Updated NICEATM Evaluation of the Reduced Murine Local Lymph Node Assay

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Abstract

To minimize the occurrence of allergic contact dermatitis (ACD), regulatory authorities require testing to identify substances that may cause ACD. Such substances must be labeled with the hazard description and precautions necessary to minimize exposure. The murine local lymph node assay (LLNA) is an alternative test method for determining the ACD hazard potential of most types of substances. Compared to guinea pig tests, it requires fewer animals, less time, and eliminates pain and distress. The reduced LLNA (rLLNA), which uses only one high dose, further reduces animal use by 40% compared to the multidose LLNA. Based on the evaluation by ICCVAM in 2009 using 471 LLNA studies, the rLLNA was included in an updated version of the OECD Test Guideline for the LLNA (TG 429) adopted in 2010. LLNA results from 1071 published and unpublished studies, representing 664 unique substances, were obtained. Accuracy for the rLLNA was 98.5% (1055/1071), with false positive and false negative rates of 0% (0/319) and 2.1% (16/752), respectively. Sixteen false negative studies encompassed 13 substances; all produced relatively weak (SI ≤ 7.7) responses. This updated analysis of the rLLNA supports the conclusions and recommendations described in the 2009 ICCVAM rLLNA test method evaluation report, including the recommendation that the rLLNA should be routinely considered and used where determined appropriate. This analysis also provides further support for the use of the updated OECD TG, which is expected to significantly refine and reduce animal use for ACD hazard testing while supporting the protection of human health.
ILS staff supported by NIEHS contract N01-ES-35504.

Introduction

- Allergic contact dermatitis (ACD) can develop upon skin exposure to an allergen **(Figure 1)**.
  - ACD causes over 7 million outpatient visits per year (Middleton et al. 1998).
  - In 2009, 8.9 million children were diagnosed with ACD (National Center for Health Statistics 2010).
  - Skin diseases account for at least 15% of all reported occupational diseases. Twenty percent of the reported skin diseases are ACD (Bureau of Labor Statistics 2010a, b).
  - ACD causes lost workdays and can significantly diminish quality of life (Bureau of Labor Statistics 2010b; Hutchings et al. 2001; Skoet et al. 2003).
- Due to the adverse impact of ACD (Hogan et al. 1990), prevention is essential.
  - Testing has identified more than 3700 contact allergens worldwide (Beltrani et al. 2006).

- Allergens must be labeled with a hazard description and precautions to minimize exposure.

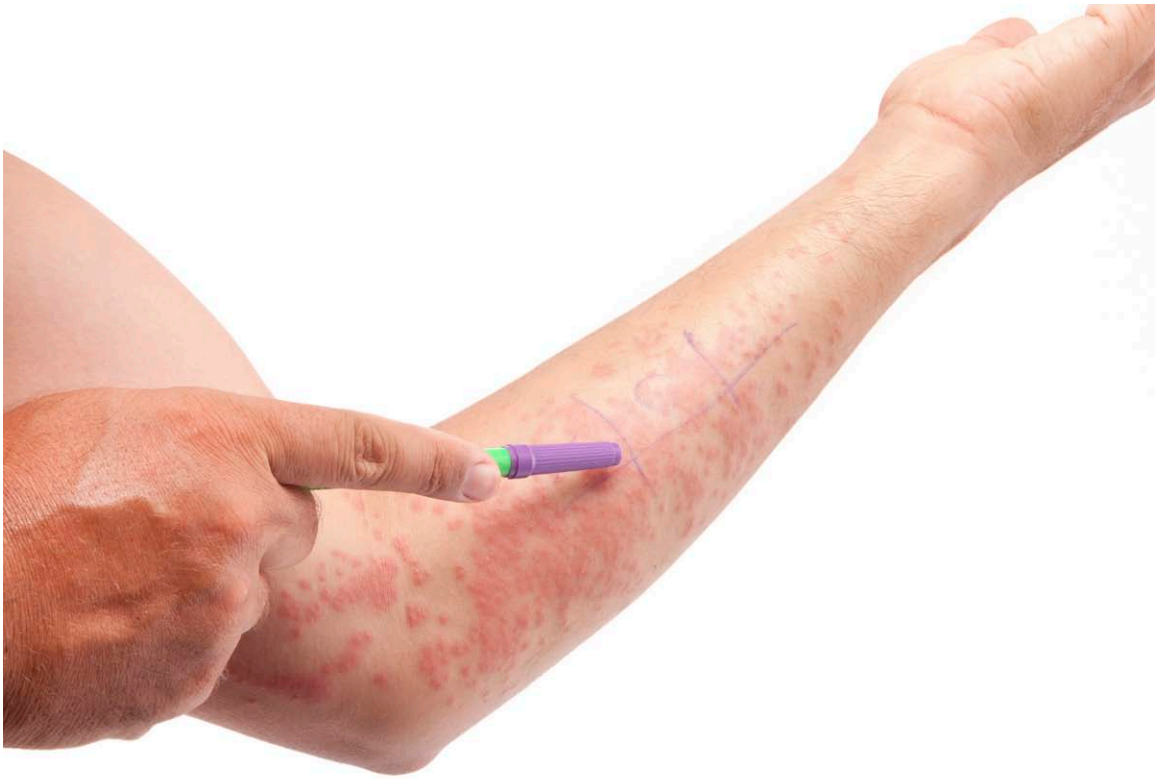
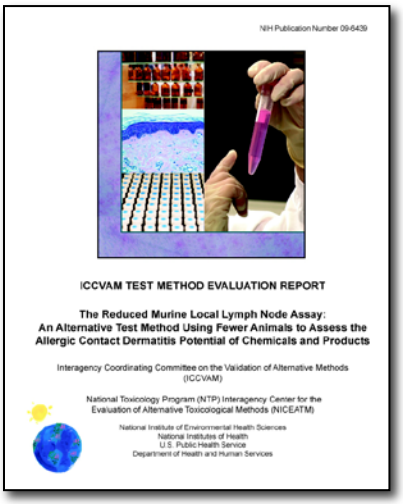


