Murine Local Lymph Node Assay (LLNA) Performance Standards

European Centre for the Validation of Alternative Methods (ECVAM)
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1. BACKGROUND AND PURPOSE OF PERFORMANCE STANDARDS

1.1 Introduction

Prior to the acceptance of new test methods for regulatory testing applications, validation studies are conducted to assess reliability (the extent of intra- and inter-laboratory reproducibility) and predictive capacity (the ability of the test methods to correctly predict or measure the biological effect of interest; also referred to as relevance).

At the OECD level (1) it is agreed that the purpose of Performance Standards is to communicate the basis by which new test methods both proprietary (i.e., copyrighted, trademarked, registered) and non-proprietary can be determined to have sufficient accuracy (in this document referred to as predictive capacity) and reliability for specific testing purposes. These Performance Standards, based on validated and accepted test methods, can be used to evaluate the accuracy and reliability of other analogous test methods (also referred to as “me-too” tests) that are based on the same or similar scientific principles and that measure or predict the same biological property or toxic effect. Performance Standards such as those described below should be provided by the Management Team of a Validation Study, and, as appropriate, used in the Test Guidelines issued for new test methods.

1.2 Elements of Performance Standards

As described in the OECD “Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment” (1), the three elements of Performance Standards are:

- **Essential Test Method Components:** These consist of essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed, mechanistically and functionally similar test method. These components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to essential test method components will help to assure that a proposed test method is based on the same concepts as the corresponding validated test method.

- **A Minimum List of Reference Chemicals:** Reference chemicals are used to assess the accuracy (predictive capacity) and reliability of a proposed mechanistically and functionally similar test method. This list will comprise commercially available...
compounds and will include substances that represent a range of the chemical and product classes for which the validated test methods is considered applicable.

- **Accuracy (Predictive Capacity) and Reliability Values**: These are the performance requisites that should be achieved or exceed by the proposed test methods when evaluated using the list of reference chemicals.

2. **BACKGROUND TO THE LLNA**

The standard local lymph node assay (LLNA) is a validated and accepted method (2) used for the identification of skin sensitising chemicals. In addition, and more recently, the LLNA has been used also for measurement of the relative skin sensitising potential of contact allergens as required for effective risk assessment (3, 4).

The LLNA identifies contact allergens as a function of proliferative responses induced in draining lymph nodes following topical exposure of mice to test chemicals. The mechanistic basis for selection of this endpoint is that the acquisition of skin sensitisation is dependent upon, and correlates quantitatively with, T lymphocyte proliferation induced in regional lymph nodes draining the site of encounter with a contact allergen. Using the standard LLNA for the purposes of hazard identification, chemicals are classified as contact allergens if they elicit, at one or more test concentrations, a three-fold or greater increase in draining lymph node cell (LNC) proliferation compared with concurrent vehicle controls (a stimulation index [SI] of 3 or more). Using the standard LLNA for the purposes of assessment of relative potency, an EC3 value is derived by linear interpolation from an LLNA dose response. The EC3 value describes the amount of chemical that is required to elicit a stimulation index of 3. The greater the potency of a skin sensitising chemical, the lower will be the EC3 value. For both these purposes proliferative activity is measured as a function of the incorporation by draining LNC of radiolabelled $[^{3}\text{H}]$ thymidine ($[^{3}\text{H}]$TdR). A source of $[^{3}\text{H}]$TdR is injected intravenously into mice and draining lymph nodes are excised 5 hours later and processed for measurement of isotope incorporation by $\beta$ scintillation counting. Measurement of this endpoint by isotope incorporation has proven to be sensitive and reproducible. Other approaches for the measurement of LNC proliferative activity in the LLNA may be considered if they are shown to be as sensitive and reproducible as the validated endpoint.
3. LLNA PERFORMANCE STANDARDS

3.1 Scope

This section describes Performance Standards for the LLNA that should be used to evaluate test methods that are functionally and mechanistically similar to the traditional LLNA.

It is important to emphasise that the Performance Standards described in this document are intended for the assessment only of those methods incorporating the essential test method components described in this section. Any modification to the standard protocol should be thoroughly documented, explained and justified. Modified LLNA protocols that do not adhere to all the essential test method components, would necessarily be subjected to a more extensive evaluation and/or validation process.

If the modified LLNA protocol, like the standard LLNA, uses a decision criterion of SI ≥ 3 to distinguish between sensitisers and non-sensitisers, then the 18 reference chemicals described in this section can be used to determine its validation status. However, it is recognised that the use of an alternative read-out for LNC proliferation may require some change in the prediction model, specifically with respect to the stimulation index (SI) that is used as threshold for classifying chemicals as being positive or negative. In such cases the concentration of test material at the revised threshold limit might be other than the EC3 used in the standard LLNA and would be referred to hereafter in this document as ECt (the estimated concentration required to elicit a SI with a threshold other than three). Alternative LLNA protocols encompassing variation in the SI should be assessed on a case-by-case basis, the implication being that testing with additional chemicals might be required.
3.2 Essential Test Method Components

The following is a detailed description of the essential test method components, for the validation of modifications to the Local Lymph Node Assay (LLNA) using these 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 in bold 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 where appropriate, positive control, 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.
• The updated ICCVAM-recommended LLNA protocol 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 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 (5).

• 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, chemical toxicity, and 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
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**EC3 values 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 (6)) and in the ICCVAM Panel Report (7). 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 protocol.

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), \textit{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 tested simultaneously with non-sensitizers then a separate positive control is not required (i.e testing in such circumstances, a known
sensitizer from the reference list serves as a positive control). If non-sensitizers are tested by themselves then 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 protocol recommends the collection of lymph nodes from individual animals. This approach allows for:
   - Detection of problems caused by technical inexperience (8).
   - 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 three in the traditional LLNA might be incorrectly classified as negative due to an outlier value, because the resulting mean SI may be less than three if an outlier is not identified and excluded
   - The assessment of inter-animal 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 to reduce 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., EPA, FDA) prefer 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 favour 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 weak skin sensitizing substances.
   - In addition, it is worth recognizing that the use of pooled nodes from four mice was the source of the great majority of the data employed in the original
validation of the assay, and 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.

3.3 Data and Reporting

Reporting should comply in most respects to OECD TG 429. Any deviations from the standard LLNA protocol should be thoroughly documented, explained and justified.

The test report should include the following information:

Test substance:
- identification data (e.g. CAS number, if available; source; purity; known impurities; lot number);
- physical nature and physicochemical properties (e.g. volatility, stability, solubility);
- if mixture, composition and relative percentages of components.

Vehicle:
- identification data (purity; concentration, where appropriate; volume used);
- justification for choice of vehicle.

Test animals:
- strain of mice used;
- microbiological status of the animals, when known;
- number, age, and sex of animals;
- source of animals, housing conditions, diet, etc.
Test conditions:

- details of test substance preparation and application;
- justification for dose selection (including results from range finding studies, if conducted); vehicle and test substance concentrations used, and total amount of substance applied;
- details of food and water quality (including diet type/source, water source).

Reliability check:

- a summary of results of latest reliability check, including information on substance, concentration and vehicle used;
- concurrent and/or historical positive and negative control data for testing laboratory

Results:

- individual weights of animals at start of dosing and at scheduled kill;
- a table of test values for each dose (including vehicle control) group;
- statistical analysis, where appropriate
- time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal.

Discussion of the results

A brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test substance should be considered a skin sensitizer.
3.4 Minimum List of Reference Chemicals

Reference chemicals are used to determine if the performance of the proposed method is comparable with that of the standard LLNA. For this purpose the minimum list of reference chemicals includes 18 substances (13 sensitisers, 5 non-sensitisers) listed in Table 1. An additional four optional substances (i.e. substances which were either false positive or false negative in the traditional LLNA with respect to human or guinea pig results) are also included to provide the opportunity for demonstrating superior performance with respect to the traditional LLNA.

Chemicals have been chosen for inclusion in this list on the basis of:

- commercial availability
- varying skin sensitising potency
- well defined chemical structure
- well characterised responses in the standard LLNA and representative of a relevant range of chemistry and chemical classes
- available guinea pig data (from either M&K or Buehler) and evidence for allergic contact dermatitis in humans.
Table 1: Reference chemicals recommended for evaluation of modified LLNA methods for skin sensitisation hazard identification.

