Appendix C

Essential Test Method Components and Other Validation Considerations for the Murine Local Lymph Node Assay[17]

[17] Based on the updated ICCVAM-recommended LLNA test method protocol in **Appendix A**.

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1.0 Essential Test Method Components

The following is a detailed description of the essential test method components for the validation of modifications to the murine local lymph node assay (LLNA) using the Interagency Committee on the Validation of Alternative Methods (ICCVAM) performance standards and the 18 required reference substances. Adherence to these essential test method components ensures that a modified test is functionally and mechanistically similar to the traditional LLNA. The essential test method components are provided as bolded text and are accompanied by additional guidance information in the bulleted text.

1. **The test substance must be applied topically to both ears of the mice.**
    - On treatment days, an appropriate volume (e.g., 25 µL) of the test substance, vehicle control, and positive control (where appropriate) should be applied to each ear.
    - Since the ear is the site of test substance application, any unique identification of the animals prior to placement in the study should not involve identification via the ear (i.e., marking, clipping, or punching of the ear).
    - The ears of all animals should be examined prior to initiation of the test to ensure there are no skin lesions present.
2. **Lymphocyte proliferation must be measured in the lymph nodes draining the site of test substance application.**
    - The basic principle underlying the LLNA is that sensitizers induce proliferation of lymphocytes during the induction phase of skin sensitization in the lymph nodes that drain the site of substance application. Test method endpoints may include cell turnover and/or cell number.
    - Under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization.
    - Since topical application of the test substance must be to the ear, the LLNA essential test method components state that measurement of lymphocyte proliferation should be from lymph nodes that drain the auricular site of test substance application.
    - **Annex I** in **Appendix A** of the ICCVAM Recommended LLNA Performance Standards describes an approach to dissection and identification of the draining auricular lymph nodes.
3. **Lymphocyte proliferation must be measured during the induction phase of skin sensitization.**
    - The LLNA measures events during the induction phase, rather than in the elicitation phase, of allergic contact dermatitis (ACD).
    - In order for a modified LLNA test method protocol to remain mechanistically and functionally similar to the LLNA, the dosing schedule should ensure that lymphocyte proliferation is only measured during the induction phase of ACD.

- Usually, the induction phase lasts eight to 15 days in humans, and five to seven days in the mouse (Saint-Mezard et al. 2003)
- Raw data and calculated results (i.e., as measured or quantified by the stimulation index [SI]) should be provided for all test substance dose levels and concurrent controls.
- Description of decision criteria for what constitutes positive and negative responses in the proposed test method and the basis for the decision criteria should be provided.
  - For example, when the threshold for a positive response is SI = 3, the test substance is regarded as a skin sensitizer when the SI for any single treatment group is ≥ 3.
  - However, the magnitude of the SI should not be the sole factor used in determining the biological significance of a skin sensitization response. Factors that could be considered in addition to the SI include: statistical analyses of individual animal data (if available), the nature of the dose-response relationship, test substance toxicity, and test substance solubility.
  - Statistical analysis of individual animal data may provide a more complete evaluation.

4. **For test substances, the highest dose selected must be the maximum soluble concentration that does not induce systemic toxicity and/or excessive local irritation. For positive control substances, the highest dose selected should exceed the known EC3 values (i.e., the estimated concentrations needed to produce an SI of 3) of the reference substances without producing systemic toxicity and/or excessive local irritation.**
   - If dose-response information is desired, then a minimum of three dose levels should be tested plus concurrent vehicle control and, where appropriate positive control. Test substance treatment dose levels should be based on the recommendations given in Kimber and Basketter (1992) and in the ICCVAM Panel Report (ICCVAM 1999). Dose levels are normally selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc.
   - Efforts should be made to identify existing information that may aid in selecting the appropriate maximum test substance dose level.
     - Guidance for determining the appropriate maximum dose based on the avoidance of excessive local irritation (indicated by erythema and/or ear swelling) and/or systemic toxicity (indicated by clinical observations) is detailed in the updated ICCVAM-recommended LLNA test method protocol (**Appendix A** of the ICCVAM Recommended LLNA Performance Standards).