Figure 1. Allergic Contact Dermatitis Rash

- An independent international scientific peer review panel evaluated the LLNA validation status (ICCVAM 1999).
  - In 1999, ICCVAM recommended the murine local lymph node assay (LLNA) as a valid substitute for the accepted guinea pig test methods (ICCVAM, 1999; Haneke et al. 2001).
  - The LLNA was incorporated into national and international test guidelines (EPA 1998; OECD 2002; ISO 2008).
- Advantages of the LLNA over guinea pig methods include the following (Dean et al. 2001; Sailstad et al. 2001):
  - Potential pain and distress are virtually eliminated.
  - Fewer animals are used.
  - Less time is required for testing.

  - Dose–response information is available.

- A reduced LLNA (rLLNA) protocol was proposed that only uses a single high dose, based on results from 211 studies (Kimber et al. 2006) **(Figure 2).**
  - In 2009, ICCVAM evaluated the rLLNA using results from 457 unique substances that had been tested in 471 multidose LLNA studies.
  - ICCVAM recommended that the rLLNA should be used routinely to determine the ACD potential of chemicals and products.
    - Substances with negative results can be classified as nonsensitizers, and those with positive results can be classified as sensitizers.
    - If dose–response information is required for a suspected contact allergen, the substance should be evaluated initially using the multidose LLNA.
  - The rLLNA was adopted internationally in the updated OECD Test Guideline 429 (OECD 2010).
- After ICCVAM evaluation of the rLLNA, NICEATM continued to compile LLNA results and now has a database that contains the results from 664 unique substances tested in 1071 multidose LLNA studies.
- Because the database has almost doubled since the original evaluation, NICEATM conducted an updated evaluation of the rLLNA based on these LLNA results.

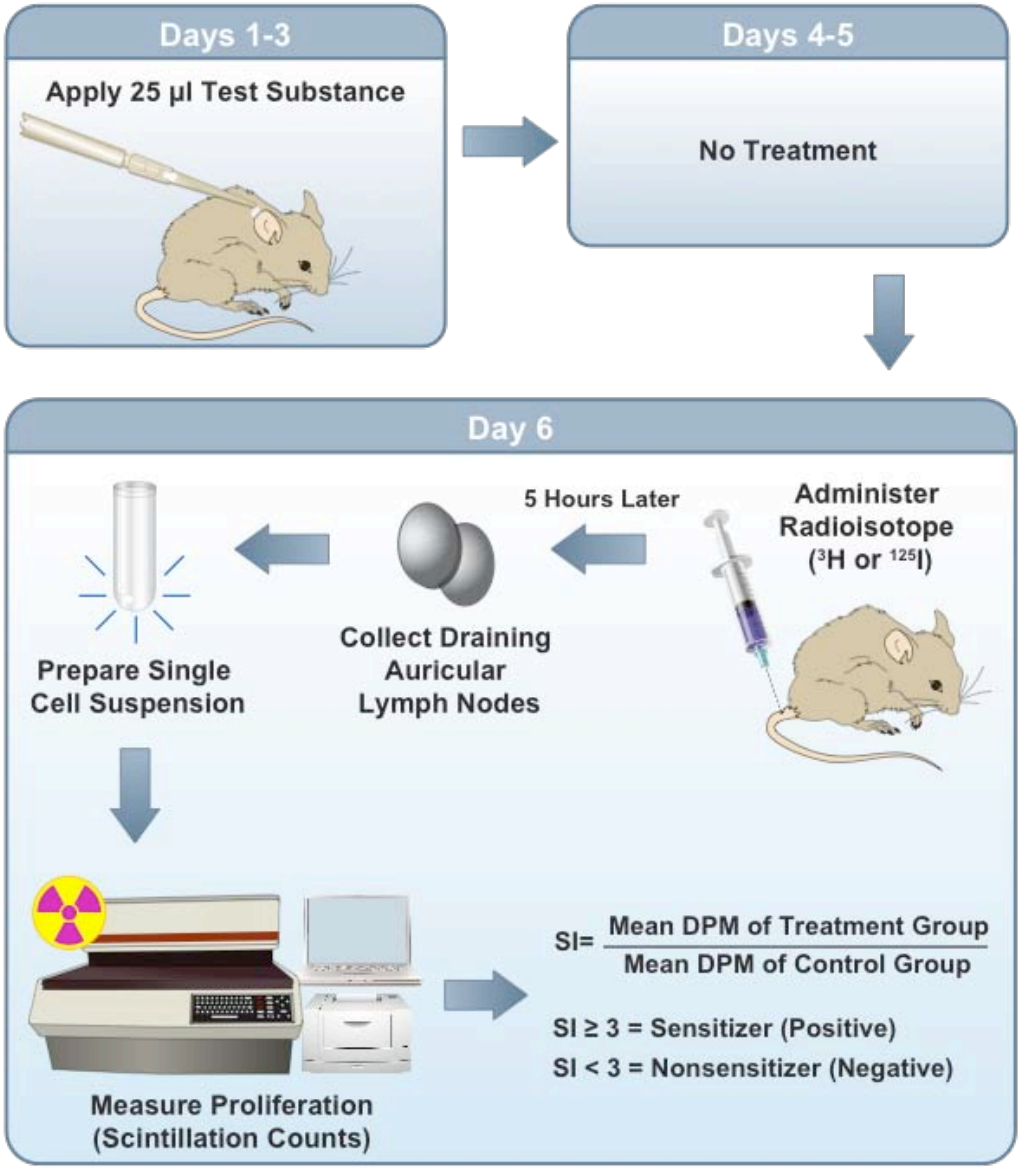


Methods

- This study evaluated 1071 independent LLNA studies of 664 unique substances.
- The only difference between the protocols for the multidose LLNA and the rLLNA **(Figure 2)** is the number of doses tested.
- In the multidose LLNA, at least three doses are tested, with the highest dose based on the maximum soluble concentration and the avoidance of excessive local irritation and/or systemic toxicity.

- Only the highest dose of a substance is tested in the rLLNA (ICCVAM 2009a; Kimber et al. 2006).

Figure 2. LLNA/rLLNA[1] Test Method Protocol



Abbreviations: DPM = disintegrations per minute; LLNA = murine local lymph node assay; SI = stimulation index.

[1] The only difference between the LLNA and the rLLNA protocols is that the LLNA tests multiple doses of test substance, while rLLNA tests one high dose.