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>CASRN</th>
<th>Physical Form</th>
<th>Vehicle</th>
<th>Potency Category 4)</th>
<th>EC3 Value*</th>
<th>n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Chloro-2-methyl-4-isothiazolin-3-one</td>
<td>26172-55-4</td>
<td>Liq</td>
<td>DMF</td>
<td>Extreme</td>
<td>0.009</td>
<td>1</td>
<td>9)</td>
</tr>
<tr>
<td>2-4 Dinitrochlorobenzene</td>
<td>97-00-7</td>
<td>Sol</td>
<td>AOO</td>
<td>Extreme</td>
<td>0.04</td>
<td>13</td>
<td>10)</td>
</tr>
<tr>
<td>4-Phenylenediamine</td>
<td>106-50-3</td>
<td>Sol</td>
<td>AOO</td>
<td>Strong</td>
<td>0.11</td>
<td>10</td>
<td>10)</td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>7646-79-9</td>
<td>Sol</td>
<td>DMSO</td>
<td>Strong</td>
<td>0.6</td>
<td>2</td>
<td>11); 12)</td>
</tr>
<tr>
<td>Isoeugenol</td>
<td>97-54-1</td>
<td>Liq</td>
<td>AOO</td>
<td>Moderate</td>
<td>1.5</td>
<td>31</td>
<td>10)</td>
</tr>
<tr>
<td>2-Mercaptobenzothiazole</td>
<td>149-30-4</td>
<td>Sol</td>
<td>DMF</td>
<td>Moderate</td>
<td>1.7</td>
<td>1</td>
<td>9)</td>
</tr>
<tr>
<td>Citral</td>
<td>5392-40-5</td>
<td>Liq</td>
<td>AOO</td>
<td>Moderate</td>
<td>9.2</td>
<td>6</td>
<td>9); 13), 14)</td>
</tr>
<tr>
<td>Hexyl cinnamic aldehyde</td>
<td>101-86-0</td>
<td>Liq</td>
<td>AOO</td>
<td>Moderate</td>
<td>9.9</td>
<td>15</td>
<td>10)</td>
</tr>
<tr>
<td>Eugenol</td>
<td>97-53-0</td>
<td>Liq</td>
<td>AOO</td>
<td>Weak</td>
<td>10.1</td>
<td>4</td>
<td>10)</td>
</tr>
<tr>
<td>Phenyl benzoate</td>
<td>93-99-2</td>
<td>Sol</td>
<td>AOO</td>
<td>Weak</td>
<td>13.6</td>
<td>1</td>
<td>9); 15); 16)</td>
</tr>
<tr>
<td>Cinnamic alcohol</td>
<td>104-54-1</td>
<td>Sol</td>
<td>AOO</td>
<td>Weak</td>
<td>21</td>
<td>1</td>
<td>9)</td>
</tr>
<tr>
<td>Imidazolidinyl urea</td>
<td>39236-46-9</td>
<td>Sol</td>
<td>DMF</td>
<td>Weak</td>
<td>24</td>
<td>1</td>
<td>9)</td>
</tr>
<tr>
<td>Methyl methacrylate</td>
<td>80-62-6</td>
<td>Liq</td>
<td>AOO</td>
<td>Weak</td>
<td>95.8</td>
<td>1</td>
<td>17)</td>
</tr>
<tr>
<td>Methyl salicylate</td>
<td>119-36-8</td>
<td>Liq</td>
<td>AOO</td>
<td>N</td>
<td>1</td>
<td>9)</td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td>67-63-0</td>
<td>Liq</td>
<td>AOO</td>
<td>N</td>
<td>1</td>
<td>9)</td>
<td></td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>69-72-7</td>
<td>Sol</td>
<td>AOO</td>
<td>N</td>
<td>1</td>
<td>9)</td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
<td>50-21-5</td>
<td>Sol</td>
<td>DMSO</td>
<td>N</td>
<td>1</td>
<td>9)</td>
<td></td>
</tr>
<tr>
<td>Chlorobenzene</td>
<td>108-90-7</td>
<td>Liq</td>
<td>AOO</td>
<td>N</td>
<td>1</td>
<td>9)</td>
<td></td>
</tr>
</tbody>
</table>

Optional Substances to Demonstrate Improved Performance Relative to the Traditional LLNA

