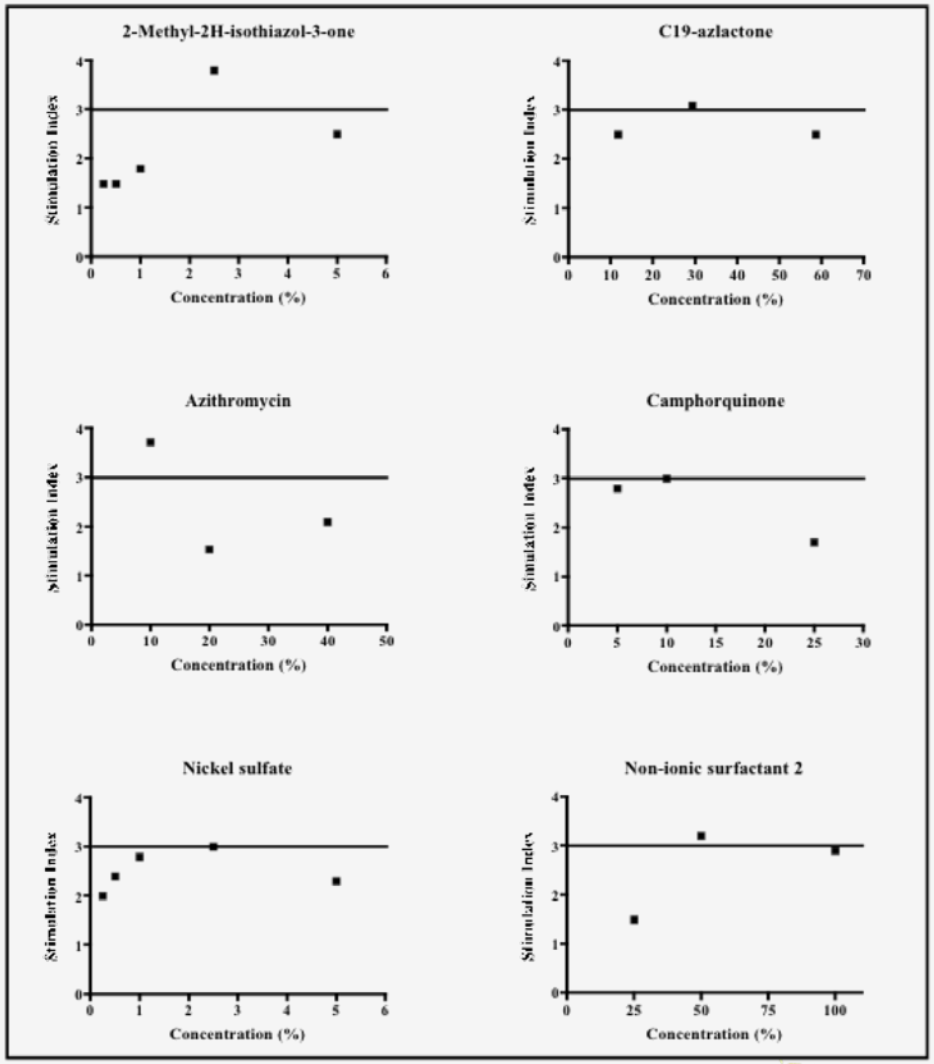
NICEATM-ICCVAM Evaluation of rLLNA – Test Method Accuracy - 1

Data	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
Kimber et al. 2006	211	98.6% (208/211)	98.2% (166/169)	100% (42/42)	0% (0/42)	1.8% (3/169)
rLLNA	471	98.7% (465/471)	98.1% (312/318)	100% (153/153)	0% (0/153)	1.9% (6/318)
rLLNA (substances repeated in same vehicle considered together)	465	98.7% (459/465)	98.1% (309/315)	100% (150/150)	0% (0/150)	1.9% (6/315)