Results

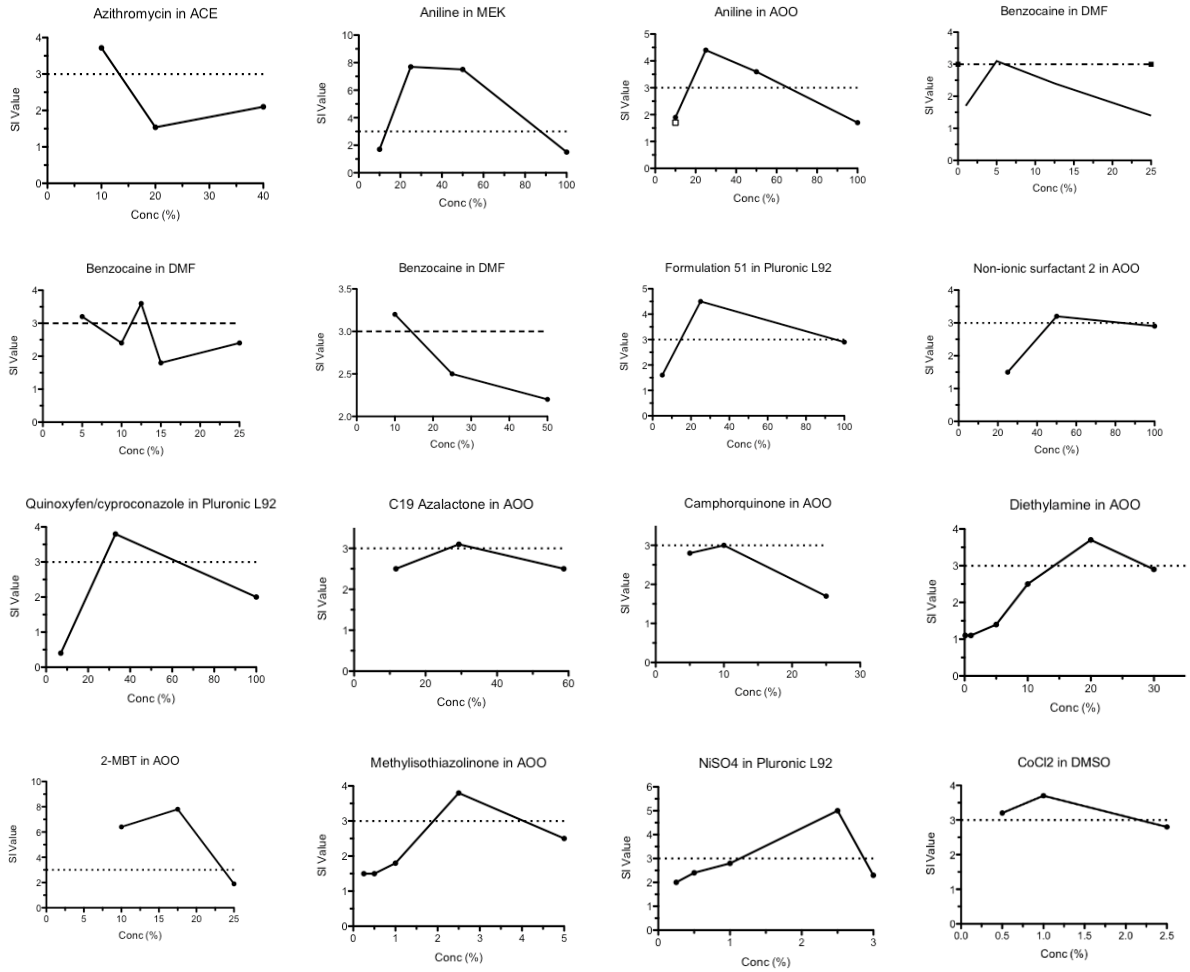
- Accuracy for the rLLNA from this analysis is nearly identical to the accuracy from the ICCVAM 2009 evaluation, despite a 5-fold increase in the number of studies **(Table 1).**
- A 2-sided Fisher's exact test confirmed that there is no statistically significant difference between the accuracy and false negative rates from the 2009 and 2011 results (p = 0.80) **(Table 1)**.
- Among 752 positive multidose LLNA studies, only 2.1% (16/752) tested false negative in the rLLNA **(Table 1).**
- No consistent patterns were discerned in the physicochemical properties of the substances that produced false negative results in the rLLNA **(Table 2)**.

Table 1. Performance of the rLLNA in Predicting Skin Sensitizers Compared to the Multidose LLNA

Data Source	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
		% (No.)	% (No.)	% (No.)	% (No.)	% (No.)
Kimber et al. (2006)	211	98.6 (208/211)	98.2 (166/169)	100 (42/42)	0 (0/42)	1.8 (3/169)
rLLNA (ICCVAM 2009a)	471	98.7 (465/471)	98.1 (312/318)	100 (153/153)	0 (0/153)	1.9 (6/318)
rLLNA (updated database) 2011	1071	98.5 (1055/1071)	97.9 (736/752)	100 (319/319)	0 (0/319)	2.1 (16/752)

Abbreviations: ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine local lymph node assay; N = number of studies; No. = numbers used to calculate percentage; rLLNA = reduced murine local lymph node assay.

