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# Validation of alternative endpoints for the LLNA: General considerations

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**Abstract**
Before being considered for regulatory use, every *in vitro* or *in vivo* method should provide evidence of its relevance and reliability in a validation exercise. Validation procedures are becoming more flexible to accommodate the different needs including the evaluation of those tests which are considered similar or equivalent to an already validated one. In the field of skin sensitisation, there is considerable interest in developing modifications to the standard murine Local Lymph Node Assay (LLNA) to encompass non-radioactive endpoints. These methods, once sufficiently advanced and standardised, will need to be validated. For this purpose, Performance Standards for the LLNA are needed to guide such appraisal. In order to decide which type of evaluation a modified method needs to undergo to prove its validity, consideration has to be given as to whether minor or major modifications have been introduced compared to the validated test.

**Keywords:** performance standards, validation, LLNA

## Introduction

The assessment of the skin sensitising potential of chemicals relies on the use of animals. Beside the conventional guinea-pig tests, such as the Buehler Test (Buehler, 1965) and the Magnusson Kligman Guinea-pig Maximisation Test (Magnusson, 1970) the LLNA (Kimber, 1992) represents an alternative method. The advantages of the LLNA with respect to the traditional guinea-pig tests include a more-quantitative and more-objective assessment of the endpoint measured. Furthermore, it provides benefits in terms of animal welfare since it reduces the number of animals required, and entails less painful and stressful procedures. Following its development and optimisation, the LLNA was subjected to an extensive evaluation, including a series of collaborative trials, and the comparisons of LLNA data with data generated from other animal models and from human studies. Based on this assessment the LLNA was peer reviewed by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM; NIH, 1999) and recommended as a stand alone method for contact sensitisation hazard assessment. The scientific validity of the LLNA was subsequently endorsed by the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) (Balls, 2000). In 2002 the LLNA was adopted by the Organisation for Economic Cooperation and Development as Test Guideline (TG) N. 429 (OECD, 2002).

In the LLNA the sensitising activity of a chemical is measured as a function of lymphocyte proliferation in the auricular lymph nodes following topical exposure to the substance. Proliferative activity is determined by analysing the incorporation of tritiated thymidine after intravenous injection in the mouse.

In recent years, to avoid the use of radioactive labelling, there has been a significant investment in the development of modifications to the standard test involving non-radioisotope endpoints. These include the measurement of bromodeoxyuridine (BrdU) incorporation (Takeyoshi, 2001) and of ATP content (Yoshimura, 2007) to assess lymph node activation. Among the other endpoints that have been investigated are changes in lymph node cellularity or lymph node weight (Ehling, 2005a, 2005b), the analysis of changes in the frequency of lymph node cells subpopulations (Gerberick, 2002; Lee, 2004), and the measurement of the expression and/or production of cytokines by lymph node cells (Hatao, 1995; Dearman, 1999). The status of development/validation of some of these approaches has been presented in September 2007 at the ECVAM Workshop on "An Evaluation of Performance Standards and Non-Radioactive Endpoints for the LLNA" (Basketter, manuscript in preparation).

In order to be considered for regulatory use, such methods need to be validated, i.e. they need to demonstrate that their predictive capacity and their reliability are at least as good as or better than those of the validated test. Depending on the type of procedural modifications with respect to the standard

 

test, the new method might need to be subjected to an extensive evaluation/validation or might be assessed by a simplified procedure. In order to guide in such appraisal both ECVAM in Europe and ICCVAM in the USA have been developing Performance Standards for the LLNA. Both organisations are interacting closely in this exercise.

**Background to Performance Standards**

The concept of Performance Standards was firstly introduced in 1997 (Balls, 1997). As currently defined by the OECD (OECD, 2005) 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 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 or toxic effect.

Performance Standards should be provided by the Management Team of a Validation Study, and, as appropriate, used in the Test Guidelines issued for new test methods. The three elements of Performance Standards include:

- Essential test method components: these consist in 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 list of recommended reference chemicals that is used to assess the accuracy and reliability of a proposed mechanistically and functionally similar test method. These chemicals are a representative subset of those used to demonstrate the reliability and the accuracy of the validated method.
- Accuracy and reliability values. These are the comparable performance requisites that should be achieved by the proposed test method when evaluated using the list of reference chemicals.

So far, Performance Standards have been developed only for *in vitro* methods i.e. for *in vitro* skin corrosion testing (OECD, 2004) and *in vitro* skin irritation testing (ECVAM, 2007). Both of them should be used to evaluate the performance of similar human skin models to those that have been validated.

**Performance Standards for the LLNA**

The development of Performance Standards for the LLNA represents the first attempt to define criteria for judging the performance of methods involving modifications with respect to a validated *in vivo* test.

**Essential test method components**

There is a general agreement in relation to the fact that the essential test method components of the LLNA are those reflected in the OECD TG 429 including the detection of T-cell proliferation in the draining lymph nodes as endpoint. The incorporation of tritiated thymidine is the endpoint measurement used in the validated method, but the substitution of this with another endpoint measurement which could be shown to provide reliable assessment of T-cell proliferation at the relevant site, would not be regarded as a major change. Nevertheless, consideration should be given on whether a proposed new or revised prediction model represents a major change to the validated method. In any case, a revised prediction model should necessarily be evaluated using a different set of chemicals than those used to generate the data to derive it.

**Reference chemicals**

A defined and relevant set of reference chemicals should be used to determine if the performance of the proposed method is comparable with that of the standard LLNA. For this purpose, the number of chemicals selected should allow for a proper evaluation of modified versions of the standard test while minimising the number of animals required for such evaluation. Ideally the selection of the reference chemicals should meet the following criteria;

- should be easily available from commercial sources;
- should represent the full range of responses in the LLNA;
- should have well characterised responses in the standard LLNA and should be representative of a relevant range of chemistry and chemical classes;
- should ideally have available guinea-pig data (from either M&K or Buehler) and evidence for allergic contact dermatitis in humans.

**Performance criteria**

Criteria are needed to establish whether the predictive capacity and reliability of a new method are at least as good as those of the validated test. In

setting such criteria one should consider not only the performance of the standard test as it was assessed at the time of its peer-review but also the experience gained from the use of the test during the years that followed the evaluation. The understanding here is that the concentration at which a chemical produces a positive response in a modified version of the LLNA should be consistent with the one of the standard test. Such criterion should be considered when assessing both the predictive capacity and the reliability of a new method.

## Conclusions

Efforts have been done in developing variations to the standard LLNA which do not involve the use of radioactive labelling. Such methods need to undergo a formal validation process/evaluation to be considered and accepted for regulatory use. An understanding of what represents a minor or a major modification to the validated test is crucial for a proper evaluation of these methods. If a new method involves a minor modification then Performance Standards are to be used for its assessment, which will speed-up the validation/evaluation process and will minimise the use of laboratory animals. Activities are ongoing both in Europe and in the USA to define Performance Standards for the LLNA to be used for an objective evaluation of those methods representing minor modifications to the validated test.

## References


Buehler, E.V. (1965). Delayed contact hypersensitivity in the guinea pig. *Archives of Dermatology* **91**, 171-177.

Magnusson B, & Kligman AM. (1970). *Allergic Contact Dermatitis in the Guinea Pig: Identification of Contact Allergens*, 141pp. Springfield, IL, USA: Charles C. Thomas.

Kimber, I. & Basketter, D.A. (1992). The murine local lymph node assay; collaborative studies and new directions: A commentary. *Food and Chemical Toxicology* **30**, 165-169.

NIH (1999). *The Murine Local Lymph Node Assay. The Results of an Independent Peer Review Evaluation Coordinated By the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICEATM). NIH Publication No. 99-4494*, 211 pp. Research Triangle Park, NC, USA.

Balls, M. & Hellsten, E. (2000). Statement on the validity of the local lymph node assay for skin sensitisation testing. ECVAM Joint Research Centre, European Commission, Ispra. *Alternatives to Laboratory Animals* **28**, 366-367.

OECD (2002). *OECD Guideline for the Testing of Chemicals No. 429*: *Skin Sensitisation: Local Lymph Node Assay,* 7pp. Paris, France: Organisation for Economic Cooperation and Development.

Takeyoshi, M., Yamasaki, K., Yakabe, Y., Takatsuki, M. & Kimber, I. (2001). Development of non-radio isotopic endpoint of murine local lymph node assay, based on 5-bromo-2'-deoxyuridine (BrdU) incorporation. *Toxicology Letters* **119**, 203-208.

Yoshimura, I., Idehara, K., Omori, T., Kojima, H., Sozu, T., Arima, K., Goto, H., Hanada, T., Ikarashi, Y., Inoda, T., Kanazawa, Y., Kosaka, T., Maki, E., Morimoto, T., Shinoda, S., Shinoda, N., Takeyoshi, M., Tanaka, M., Uratani, M., Usami, M., Yamanaka, A., Yoneda, T. & Yuasa, A. Validation of LLNA-DA assay for assessing skin sensitisation potential. *Poster.* 46th SOT Meeting. 25-29 March, 2007. Charlotte, North Carolina, USA.

Ehling, G., Hecht, M., Heuser, A., Huesler, J., Gamer, A.O., Van Loveren, H., Maurer, T., Riecke, K., Ullmann, L., Ulrich, P., Vandebriel, R. & Vohr, H.W. (2005). An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay First round. *Toxicology* **212**, 60-68.

Ehling, G., Hecht, M., Heuser, A., Huesler, J., Gamer, A.O., Van Loveren, H., Maurer, T., Riecke, K., Ullmann, L., Ulrich, P., Vandebriel, R. & Vohr, H.W. (2005). An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay 2nd round. *Toxicology* **212**, 69-79.

Gerberick GF, Cruse LW, Ryan CA, Hulette BC, Chaney JG, Skinner RA, Dearman RJ,Kimber I. (2002). Use of a B cell marker (B220) to discriminate between allergens and irritants in the local lymph node assay. *Toxicological Sciences* **68**, 420-8.

Lee, K.J., Park, S.H., Byun, J.A., Kim, H. S. & Oh, HY. (2004). Evaluation of lymphocyte subpopulations in draining lymph node cells following allergen and irritant. *Environmental Toxicology and Pharmacology* **17**, 95-102.

Hatao, M., Hariya, T., Katsumura, Y. & Kato, S. (1995). A modification of the local lymph node assay for contact allergenicity screening: measurement of interleukin-2 as an alternative to radioisotope-dependent proliferation assay. *Toxicology* **12**, 15-22.

Dearman, R.J., Hilton, J., Basketter, D.A & Kimber, I. (1999). Cytokine endpoints for the local lymph node assay: consideration of interferon-gamma and interleukin 12. *Journal of Applied Toxicology*, **19**, 149-155.

Basketter, D., Cockshott, A., Corsini, E., Gerberick, F., Idehara, K., Kimber, I., van Loveren, H., Takeyoshi, M., Matheson, J., Mehling, A., Omori, T., Rovida, C., Sozu, T., Stokes, W. and Casati S. An Evaluation of Performance Standards and Non-Radioactive Endpoints for the Local Lymph Node Assay. The Report and Recommendations of ECVAM Workshop XX. *Manuscript in preparation.*

Balls, M. 1997. Defined structural and performance criteria would facilitate the validation and acceptance of alternative test procedures. ATLA **25**:483-484.

OECD (2005). OECD Series on Testing and Assessment *No. 34. Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment*, 96pp. Paris, France: Organisation for Economic Cooperation and Development.

OECD (2004). *OECD Guideline for the Testing of Chemicals No. 431*: *In Vitro Skin Corrosion: Human Skin Model Test,* 8pp. Paris, France: Organisation for Economic Cooperation and Development.

ECVAM (2007). Performance Standards for Applying Human Skin Models to In Vitro Skin Irritation Testing, 13pp. Web site: http://ecvam.jrc.it/index.htm (Accessed 12.12.07).