# Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluated the validation status of the reduced murine local lymph node sssay (rLLNA), a test method for assessing the potential of substances to cause allergic contact dermatitis (ACD). ACD is an allergic skin reaction characterized by redness, swelling, and itching that can result from contact with a sensitizing chemical or product. This Test Method Evaluation Report provides ICCVAM's recommendations regarding the usefulness and limitations of the rLLNA as an alternative to the traditional murine local lymph node assay (LLNA). When deemed appropriate for use, the rLLNA can reduce by 40% the number of animals used for each test compared to the traditional LLNA. This report also includes the updated ICCVAM-recommended LLNA test method protocol, the final rLLNA background review document (BRD), and recommendations for future studies and performance standards.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), ICCVAM, and the ICCVAM Immunotoxicity Working Group prepared a draft BRD and draft test method recommendations, which were provided to an international independent scientific peer review panel (Panel) and the public for comment. The BRD evaluated data from 471 traditional LLNA studies, including the 211 substances from the 1998 ICCVAM evaluation of the traditional LLNA (ICCVAM 1999), and 246 from the peer-reviewed literature and submissions to NICEATM in response to a May 17, 2007, *Federal Register* request for comments (72 FR 27815[7]). A detailed timeline of the rLLNA test method evaluation is included with this report.

The Panel met in public session on March 4–6, 2008, to discuss their peer review of the ICCVAM draft BRD and to provide conclusions and recommendations on the current validation status of the rLLNA test method. The Panel also reviewed how well the information contained in the draft BRD supported ICCVAM's draft test method recommendations. In finalizing this Test Method Evaluation Report and the BRD, which is included as an appendix, ICCVAM considered the conclusions and recommendations of the Panel and comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods and the public.

## ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the scientific validity of the rLLNA has been adequately evaluated and that the performance of the rLLNA, when conducted in accordance with the updated ICCVAM-recommended LLNA protocol, is sufficient to distinguish between skin sensitizers and non-sensitizers in cases that do not require dose-response information. ICCVAM also concludes that, compared to the traditional LLNA, the rLLNA will reduce animal use by 40% for each test. Accordingly, ICCVAM recommends that the rLLNA test method should be used routinely to determine the ACD potential of chemicals and products before conducting the traditional LLNA. Negative substances can be classified as non-sensitizers, and positive substances can be classified as sensitizers.

In cases that require dose-response information, positive substances must be tested in the traditional multiple-dose LLNA. Therefore, if dose-response information is required for a

[7] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

substance that, after consideration of all available information, is also suspected of having the potential to produce ACD, it should be evaluated initially using the traditional LLNA.

There is a small possibility of a false negative result (1.9% [6/318]) in the rLLNA compared to the traditional LLNA. This information should be considered when evaluating results from the rLLNA, and negative results should always prompt a weight-of-evidence evaluation of supplemental information (e.g., possibility of downturn in response at the high dose, test results with similar substances, peptide-binding activity, molecular weight, other testing data). If false negative results are suggested, confirmatory testing in the traditional LLNA or another accepted skin sensitization test method should be considered.

**ICCVAM Recommendations: Test Method Protocol**

The updated LLNA test method protocol recommended by ICCVAM is included as an appendix to this report. In the traditional LLNA, at least three dose levels of each test substance are evaluated. The rLLNA evaluates only the highest dose of the test substance along with the concurrent vehicle- and positive-control groups. ICCVAM recommends testing only the highest concentration, defined as the maximum soluble concentration that does not induce excessive local irritation and/or overt systemic toxicity.

ICCVAM recommends that individual animal data should be collected in order to permit identification and exclusion of outlier values that could cause false negative or false positive results. Collection of individual animal data (versus pooled) also allows for statistical analysis to determine whether the test-substance response is significantly different from that of the vehicle control.

The ICCVAM-recommended LLNA test method protocol has been revised to require a minimum of four animals per dose group. Data analysis indicated that reducing dose groups from five animals to four is unlikely to significantly affect the results of an LLNA study. Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429 for the LLNA currently requires at least five animals per dose group if individual animal data are collected but only four animals in each dose group if lymph nodes from all animals in the group are pooled into one sample for data collection (OECD 2002). To determine if these requirements could be harmonized without diminishing accuracy, NICEATM evaluated data from 83 LLNA studies (275 dose groups) from six different laboratories. This revision is important because many national regulations and policies require that the minimum number of animals be used for studies. Therefore, once TG 429 is updated with the revision, the collection of individual animal data will be consistent with this requirement.

ICCVAM also recommends including a positive-control substance with each test to ensure that all protocol procedures are conducted properly and all aspects of the test system work properly such that they can produce a positive response. However, similar to OECD TG 429, the updated ICCVAM-recommended LLNA test method protocol states that laboratories that conduct the LLNA at least once per month and that have a history of and a documented proficiency for obtaining consistent results with positive controls may consider testing positive control substances at intervals of no more than six months.

### ICCVAM Recommendations: Future Studies

ICCVAM recommends additional studies to further characterize and potentially improve the usefulness and applicability of the rLLNA for identifying potential skin sensitizers.

