

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



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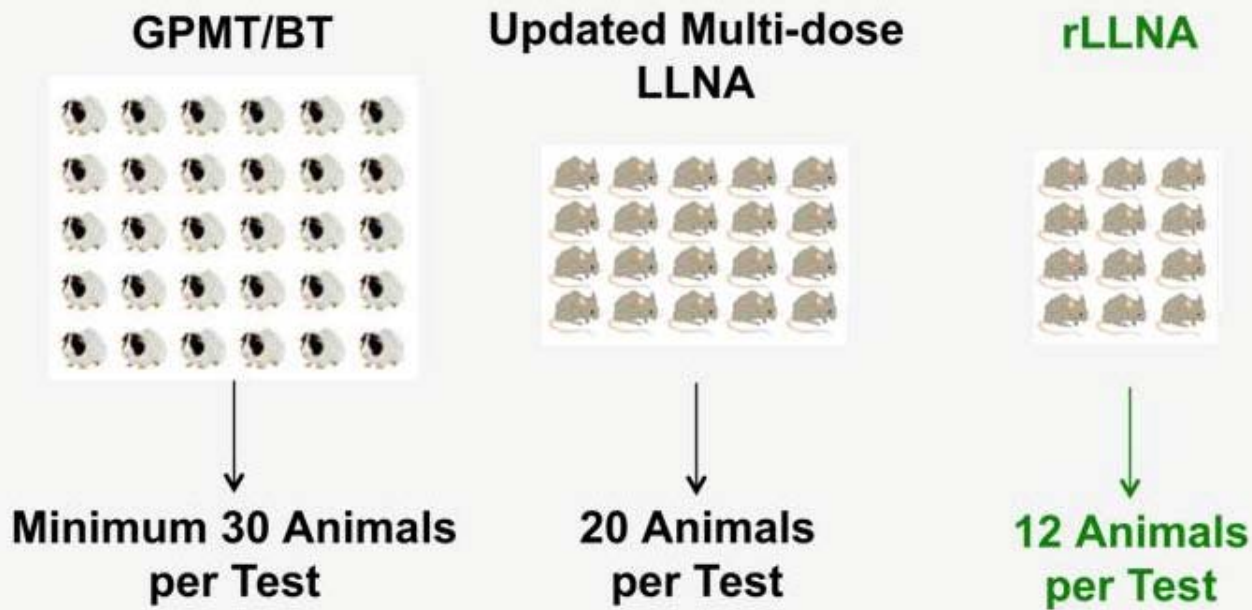
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Allergic Contact Dermatitis

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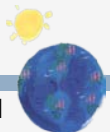
William H. Natcher Conference Center
National Institutes of Health
Bethesda, MD

