Executive Summary

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (traditional LLNA[4]) as a valid test method to assess most types of substances for their potential to cause skin sensitization (ICCVAM 1999). United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for ACD testing. It is now commonly used around the world.

Before a new test method such as the LLNA is accepted for regulatory testing, validation studies are conducted to assess its reliability (how well its results are reproduced within and across among laboratories (intra- and interlaboratory reproducibility) and its relevance (ability to correctly predict or measure the biological effect of interest) (OECD 1996, 2005; ICCVAM 1997, 2003). When a new test method is considered to have adequate relevance and reliability for regulatory testing purposes, ICCVAM develops and recommends performance standards based on the adequately validated reference test method. Such performance standards provide criteria for more efficiently evaluating the validity of test methods that are similar in function and mechanism to the reference test method.

When ICCVAM evaluated the LLNA in 1999, the concept of performance standards had not yet been developed. ICCVAM subsequently defined performance standards and described a process for their development (ICCVAM, 2003). Recognizing a need for LLNA performance standards, ICCVAM recently completed the development of performance standards for the LLNA so they can be used to evaluate the validity of modified versions of the traditional LLNA. The performance standards consist of (1) essential test method components, (2) reference substances, and (3) standards for accuracy and reliability that the proposed test method should meet or exceed.

ICCVAM recently updated its recommended LLNA test method protocol, and this was the key reference used to establish these LLNA performance standards. The updated LLNA test method protocol is appended to this document. ICCVAM revised the original ICCVAM protocol to include (1) guidance on when it may be appropriate to reduce the number of positive control animals, including statistical analysis to justify the reduction; (2) reduction in the minimum number of animals per dose group to four rather than the previous minimum of five; and (3) detailed guidance on evaluating local irritation and systemic toxicity to ensure that the appropriate highest dose is tested. The updated ICCVAM-recommended test method protocol for the LLNA is based on evaluation of extensive additional data and experience gained since the original evaluation in 1998.

Essential Test Method Components

To be considered functionally and mechanistically similar to the traditional LLNA, a modified LLNA test method protocol must include the following components to ensure that the same biological effect is being measured:

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[4] "Traditional LLNA" refers to the validated ICCVAM-recommended LLNA test method protocol (ICCVAM 1999), which measures lymphocyte proliferation based on incorporation of tritiated methyl thymidine into the cells of lymph nodes draining the site of test substance application.

- The test substance must be applied topically to both ears of the mouse.
- Lymphocyte proliferation must be measured in the lymph nodes draining the site of test substance application.
- Lymphocyte proliferation must be measured during the induction phase of skin sensitization.
- The highest dose selected must be the maximum soluble concentration that does not induce systemic toxicity and/or excessive local irritation.
- A vehicle control must be included in each study, and, where appropriate, a positive control should also be used.
- A minimum of four animals per dose group must be included.
- Either individual or pooled animal data may be collected. [Note: Collection of individual animal data is recommended by ICCVAM, and also required by several regulatory authorities]

If any of these criteria are not met, then these performance standards cannot be used for validation of the modified test method.

These essential test method components have been internationally harmonized for the validation of modifications to the traditional LLNA. Test method users should be aware that certain national regulatory authorities might differ in their requirements for use of a modified LLNA test method to support regulatory submissions. For example, U.S. regulators require the following:

- As the high dose, the maximum soluble concentration that does not produce systemic toxicity and/or excessive local irritation
- Collection of individual animal data
- A concurrent positive control included in each LLNA study

The performance standards provided in this document apply to the traditional LLNA test method protocol and to LLNA test method protocols with modifications that do not affect their functional and mechanistic similarity to the traditional LLNA test method protocol. However, the modified test method protocol must incorporate the essential components listed above. Modifications must be detailed and scientifically rationalized and justified; and the modified test method must perform as well as or better than the traditional LLNA. Rationale for such changes should include descriptions of and the basis for the criteria used to distinguish between sensitizers and nonsensitizers.

Reference Substances

Using established selection criteria, ICCVAM narrowed the initial database of more than 200 substances to a final list of 18 mimimum reference substances for the LLNA performance standards. The criteria were: (1) the substances should be be readily available from a commercial source; (2) LLNA data from guinea pig tests and (where possible) data from humans should be available for each substance; and (3) the minimum list of reference substances represent the types of substances typically tested for skin sensitization potential and the range of responses that can be assessed in the LLNA. Reference substances proposed

in draft LLNA Performance Standards by the European Centre for the Validation of Alternative Methods or included in validation studies by the Japanese Center for the Validation of Alternative Methods were also considered. To provide the opportunity to demonstrate performance equal to or better than that of the traditional LLNA, ICCVAM included four optional substances (substances that produced either false positive or false negative results in the LLNA when compared to either human or guinea pig results).

Test Method Performance Standard: Accuracy

The accuracy of a modified LLNA test method should meet or exceed that of the traditional LLNA when evaluated using the 18 minimum recommended reference substances, which have data from traditional LLNA and guinea pig tests. The proposed test method should result in the correct classification based on a "yes/no" decision. However, the modified test method might not correctly classify all of the reference substances on the minimum list. If, for example, one of the weak sensitizers were misclassified, a rationale for the misclassification and appropriate additional data (e.g., test results that provide correct classifications for other substances with physical, chemical, and sensitizing properties similar to those of the misclassified reference substance) could be considered to demonstrate equivalent performance. Under such circumstances, the validation status of the modified LLNA would be evaluated on a case-by-case basis.

Test Method Performance Standard: Reliability

Test method reliability is the degree to which a test method can be performed consistently/uniformly within (*intralaboratory reproducibility*) and among (*interlaboratory reproducibility*) laboratories over time. To determine intralaboratory reproducibility, a modified LLNA test method should be assessed using a sensitizing substance that is well characterized in the traditional LLNA. Therefore, the LLNA performance standard is based on the variability of results from repeated tests of hexyl cinnamic aldehyde (HCA).

Assessing the reliability of a modified test method requires calculating the estimated concentration needed to produce a stimulation index with a specific threshold value (an ECt value) in order to distinguish between sensitizers and non-sensitizers. To assess intralaboratory reliability, ECt values for HCA should be derived on four separate occasions with at least one week between tests. Acceptable intralaboratory reproducibility is indicated by a laboratory's ability to obtain, in each HCA test, ECt values between 5% and 20%, which represents the range of 0.5x to 2.0x the mean EC3 specified for HCA (10%) in the traditional LLNA.

Interlaboratory reproducibility of a modified LLNA test method should be assessed using two sensitizing substances that are well characterized in the traditional LLNA. The LLNA performance standard is based on the variability of results from tests of HCA and 2,4-dinitrochlorobenzene (DNCB) in different laboratories. ECt values should be derived independently from a single study conducted in at least three separate laboratories. To demonstrate acceptable interlaboratory reproducibility, each laboratory must obtain ECt values of 5% to 20% for HCA and 0.025% to 0.1% for DNCB, which represents the range of 0.5x to 2.0x the mean EC3 concentrations specified for HCA (10%) and DNCB (0.05%), respectively, in the traditional LLNA.

Using the Performance Standards

Test method developers are encouraged to consult directly with ICCVAM before using these performance standards to conduct a validation study of a modified LLNA test method. Developers are also encouraged to submit results of validation studies to ICCVAM for an evaluation of the validation status. Upon completing its evaluation in accordance with the ICCVAM Authorization Act (Public Law 106-545, 42 United States Code 285*l*-3[5]), ICCVAM will forward recommendations to ICCVAM agencies regarding the usefulness and limitations of the test method.

[5] Available at http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf