ICCVAM Performance Standards for the Murine Local Lymph Node Assay (LLNA)

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Abstract

ICCVAM develops performance standards to facilitate the efficient validation of modified versions of adequately validated alternative test methods. ICCVAM recently developed performance standards based on the ICCVAM-recommended LLNA protocol (ICCVAM 1999). The protocol was revised recently to reduce the minimum number of mice per dose group from five to four, and to provide guidance on reducing the number of positive control animals and determining the appropriate highest test dose. The performance standards include essential test method components, a minimum list of reference substances, and standards for accuracy and reliability. Essential test method components are the structural, functional, and procedural elements of a validated test method that must be included in a modified method in order for it to be evaluated using the established performance standards. Essential components of the LLNA include topical application of the test substance to the ears of mice, measurement of lymphocyte proliferation in the lymph nodes draining the area of test substance application, and use of the maximum soluble dose that does not result in systemic toxicity or excessive local irritation. The minimum list of reference substances for these LLNA performance standards includes 13 sensitizers and 5 non-sensitizers. The accuracy and reliability standards to be achieved by a modified LLNA are based on the performance of the traditional LLNA. These LLNA performance standards will facilitate rapid and efficient validation of modified LLNA protocols, such as those using non-radioactive markers of lymphocyte proliferation. New versions of the LLNA that provide improved performance or other advantages are expected to result in broader use of the LLNA, which will further reduce and refine animal use for allergic contact dermatitis assessments while ensuring human safety. ILS staff contributing to this abstract supported by NIEHS contract N01-ES-35504.

Introduction

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is charged by the ICCVAM Authorization Act of 2000[1] with evaluating the scientific validity of new, revised, and alternative toxicological test methods applicable to U.S. Federal agency safety testing requirements. ICCVAM is also required to provide recommendations to U.S. Federal agencies regarding the usefulness and limitations of such test methods. In 1999, ICCVAM recommended the murine (mouse) local lymph node assay (LLNA) 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"[2] as an alternative test method for skin sensitization testing. It is now commonly used around the world.

The purpose of performance standards is to communicate the basis by which new test methods have been determined to have sufficient accuracy and reliability for a specific testing purpose. When ICCVAM evaluated the LLNA in 1999, the concept of performance standards had not yet been developed. Therefore, ICCVAM is now providing performance standards for the LLNA so that modified versions of that are mechanistically and functionally similar can be effectively and efficiently evaluated for their validity. The updated ICCVAM-recommended test method protocol (Appendix A of ICCVAM 2009) is the key reference used for establishing these performance standards. ICCVAM revised the original ICCVAM protocol to include:

1. Guidance on reducing the number of positive control animals, including statistical analysis to justify the reduction
2. An extensive discussion of collecting data for individual animals and of the recommended number of animals per dose group; and
3. Detailed guidance on evaluating local irritation and systemic toxicity to ensure that the appropriate highest dose is tested.

The three elements of performance standards are:

1. Essential test method components
2. A minimum list of reference substances, and
3. The comparable accuracy and reliability that should be achieved or exceeded by a modified test method.

[1] 42 U.S.C. § 285 f-2. 2851-5 (2000). The ICCVAM Authorization Act is available on the NICEATM-ICCVAM website at http://iccvam.niehs.nih.gov/about/PL106545.pdf.

[2] The traditional LLNA refers to the validated ICCVAM LLNA test method protocol (ICCVAM 1999), which measures lymphocyte proliferation based on incorporation of tritiated methyl thymidine into the cells of the draining lymph nodes

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Timeline for the Development of ICCVAM Performance Standards for the LLNA