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



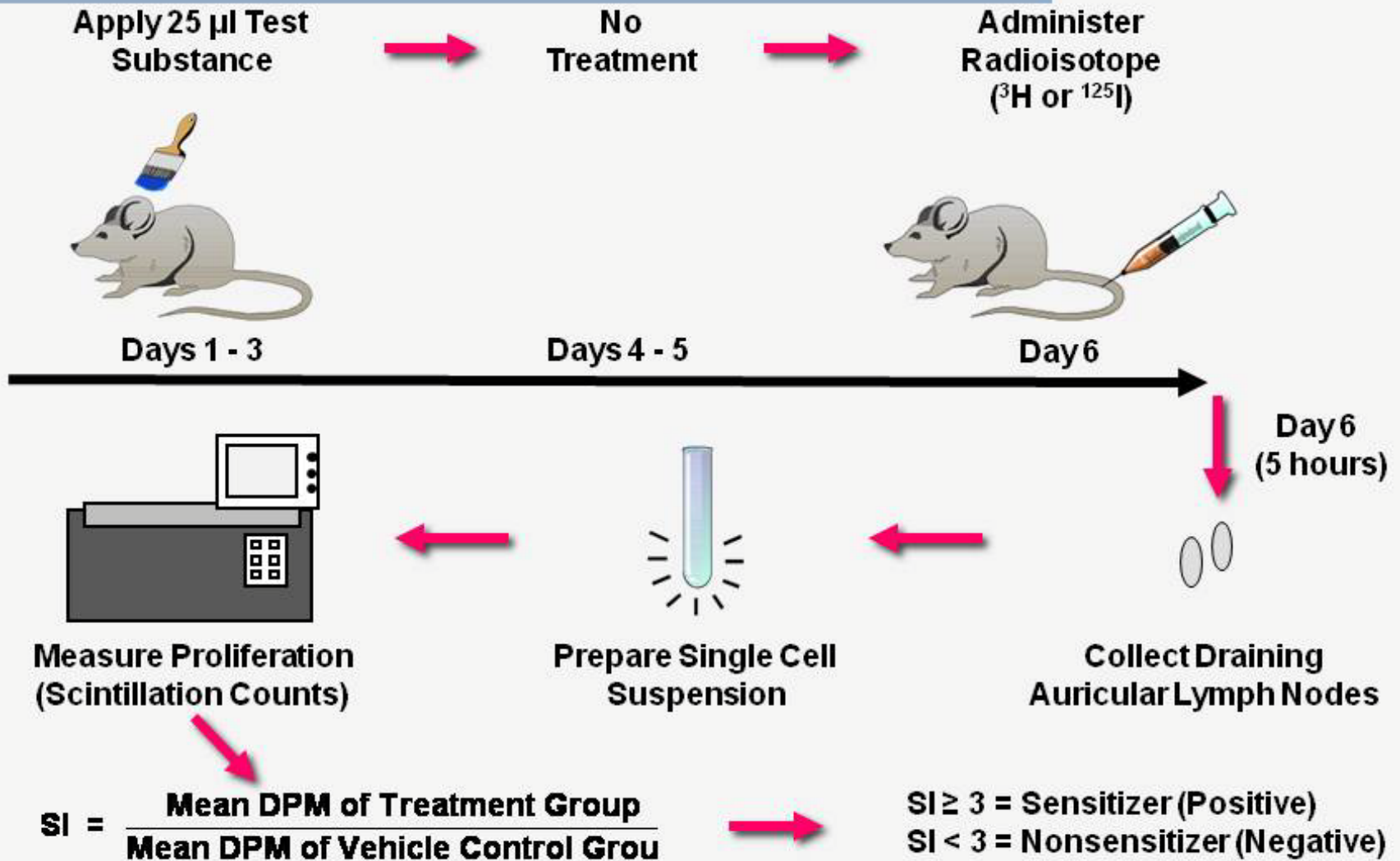
Abbreviations: GPMT/BT = guinea pig maximization test/Buehler test