Figure 2. Dose–Response Curves for 13 Substances Identified as Sensitizers by the Multidose LLNA but as Nonsensitizers by the rLLNA



Note: The dotted line in each figure indicates a stimulation index of 3, which is the threshold for a positive response in the multidose LLNA and the rLLNA. Points on or above this line indicate a positive response, while points below this line indicate a negative response.

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1 by volume); Conc. = concentration; DMF = dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4 dinitrochlorobenzene; LLNA = murine local lymph node assay; MBT = mercaptobenzothiazole; MEK = methyl ethyl ketone; rLLNA = reduced murine local lymph node assay; SI = stimulation index.

Table 2. Summary of Physicochemical Properties for 13 Substances that Tested False Negative by the rLLNA

Substance	CASRN	Vehicle	Molecular Weight (g/mol)	K_{OW}[1]	MeSH® Chemical Categories	Correct rLLNA studies/Total no. studies
Cobalt chloride	1332-82-7	DMSO	58.90	NA	Metals	2/3
Diethylamine	109-89-7	AOO	73.14	0.81[2]	Amines	0/1
Aniline	62-53-3	AOO	93.13	1.56[2]	Amines	7/9
2-Methyl-2H-isothiazol-3-one	2682-20-4	AOO	115.15	0.68[3]	Heterocyclic compounds; Sulfur compounds	1/2
Nickel sulfate	7786-81-4	PLU	154.76	-0.17[2]	Metals	11/12
Benzocaine	94-09-7	DMF	165.19	1.52[2]	Carboxylic acids	18/21
Camphorquinone	465-29-2	AOO	166.22	2.15[3]	Hydrocarbons, other	0/1
2-Mercaptobenzothiazole	149-30-4	AOO	167.25	1.8[2]	Heterocyclic compounds	9/10
Quinoxyfen/ cyproconazole	124495-18-7 /13096-99-4	PLU	308.134/ 291.776	5.69/ 3.25[2]	Heterocyclic compounds	5/6
C19-azlactone	NA	AOO	379.63	5.21[3]	Heterocyclic compounds; Lactones	0/1
Azithromycin	83905-01-5	ACE	748.99	3.24[2]	Glycosides; Lactones; Polycyclic compounds	0/1
Formulation 51	40487-42-1 /422556-08-9	PLU	NA	NA	NA	0/1
Non-ionic surfactant 2	NA	AOO	NA	NA	NA	0/1

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1 by volume); CASRN = CAS Registry Number® (American Chemical Society); DMF = dimethylformamide; DMSO = dimethyl sulfoxide; MeSH® = Medical Subject Headings (U.S. National Library of Medicine); NA = not available; PLU = 1% Pluronic L92.

[1] K_{OW} represents the estimated octanol–water partition coefficient (expressed on log scale).