5. **A vehicle control must be included in each study and, where appropriate, a positive control should be used.**

   Vehicle control

   - The response of the vehicle control group is used as the reference value against which the SI is calculated and therefore, a vehicle control must be included in each experiment.
   - The choice of vehicle should be informed by the relevant literature.
   - Other vehicles may be used if appropriate justification is provided. This may necessitate the use of additional controls in order to demonstrate that the alternative vehicle does not adversely impact the outcome of a test substance. Recommended vehicles are acetone: olive oil (4:1 v/v), N,N-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide.

   Positive control

   - The purpose of the positive control substance is to demonstrate that the test method is responding with adequate sensitivity to a sensitizing substance for which the magnitude of the response is well characterized.
   - If sensitizer(s) are run with non-sensitizers, no positive control is required (i.e., for any test, a known sensitizer from the reference substance list may serve as a positive control). If non-sensitizers are run by themselves, a positive control is required.

6. **A minimum of four animals per dose group is required.**

7. **Either individual or pooled animal data may be collected.**

   Individual animal data

   The updated ICCVAM-recommended LLNA test method protocol recommends the collection of lymph nodes from individual animals. This approach allows for:

   - Detection of problems caused by technical inexperience (Cockshott et al. 2006)
   - Identification of potential outlier responses that may aid in avoiding false negative results for weaker sensitizers (i.e., substances that normally would induce an SI just above 3 in the traditional LLNA might be incorrectly classified as negative due to an outlier value because the resulting mean SI may be less than 3 if the outlier is not identified and excluded)
   - The assessment of interanimal variability
   - Statistical comparison of the difference between test substance and vehicle control group measurements and an assessment of statistical power associated with the number of animals per group
   - Evaluation of the possibility of reducing the number of animals in the positive control group, which is only feasible when individual animal data are collected

- Recognition that certain regulatory authorities (e.g., U.S. Environmental Protection Agency [EPA], U.S. Food and Drug Administration [FDA]) require data from single animals

Pooled animal data

- The use of pooled nodes has the advantage of technical simplicity. It is the view of those who favor this approach that pooling of nodes serves to minimize variability and also serves to minimize the inevitable loss of material associated with the handling and processing of very small amounts of tissue. Although this may be of little impact generally, it may be of importance in relation to the detection of the weakest skin-sensitizing substances.
- In addition, it is worth recognizing that the great majority of the data employed in the original validation of the assay was drawn from experiments using pooled nodes from four mice, and that data generated in this manner still represents the greater part of the published data.

Assessment of lymphocyte proliferation and interpretation of results

- Lymphocyte proliferation should be expressed in the units obtained from the method (e.g., disintegrations per minute for methods using radioactive reagents; absorbance at a specified wavelength for methods using colorimetric reagents). Results should be provided for all test substance dose levels and concurrent positive and vehicle controls.

2.0 Other Validation Considerations

The following should also be considered during the validation of a modified LLNA test method using the ICCVAM LLNA performance standards and the 18 required reference substances.

1. **Use of the positive control**
    - Consideration should be given to concurrently running a mix of negative, weakly, and strongly positive substances from the reference substance list so that the strongly positive substance can act as a positive control for the weaker skin sensitizer.
2. **Group housing is recommended; otherwise animal selection, preparation, housing, and feeding should be in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 429 (OECD 2002) in compliance with other relevant regulatory requirements (e.g., animal care and use).**
3. **Appropriate quality assurance systems (e.g., Good Laboratory Practice guidelines e.g., OECD 1998; EPA 2006a, 2006b; FDA 2006) are required.**
    - Collection, recording and retention of raw and processed data
    - Data available upon request

4. **The study should be conducted according to international validation principles (e.g., OECD Guidance Document 34; OECD 2005) and in compliance with other relevant regulatory requirements (e.g., animal care and use).**