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 tests¹

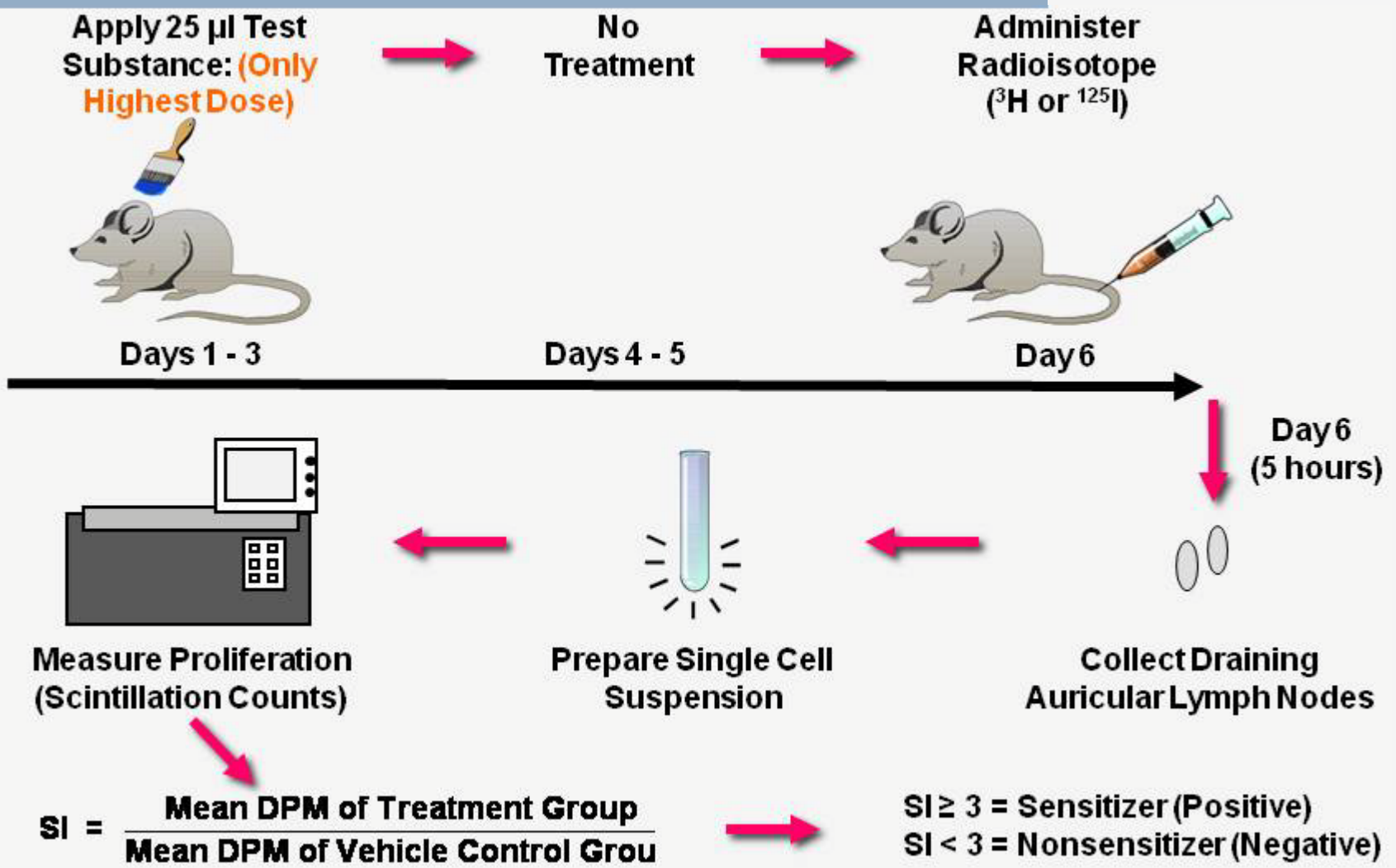
LLNA Test Method Protocol



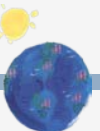
Abbreviations: DPM = disintegrations per minute; SI = stimulation index



rLLNA Test Method Protocol - 1

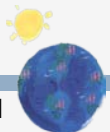


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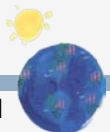
rLLNA Test Method Protocol - 2

- 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



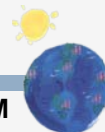
rLLNA Test Method Protocol – ICCVAM Recommendations - 1

- 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)



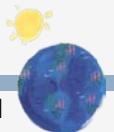
rLLNA Test Method Protocol – ICCVAM Recommendations - 2

- 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



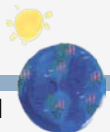
NICEATM-ICCVAM Evaluation of rLLNA – Validation Database - 1

- 471 traditional LLNA studies
 - Published reports and unpublished data in response to May 17, 2007 Federal Register (FR)¹
 - 318 sensitizers (SI \geq 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 combinations²
 - 315 sensitizers (SI \geq 3)
 - 150 nonsensitizers (SI $<$ 3)
 - 211 substances in the original ICCVAM LLNA evaluation³

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	471¹	

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			



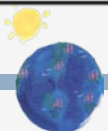
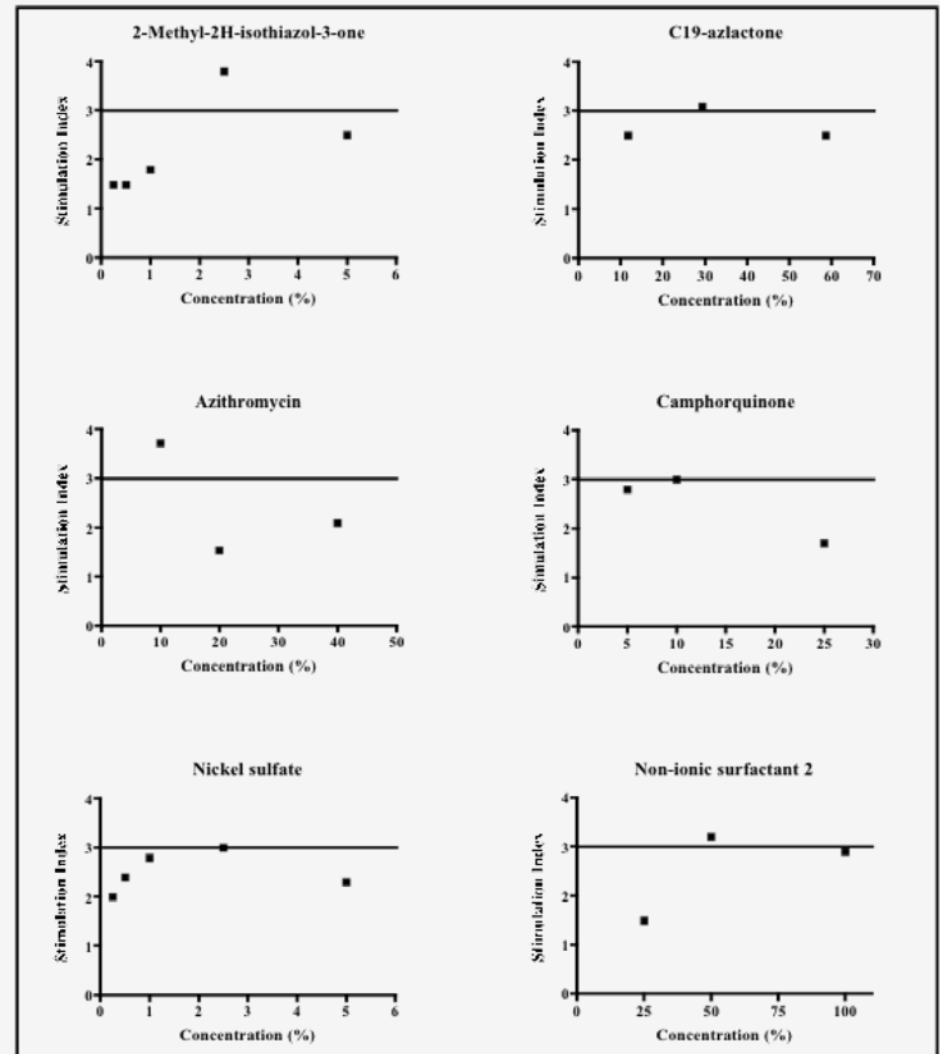
NICEATM-ICCVAM Evaluation of rLLNA – Test Method Accuracy - 1

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

¹ICCVAM. 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.

²Haneke et al. 2001. Reg Tox Pharm 34:274-286.



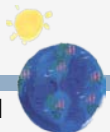
ICCVAM Test Method Recommendations for rLLNA – Usefulness and Limitations