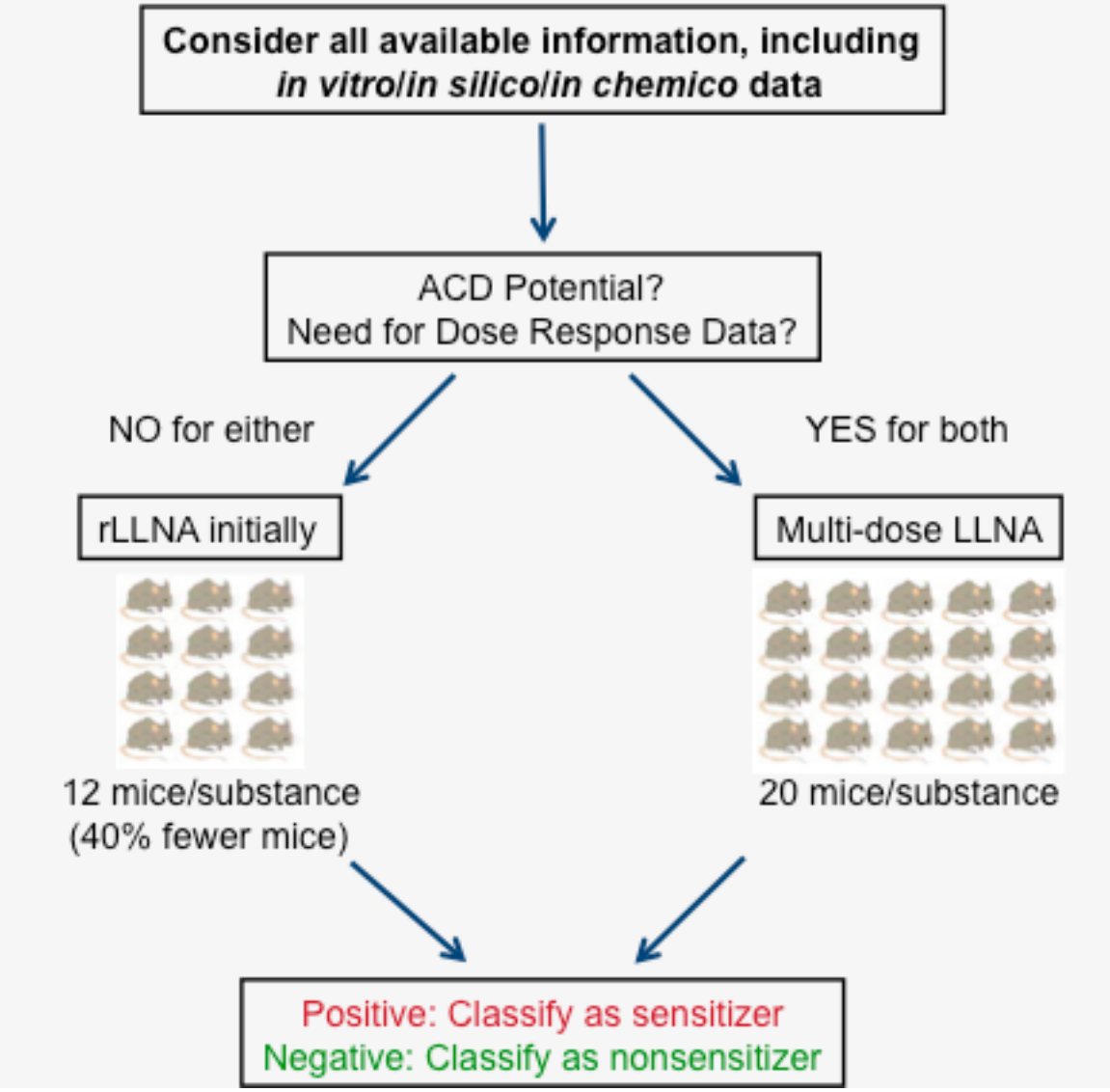
[2] K_{OW} calculated by the method of Meylan and Howard (1995) and obtained from the website http://www.srcinc.com/what-we-do/databaseforms.aspx?id=385.

[3] K_{OW} calculated by the method of Moriguchi et al. (1994) and provided in Gerberick et al. (2005).

Conclusions

- This updated analysis of the rLLNA further supports the conclusions and recommendations described in the ICCVAM rLLNA test method evaluation report (ICCVAM 2009a).
- ICCVAM has recommended that the rLLNA should always be used as the initial test to determine the skin sensitization hazard potential of chemicals and products when a negative result is expected or dose-response information is not required (ICCVAM 2009a).
- The rLLNA reduces animal use by up to 40% for each test, compared to the multidose LLNA. A decision strategy for use of the rLLNA is shown in **Figure 3**.

Figure 3. Decision Strategy for Using the rLLNA



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Acknowledgements

We acknowledge the following companies and organizations and their representatives that submitted LLNA data to NICEATM for the evaluation of new applications and modifications of the LLNA:

- BASF – The Chemical Company, USA (Mr. Charles Hastings)
- The German Institute for Occupational Safety and Health, Germany (Ms. Heidi Ott)
- Dow AgroSciences, USA (Dr. Michael Woolhiser)
- DuPont, USA (Dr. Fredrick O'Neal)
- European Crop Protection Association, Belgium (Dr. Philip Botham)
- European Federation for Cosmetic Ingredients, Belgium (Dr. Peter Ungeheuer)

The Intramural Research Program of the National Institute of Environmental Health Sciences (NIEHS) supported this poster. Technical support was provided by ILS, Inc., under NIEHS contract N01-ES 35504.