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Physical Form</th>
<th>Vehicle</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium lauryl sulphate</td>
<td>151-21-3</td>
<td>Sol</td>
<td>DMF</td>
<td>False positive</td>
</tr>
<tr>
<td>Nickel chloride</td>
<td>7718-54-9</td>
<td>Sol</td>
<td>DMSO</td>
<td>False negative</td>
</tr>
<tr>
<td>Ethylene glycol dimethacrylate</td>
<td>97-90-5</td>
<td>Liq</td>
<td>MEK</td>
<td>False positive**</td>
</tr>
<tr>
<td>Xylene</td>
<td>1330-20-7</td>
<td>Liq</td>
<td>AOO</td>
<td>False positive***</td>
</tr>
</tbody>
</table>

N = negative in standard LLNA
AOO = acetone/olive oil (4:1 v/v)
DMF = N,N-dimethylformamide
DMSO = dimethyl sulfoxide
MEK = methyl ethyl ketone
* number of LLNA studies from which the EC3 value reported was derived
** with respect to guinea pig results
*** with respect to human results
The proposed reference chemicals should be used to evaluate the performance of the proposed method. The selection of test concentrations should be guided by the EC3 value reported in Table 1. If data on additional chemicals to those proposed in the list are already available and for which adequate reference data are published, these will be used to evaluate more comprehensively the proposed test method.

3.5 Predictive Capacity and Reliability

When evaluated using the list of recommended reference chemicals (Table 1), the proposed test method should provide performance characteristics (predictive capacity and reliability) that at least meet or exceed those of the validated reference method.

To demonstrate a predictive capacity as good than that provided by the standard LLNA, the proposed test method should be able to correctly classify the 13 sensitisers and the 5 non-sensitisers (yes/no answer).

Whilst it would be expected that the alternative assay would achieve correct classification of all the chemicals in Table 1, where there was a small discrepancy (eg a misclassification of one of the seven weak sensitisers), then provision of a rationale and appropriate additional data would be assessed on a case by case basis to determine acceptability.

Furthermore, to show improved performance with respect to the standard test, four additional substances (three false positive and a false negative in the standard LLNA) may be tested.

The reliability of the proposed test method should be as good as or better than that provided by the validated reference method. For the intra-laboratory reproducibility the recommendation is that ECt values for hexyl cinnamic aldehyde should be derived on 4 separate occasions over a period of at least 12 weeks. In each instance the ECt values obtained should be between 5% and 20% (that is within the range of 0.5x and 2.0x the target EC3 for hexyl cinnamic aldehyde of approximately 10%).

For the inter-laboratory reproducibility the guideline is that at least 2 sensitising chemicals with well characterised activity in the standard LLNA should be tested independently in each of at least three separate laboratories. The chemicals selected for this purpose are shown in Table 2.
### Table 2: Reference chemicals recommended for the assessment of the inter-laboratory reproducibility of new test method

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>CASRN</th>
<th>Physical Form</th>
<th>Vehicle</th>
<th>Potency Category (4)</th>
<th>EC3 Value</th>
<th>n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-4 Dinitrochlorobenzene</td>
<td>97-00-7</td>
<td>Sol</td>
<td>AOO</td>
<td>Extreme</td>
<td>0.04</td>
<td>13</td>
<td>10)</td>
</tr>
<tr>
<td>Hexyl cinnamic aldehyde</td>
<td>101-86-0</td>
<td>Liq</td>
<td>AOO</td>
<td>Moderate</td>
<td>9.9</td>
<td>15</td>
<td>10)</td>
</tr>
</tbody>
</table>

The requirement is that all participating laboratories are able to demonstrate with at least the two suggested chemicals derivation of ECt values within the range of 0.5x and 2.0x the expected EC3 values reported in Table 2.

### 4 Other Validation Considerations

The following is a description of important points to consider during the validation of a modified LLNA test method using the Performance Standards and the 18 required reference substances.

1. **Use of the positive control**
   - Consideration should be given to testing concurrently 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 OECD TG 429 in compliance with other relevant regulatory requirements (e.g., animal care and use).**

3. **Appropriate quality assurance systems (e.g., GLP) 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 (OECD GD 34) and in compliance with other relevant regulatory requirements (e.g., animal care and use).**
5.0 REFERENCES