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

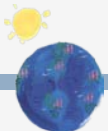
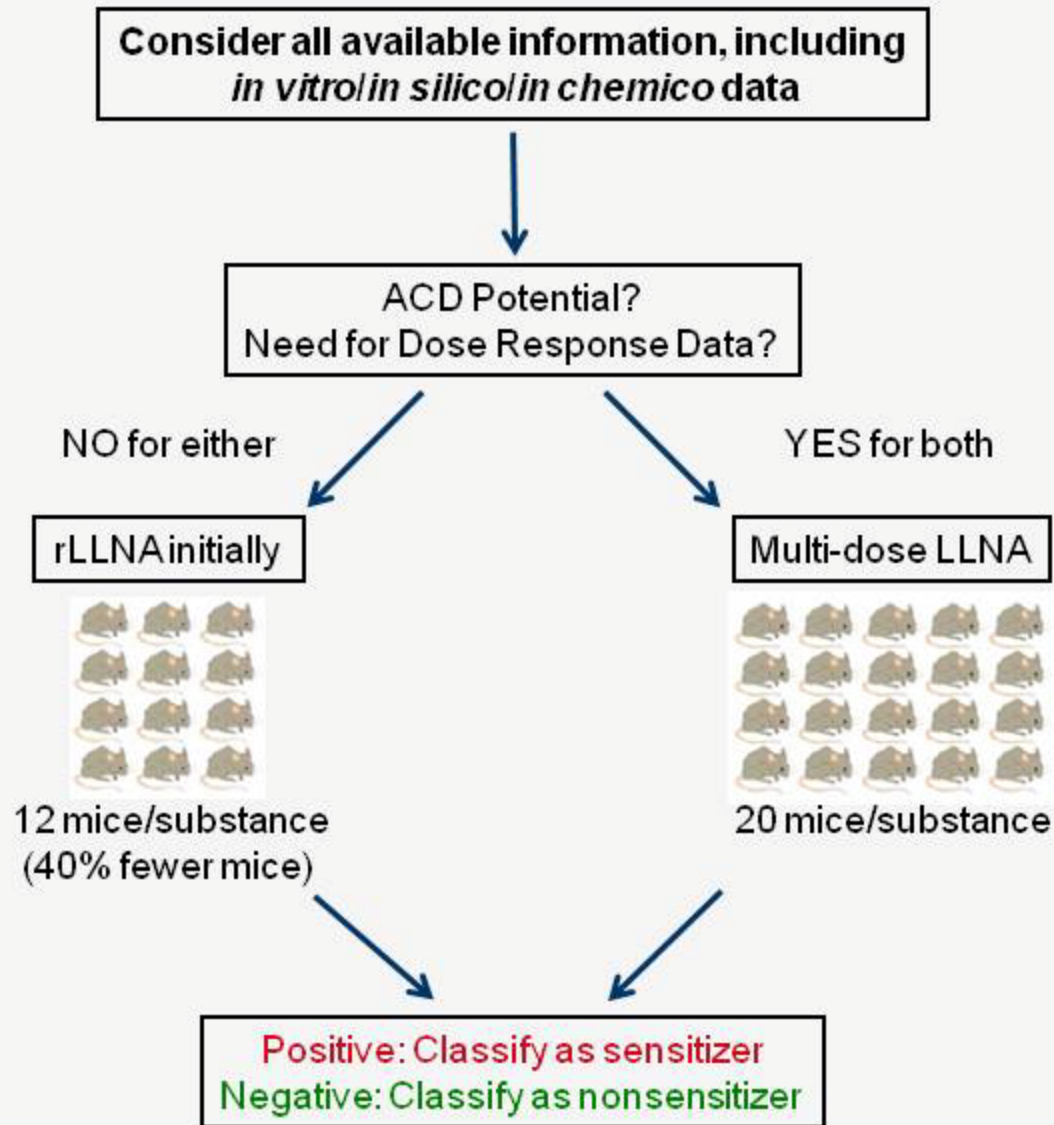


Criteria for Deciding to Use the rLLNA

- 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

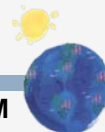


Decision Strategy for Using rLLNA



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):

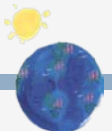
ICCVAM Evaluation and International Acceptance of the Reduced LLNA: an Alternative Test Method Using Fewer Animals to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products

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³ILS, Inc., Contractor Supporting NICEATM, RTP, NC;

⁴NICEATM/NTP/NIEHS/NIH/DHHS, RTP, NC



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