N = number of studies

NICEATM-ICCVAM Evaluation of rLLNA – Test Method Accuracy - 2

- Six substances were positive in the LLNA (SI ≥ 3) at a dose other than the highest dose
- Since the rLLNA only evaluates the highest dose, all six substances were incorrectly identified as nonsensitizers



NICEATM-ICCVAM Evaluation of rLLNA – Test Method Accuracy - 3

- Summary of available physicochemical properties for six false negatives

Substance	CASRN	Vehicle	Molecular Weight	K_{OW}[1]
2-Methyl-2H-isothiazol-3-one	2682-20-4	Acetone: olive oil	115.15	0.68
Nickel sulfate	7786-81-4	Pluronic L92 (1%)	154.76	-0.17
Camphorquinone	465-29-2	Acetone: olive oil	166.22	2.15
C19-azlactone	--	Acetone: olive oil	379.63	5.21
Azithromycin	83905-01-5	Acetone	748.99	3.24
Non-ionic surfactant 2	--	Acetone: olive oil	---	--

Abbreviations: CASRN = Chemical Abstracts Service Registry Number.
[1] K_{OW} represents the octanol-water partition coefficient (expressed on log scale).

NICEATM-ICCVAM Evaluation of rLLNA – Test Method Reproducibility

- Since the rLLNA and multi-dose LLNA use identical protocols and similar data sets, the intra- and inter-laboratory reliability of the rLLNA was deemed similar to that of the multi-dose LLNA
- 1999 ICCVAM evaluation of multi-dose LLNA reproducibility[1, 2]:
  - 2,4-dinitrochlorobenzene tested twice in each of five laboratories
  - Hexyl cinnamic aldehyde tested six times in each of two laboratories
  - Analyses indicated a lack of significant intra- and inter-laboratory variability
  - LLNA repeatability and reproducibility was considered acceptable
- Additional data in 2008 (n = 5 chemicals)
  - Analyses consistent with 1999 ICCVAM reproducibility evaluation for the multi-dose LLNA

[1]ICCVAM. 1999. The Murine Local Lymph Node Assay: A Test Method for Assessing The Allergic Contact Dermatitis Potential of Chemical/Compounds. NIH Publication No. 99-4494. Research Triangle Park, NC: National Toxicology Program.
[2]Haneke et al. 2001. Reg Tox Pharm 34:274-286.

ICCVAM Test Method Recommendations for rLLNA – Usefulness and Limitations

Usefulness

- Sufficient to distinguish between skin sensitizers and nonsensitizers
- Should be used routinely to determine the 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

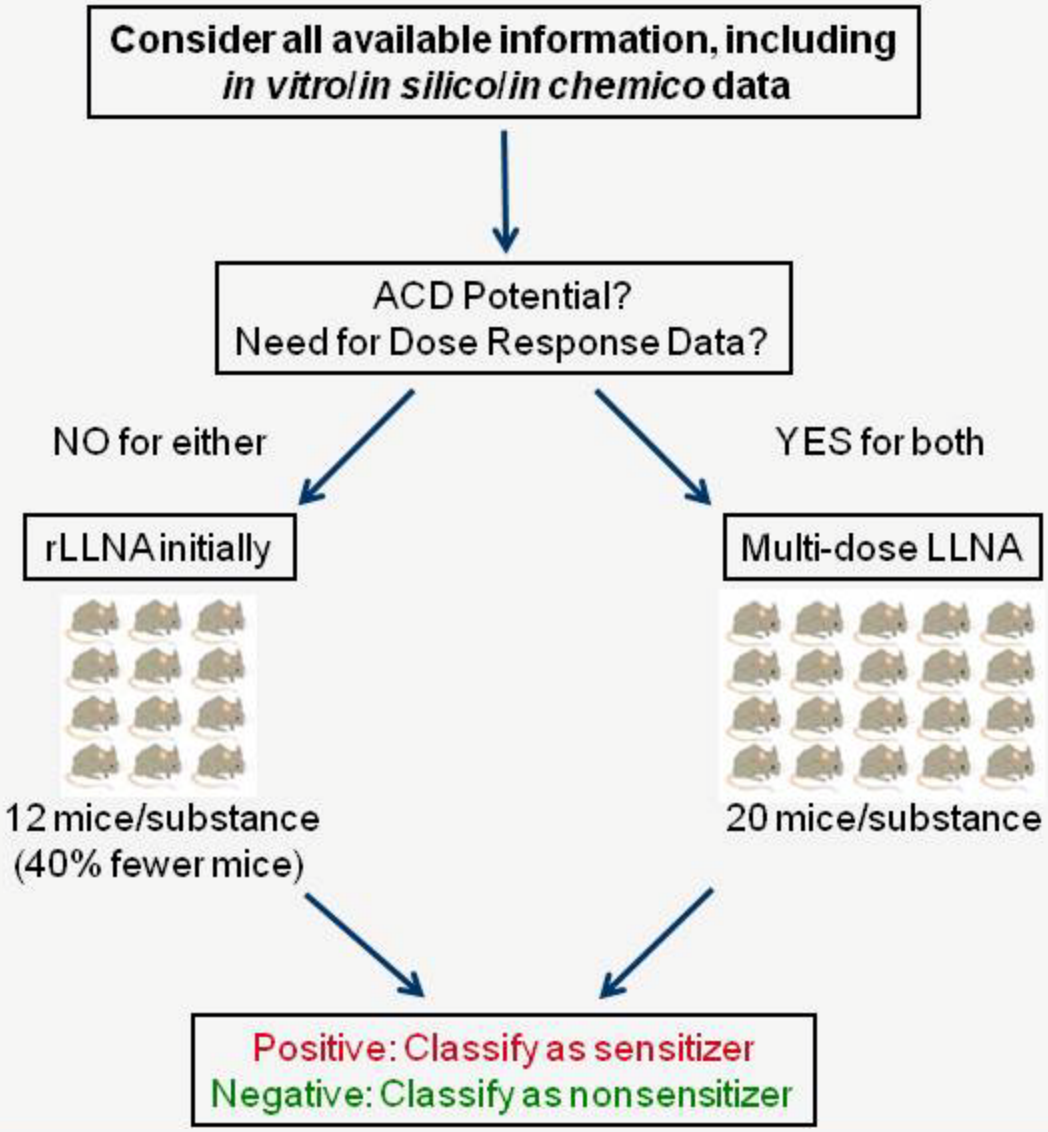
Limitations

- Does not provide dose-response information
  - EC3 cannot be calculated
- Small possibility of false negatives (1.9% [6/318]) compared to LLNA validation database
  - When rLLNA conducted for suspected positives, and a negative result is obtained confirmatory testing in the multi-dose LLNA might be considered

Criteria for Deciding to Use the rLLNA

- Use rLLNA routinely to determine the ACD hazard potential of chemicals and products unless there is a likelihood that it is a sensitizer and dose response information is needed
- Available information and data about the chemical/product to consider include:
  - Physicochemical properties
  - Structural relationship to known skin sensitizers
    - Structural alerts/QSAR
  - *In vitro*/*in silico*/*in chemico* data
  - Human data
  - Test results for similar substances
  - Toxicogenomic data

Decision Strategy for Using rLLNA



rLLNA International Acceptance

- rLLNA approach provided as an option in updated OECD TG 429 Skin Sensitization: Local Lymph Node Assay
  - Adopted July 22, 2010
  - Available at http://www.oecd-ilibrary.org/environment/test-no-429-skin-sensitisation_9789264071100-en
  - Based on ICCVAM-recommended LLNA protocol
  - Expected to further reduce animal use for ACD 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 Reduced LLNA: an Alternative Test Method Using Fewer Animals to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products

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

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

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