- Additional efforts should be made to understand the basis for abnormal dose responses for six substances in this evaluation that would have resulted in false negative results using the rLLNA compared to the traditional LLNA. This information should help identify ways to improve the accuracy of the rLLNA compared to the traditional LLNA. Efforts should also be made to identify data from guinea pigs and humans for substances that exhibit abnormal dose responses in the traditional LLNA. Information from post-marketing surveillance and/or occupational exposures should be collected and assessed.
- All future traditional LLNA and rLLNA studies should collect individual animal data. This will allow detection of outliers and avoidance of false negative results that can occur from pooling data that include one or more abnormally low values. Existing LLNA studies using data pooled from all animals in a dose group, such as four of the six false negative rLLNA results in this evaluation, should be evaluated further with data obtained from individual animals within each dose group to determine if pooling of data may have led to false negative outcomes.
- Data from individual animals should be collected and analyzed to identify opportunities to use fewer animals per dose group without compromising test method accuracy. The updated ICCVAM-recommended LLNA test method protocol incorporates statistical procedures necessary for such determinations. This includes evaluating the laboratory's historical positive-control database to determine if the number of animals in the concurrent positive-control group can be reduced.

### ICCVAM Recommendations: Performance Standards

The ICCVAM-recommended test method performance standards for the traditional LLNA[8] may be used to evaluate the performance of modified test methods, including the rLLNA, that are functionally and mechanistically similar to the traditional LLNA. Modified protocols for the rLLNA that adhere to the traditional LLNA performance standards would be considered acceptable for hazard identification purposes.

### Validation Status of the rLLNA Test Method

ICCVAM (1999) compared the accuracy and reliability of traditional LLNA results to results from guinea pig tests (EPA 2003) and results obtained from the human maximization test and sensitizing substances included in human patch test allergen panels. ICCVAM concluded that the LLNA was a valid alternative to currently accepted guinea pig test methods for most testing situations and that the LLNA reduces the number of animals required for testing while also refining the procedure by eliminating animal pain and distress. The LLNA was subsequently accepted by U.S. regulatory agencies as an alternative to the guinea pig tests (e.g., Guinea Pig Maximization Test and Buehler Test) for assessing the potential of substances to cause ACD.

[8] Available at http://iccvam.niehs.nih.gov/methods/immunotox/PerfStds/llna-ps.htm

The only difference between the test method protocols for the traditional LLNA and the rLLNA is the number of dose levels tested for a test substance. In the traditional LLNA, at least three dose levels are tested for each substance, with the highest dose based on maximum solubility and the avoidance of excessive local irritation and/or systemic toxicity. In contrast, only the highest dose of a substance is tested in the rLLNA (Kimber et al. 2006). Because the criteria for choosing the highest dose in the traditional LLNA and in the rLLNA are the same, the maximum dose level tested in the traditional LLNA and that tested in the rLLNA should be the same. Thus, the accuracy and reliability of the rLLNA test method should be similar for the same substances tested in the traditional LLNA, although the accuracy was slightly different based on available data described below.

**Accuracy and Reliability of the rLLNA**

The accuracy of the rLLNA for identifying potential skin sensitizers was compared to that of the traditional LLNA. In the 471 traditional LLNA studies, 318 results were positive and 153 were negative. When studies in which substances were tested more than once in the same vehicle were combined to yield an overall skin sensitization classification, 465 studies with unique combinations of substances and vehicles were evaluated, with 315 classified as sensitizers and 150 classified as non-sensitizers.

As shown in **Table 1**, compared to the traditional LLNA, the rLLNA has an accuracy of 98.7% (465/471), a sensitivity of 98.1% (312/318), a specificity of 100% (153/153), a false positive rate of 0% (0/153), and a false negative rate of 1.9% (6/318). When only unique combinations of substances and vehicles are considered, the rLLNA has an accuracy of 98.7% (459/465), a sensitivity of 98.1% (309/315), a specificity of 100% (150/150), a false positive rate of 0% (0/150), and a false negative rate of 1.9% (6/315).

**Table 1 Performance of the rLLNA in Predicting Skin Sensitizers Compared to the Traditional LLNA**

| Data | N | Accuracy | Sensitivity | Specificity | False Positive | False Negative |
|---|---|---|---|---|---|---|
| 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 the same vehicle considered together) | 465 | 98.7% (459/465) | 98.1% (309/315) | 100% (150/150) | 0% (0/150) | 1.9% (6/315) |

Abbreviation: N = number of tests
*Accuracy* = the percentage of correct outcomes (positive and negative) of a test method
*Sensitivity* = the percentage of all positive substances that are classified as positive
*Specificity* = the percentage of all negative substances that are classified as negative
*False positive rate* = the percentage of all negative substances that are falsely identified as positive
*False negative rate* = the percentage of all positive substances that are falsely identified as negative

Interlaboratory reproducibility of the rLLNA was assessed with traditional LLNA data for five substances tested independently in the same vehicle at two or three laboratories: dinitrochlorobenzene (DNCB), hexyl cinnamic aldehyde (HCA), linalool alcohol, methyl

salicylate, and potassium dichromate. All studies classified DNCB, methyl salicylate, and potassium dichromate as sensitizers or non-sensitizers (i.e., 100% concordance). HCA and linalool alcohol, which were tested independently in two laboratories, were classified as sensitizers by one traditional LLNA study and as non-sensitizers by the other study. Review of these two studies indicates that the discordant results were due to differences in the highest dose levels tested. However, because the rLLNA and traditional LLNA use identical protocols and the data sets used to evaluate their accuracy are similar, the intra- and interlaboratory reliability of the rLLNA is deemed to be similar to that of the traditional LLNA.