Date	Event
January 10, 2007	ICCVAM receives nomination from the CPSC for six LLNA review activities.
January 2007	ICCVAM IWG re-established to work with NICEATM to carry out LLNA evaluation.
January 24, 2007	ICCVAM endorses the six CPSC-nominated LLNA review activities with a high priority and also recommends the development of ICCVAM LLNA performance standards.
May 17, 2007	*Federal Register* notice (72 FR 27815) – *The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data.*
June 12, 2007	SACATM endorses with high priority the six nominated LLNA review activities and the development of ICCVAM LLNA performance standards.
August 14, 2007	IWG endorses release of the draft ICCVAM LLNA Performance Standards for public comment.
September 12, 2007	*Federal Register* notice (72 FR 52130) – *Announcement of Draft ICCVAM Performance Standards for the Murine Local Lymph Node Assay: Request for Comments.*
January 8, 2008	*Federal Register* notice (73 FR 1360) – *Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents: Request for Comments*
March 4-6, 2008	Independent Peer Review Panel Meeting, CPSC Headquarters, Bethesda, MD; public meeting with opportunity for oral public comments.
May 7-8, 2008	Meeting of the ECVAM Scientific Advisory Committee to discuss ECVAM LLNA performance standards and to consider ICCVAM's request for ECVAM and ICCVAM to develop harmonized LLNA performance standards.
May 20, 2008	*Federal Register* notice (73 FR 29136) – *Announcement of the Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments.*
June 18-19, 2008	SACATM public meeting for comments on the Panel report.
October 29, 2008	ICCVAM endorses the ICCVAM LLNA performance standards.

Abbreviations: CPSC = U.S. Consumer Product Safety Commission; ECVAM = European Centre for the Validation of Alternative Methods; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; IWG = ICCVAM Immunotoxicity Working Group; LLNA = murine local lymph node assay; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; SACATM = Scientific Advisory Committee on Alternative Toxicological Methods

LLNA Peer Review Panel Meeting

A public meeting of an independent scientific peer review panel organized by ICCVAM and NICEATM was held at the Consumer Product Safety Commission in Bethesda, MD, on March 4-6, 2008.

Charge to the Peer Review Panel Regarding LLNA Performance Standards

Are the performance standards adequate for assessing the accuracy and reliability of test method protocols that are based on similar scientific principals and that measure the same biological effect as the traditional LLNA?

Highlights of the Peer Review Panel Conclusions

Essential Test Method Components

- During validation of a modified LLNA, data must be collected at the level of individual animals to allow an estimate of the variance within control and treatment groups.
- The proposed test method must measure the induction phase of the immune response only.
- A concurrent positive control should be run with each test substance to ensure that the system is operating as expected and technical errors are not occurring. If a known sensitizer is being evaluated during the validation effort, a concurrent positive control might not be needed.
- Once the revised test method has been adequately validated, a concurrent positive control is recommended unless the laboratory has extensive historical data indicating that the positive control consistently yields statistically bioequivalent results in the modified LLNA assay under testing. Then, on a regular periodic basis, evaluation of a positive control should be recommended.

Accuracy Standards

- Ideally, the performance of an alternative LLNA protocol should be equivalent to the traditional LLNA. However, with the small number of reference substances available, establishing equivalence will be extremely difficult. Therefore, it may not be necessary to reach the same level of accuracy if appropriate rationale for any discordance is provided.
- However, the sensitizers on the list should be weighted such that the strongest sensitizers will always be identified.
- Considerable weight should be given to the balance between animal welfare and human safety when considering the adequacy of test method accuracy.

Reliability Standards

- Using an ECt range is appropriate for the intra- and inter-laboratory reproducibility analysis because a large database of LLNA studies is available for HCA and DNCB from which to determine the appropriateness of the range.

Updated ICCVAM-recommended Test Method Protocol for the LLNA (2008)

Key Elements

- The highest dose tested should be the maximum soluble concentration that does not produce systemic toxicity and/or excessive local irritation.
- Individual animal data is collected.
- A concurrent positive control is included in each LLNA study.
- A minimum of four individual animals rather than five individual animals per group is required. This was based on an evaluation of data from 83 LLNA studies (275 dose groups) from six different laboratories, which indicated that a reduction in the sample size from five to four animals per group is unlikely to have a significant impact on the results of an LLNA study. This change is important since most animal-use regulations require that the minimum number of animals be used in studies. Because OECD TG 429 specifies four animals per group when pooled data are collected and five animals per group when individual animal data are collected, only pooled data have been collected in many countries.

LLNA Protocol

Days 1 – 3. Apply 25 µL test substance in appropriate vehicle to dorsum of both ears of each of four mice in each treatment or control group.

↓

Days 4 – 5. No treatment

↓

Day 6. Inject 20 µCi ^{3}H-methyl thymidine or 2 µCi ^{125}I-iododeoxyuridine and 10^{-5} M fluorodeoxyuridine into the tail vein of each mouse.

↓

After 5 hours, harvest lymph nodes, crush, and prepare a single-cell suspension.

↓

Wash the single-cell suspension twice with phosphate buffered saline and then precipitate the DNA with 5% trichloroacetic acid at 4°C for 18 hours.

↓

Prepare for counting radioactivity by resuspending the pellet in trichloroacetic acid and adding scintillation fluid (for ^{3}H), or by adding resuspended pellets to gamma counting tubes (for ^{125}I).

↓

Count radioactivity. Average dpm for control group and treatment groups. Calculate stimulation index (SI):

SI = Treatment group mean dpm / control group dpm

SI ≥ 3 classifies substances as sensitizers.
SI < 3 classifies substances as nonsensitizers.

Independent Scientific Peer Review Panel

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Reference Substances

ICCVAM narrowed the initial LLNA database of more than 200 substances to a final list of reference substances for the LLNA performance standards. The criteria for selection included the following:

- Are readily available commercially
- Have available LLNA, guinea pig, and (where possible) human data/experience
- Represent a full range of responses from in the traditional LLNA, from negative to weak to strong, based on EC3 and SI ranges. In addition, the list was to reflect types of substances typically tested for skin sensitization potential
- Represent a relevant range of chemistry and chemical classes
- Have an approximately equal distribution of solids and liquids

The final list of minimum reference substances consists of 18 substances and four "optional" substances. The optional substances are either false positive or false negative in the traditional LLNA when compared to either human or guinea pig results. These substances provide the opportunity to demonstrate performance equal to or better than that of the traditional LLNA.

ICCVAM-recommended Performance Standards Reference Substances for the LLNA

Substance	Form	Vehicle	EC3 (%)[1]	N[2]	0.5x - 2.0x EC3	LLNA vs. GP	LLNA vs. Human
5-Chloro-2-methyl -4-isothiazolin-3-one	Liq	DMF	0.009	1	0.0045-0.018	+/+	+/+
DNCB	Sol	AOO	0.049	15	0.025-0.099	+/+	+/+
4-Phenylene-diamine	Sol	AOO	0.11	6	0.055-0.22	+/+	+/+
Cobalt chloride	Sol	DMSO	0.6	2	0.3-1.2	+/+	+/+
Isoeugenol	Liq	AOO	1.5	47	0.77-3.1	+/+	+/+
2-Mercapto-benzothiazole	Sol	DMF	1.7	1	0.85-3.4	+/+	+/+
Citral	Liq	AOO	9.2	6	4.6-18.3	+/+	+/+
HCA	Liq	AOO	9.7	21	4.8-19.5	+/+	+/+
Eugenol	Liq	AOO	10.1	11	5.05-20.2	+/+	+/+
Phenyl benzoate	Sol	AOO	13.6	3	6.8-27.2	+/+	+/+
Cinnamic alcohol	Sol	AOO	21	1	10.5-42	+/+	+/+
Imidazolidinyl urea	Sol	DMF	24	1	12-48	+/+	+/+
Methyl methacrylate	Liq	AOO	90	1	45-100	+/+	+/+
Chlorobenzene	Liq	AOO	NA	1	NA	-/-	-/*
Isopropanol	Liq	AOO	NA	1	NA	-/-	-/+
Lactic acid	Liq	DMSO	NA	1	NA	-/-	-/*
Methyl salicylate	Liq	AOO	NA	9	NA	-/-	-/-
Salicylic acid	Sol	AOO	NA	1	NA	-/-	-/-
Optional Substances to Demonstrate Improved Performance Relative to the Traditional LLNA							
Sodium lauryl sulfate	Sol	DMF	8.1	5	4.05-16.2	+/-	+/-
Ethylene glycol dimethacrylate	Liq	MEK	28	1	14-56	+/-	+/+
Xylene	Liq	AOO	95.8	1	47.9-100	+/**	+/-
Nickel chloride	Sol	DMSO	NA	2	NA	-/+	-/+

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; EC3 = estimated concentration needed to produce a stimulation index of 3; GP = guinea pig test result; HCA = hexyl cinnamic aldehyde; Liq = liquid; LLNA = murine local lymph node assay result; MEK = methyl ethyl ketone; NA = not applicable since stimulation index <3; NC = not calculated since data was obtained from a single study; No. = number; Sol = solid.

[1] Mean value where more than one EC3 value was available

[2] Number of LLNA studies from which data were obtained

[*] Presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located.

[**] = GP data not available

References

ICCVAM 1999. The Murine Local Lymph Node Assay: A Test Method for Assessing The Allergic Contact Dermatitis Potential of Chemical/Compounds. NIH Publication No. 99-4494. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf.

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Essential Test Method Components

- Essential test method components are the structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed test method that is mechanistically and functionally similar to the validated method.
- These include unique characteristics of the test method, critical procedural details, and quality control measures.
- In order for a modified LLNA test method protocol to be considered functionally and mechanistically similar to the traditional LLNA, the characteristics listed below are essential to ensure that the same biological effect is being measured accurately:
  1. The test substance must be applied topically to both ears of the mice.
  2. Lymphocyte proliferation must be measured in the lymph nodes draining the site of test substance application.
  3. Lymphocyte proliferation must be measured during the induction phase of skin sensitization.
  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.
  5. A vehicle control must be included in each study and, where appropriate, a positive control should be used.
  6. A minimum of four animals per dose group is required.
  7. Either individual or pooled animal data may be collected.

If any of the criteria are not met, then these performance standards are not applicable to 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 have requirements that differ from these essential test method components for the prospective use of a modified LLNA test method in support of regulatory submissions. For example, U.S. regulators require the following:

1. The maximum soluble concentration that does not produce systemic toxicity and/or excessive local irritation is used as the high dose.
2. Individual animal data is collected.
3. A concurrent positive control is included in each LLNA study

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.
- 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 recommended list. If, for example, one of the weak sensitizers were misclassified, a rationale for the misclassification and inclusion of 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. Assessing the reliability of a modified test method requires calculating the estimated concentration needed to produce a stimulation index for the specific threshold value (an EC† value) used to distinguish between sensitizers and nonsensitizers.
- To determine intralaboratory reproducibility, a modified LLNA test method should be evaluated using a sensitizing substance that is well characterized in the traditional LLNA. EC† values for hexyl cinnamic aldehyde (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, EC† values between 5% and 20%, which represents 0.5x to 2.0x the mean EC3 for HCA (10%) in the traditional LLNA.
- Interlaboratory reproducibility of a modified LLNA test method should be evaluated using two sensitizing substances that are well characterized in the traditional LLNA. Assessment of interlaboratory variability is evaluated using EC† values from tests of HCA and 2,4-dinitrochlorobenzene (DNCB) in different laboratories. EC† 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 EC† values of 5% to 20% for HCA and 0.025% to 0.1% for DNCB. This range was based on 0.5x to 2.0x the mean EC3 concentrations for HCA (10%) and DNCB (0.05%), respectively, in the traditional LLNA.

Conclusions

- ICCVAM's Recommended Performance Standards for the LLNA provide criteria that can be used to more efficiently and more rapidly evaluate the validity of similar new test methods.
- Test method developers are encouraged to consult directly with ICCVAM prior to conducting a validation study on modified LLNA test methods to discuss the appropriateness of using the LLNA performance standards.
- Following completion of a validation study using the LLNA performance standards, developers are encouraged to submit results to ICCVAM for an evaluation of the validation status. ICCVAM will forward recommendations on the validity of the test method to ICCVAM agencies.

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