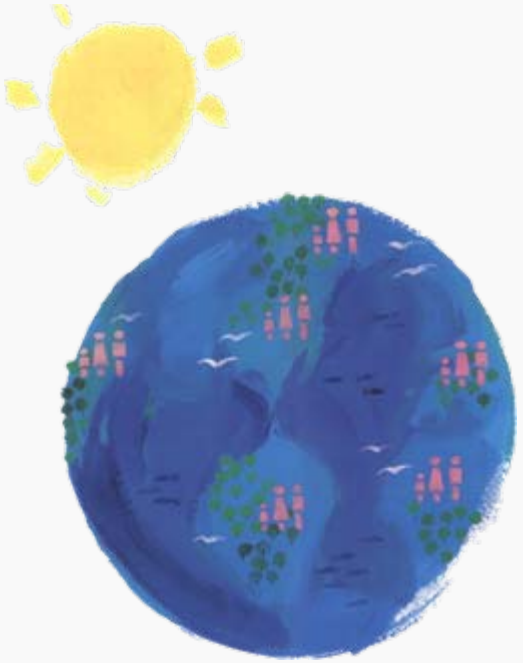
NICEATM

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

ICCVAM

Interagency Coordinating Committee on the Validation of Alternative Methods



Report on the Independent Scientific Peer Review Meeting: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA), a Test Method for Assessing the Contact Dermatitis Potential of Chemicals and Products

Introduction and Overview

Joanna Matheson, Ph.D.

Co-Chair, ICCVAM Immunotoxicity Working Group

Presented by Marilyn Wind, Ph.D.

June 19, 2008

SACATM Meeting

Research Triangle Park, NC

National Toxicology Program

NIEHS

National Institutes of Health

Disclaimer

This presentation reflects the views of the author, has not been reviewed or approved by, and may not necessarily reflect the views of the U.S. Consumer Product Safety Commission.

Timeline for ICCVAM Evaluations

2007

Jan 10	Nomination from the Consumer Product Safety Commission (CPSC) for LLNA review activities
Jan 24	ICCVAM endorses the CPSC nomination as high priority
May 17	*Federal Register Notice*: request for comments, nominations of scientific experts, and submission of data
Jun 12	SACATM endorses ICCVAM and IWG recommendations for peer review
Sep 12	*Federal Register Notice*: draft performance standards for the LLNA - request for comments

2008

Jan 08	*Federal Register* Notice: announcement of independent scientific peer review panel meeting on the LLNA; availability of documents; request for comments
Mar 4-6	Public LLNA Peer Review Panel meeting
May 19	Peer Review Panel Report available for public comment
June 18-19	***SACATM Meeting: SACATM comments***

New/Updated LLNA Applications and Protocols Reviewed by the Peer Review Panel

1. LLNA limit dose procedure
2. LLNA for testing mixtures, metals, and aqueous solutions
3. Non-radioactive LLNA: DA Method
4. Non-radioactive LLNA: BrdU-FC Method
5. Non-radioactive LLNA: BrdU-ELISA Method
6. Draft ICCVAM LLNA performance standards
7. LLNA for potency determinations

Documents Prepared by NICEATM and the ICCVAM Immunotoxicity Working Group

- Draft Background Review Document (BRD)
  - Comprehensive review of available data and information
- Draft ICCVAM test method recommendations
  - Usefulness and limitations
  - Recommended protocol
  - Future studies
- Questions for the Peer Review Panel

Overview of the Murine Local Lymph Node Assay (LLNA) Test Method Protocol

- The LLNA protocol was initially described by Kimber et al. (1986).
- The purpose of the LLNA is to identify chemical sensitizers through quantification of lymphocyte proliferation.
- The LLNA uses a minimum of three dose levels. The highest dose level should be the maximum soluble concentration that does not cause systemic toxicity or excessive local irritation.
- A Stimulation Index (SI) is calculated as the ratio of radioactivity incorporated into the cells of auricular lymph nodes of the treated animals to that of the vehicle control animals.
  - The threshold for classifying a substance as a skin sensitizer is an SI ≥ 3.
  - In order for an LLNA study to be considered acceptable, the concurrent positive control must yield an SI ≥ 3.

LLNA Test Method Protocol

Test substance applied to mouse ears on Days 1, 2, and 3

On Day 6, mice injected with radiolabeled thymidine (or an analogue of thymidine)
Radiolabeled thymidine incorporated into the DNA of proliferating cells
Lymph nodes removed from the mouse ear

Amount of radiolabeled thymidine in the lymph nodes determined as a measure of lymphocyte proliferation
Ratio of incorporated radioactivity in the auricular lymph nodes of treated vs. control mice (i.e., Stimulation Index [SI]) calculated

Negative

Sensitizer

1. LLNA Limit Dose Test Method Protocol

- The sole difference between the LLNA limit dose test method protocol and that of the traditional LLNA protocol is that only a single dose, the highest dose that does not induce systemic toxicity or excessive local irritation, is used.

LLNA Limit Dose Test Method Database

- Information included in the BRD is based on a retrospective review of traditional LLNA data that were either submitted as part of the original LLNA evaluation (ICCVAM 1999), extracted from peer-reviewed publications, or submitted to NICEATM in response to an *FR* notice requesting available data and information.
- Data from 471 studies representing 466 unique substances.
  - 211 substances were included in the 1998 ICCVAM evaluation of the traditional LLNA.

LLNA Limit Dose Test Method Performance Compared to Traditional LLNA

- Results with the LLNA limit dose test procedure almost always agree with results from the traditional LLNA.
  - Kimber et al. (2006): 98.6% accuracy (211 substances)
  - ICCVAM (2008): 98.9% accuracy (466 substances)

Draft ICCVAM Recommendations for the LLNA Limit Dose Test Method

- The LLNA limit dose procedure should be used for the hazard identification of skin sensitizing substances if dose response information is not needed
  - Use all other LLNA protocol specifications recommended by ICCVAM (ICCVAM 1999, Dean et al. 2001).
- Users should be aware that
  - The limit dose is the highest soluble concentration that does not induce overt systemic toxicity and/or excessive local irritation.
  - A small possibility of a false negative result exists (1.6% [5/313]) when compared to the traditional LLNA.

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 Toxicology Program.

Dean J, Twerdok L, Tice R, Sailstad D, Hattan D, and Stokes WS. 2001. ICCVAM evaluation of the murine local lymph node assay. Conclusions and recommendations of an independent scientific peer review panel. Regul Toxicol Pharmacol 34:258-273.

2. Updated Assessment of the Validity of the LLNA for Mixtures, Metals, and Aqueous Solutions

- A comprehensive update of available data and information regarding the current usefulness and limitations of the LLNA for assessing the skin sensitizing potential of mixtures, metals, and substances tested in aqueous solutions
- Information in the addendum is based on a retrospective review of traditional LLNA data that were either submitted as part of the original LLNA evaluation (ICCVAM 1999), extracted from peer-reviewed publications, or submitted to NICEATM in response to a *Federal Register* notice requesting available data and information (Vol. 72, No. 95, 27815-27817, May 17, 2007).
  - Current database of LLNA studies represents over 500 substances.

Substances Used for the Updated Evaluation of the Applicability Domain for LLNA

- Total of 18 mixtures
  - Ten are pesticide formulations and four are dyes.
  - The remaining four were not identified.
  - Eleven were tested in aqueous vehicles.
- Total of 17 metal compounds represented by 13 different metals
  - Aluminum, beryllium, cobalt (3), copper, gold, lead, manganese, mercury, nickel (3), platinum, potassium, tin, zinc
- Total of 21 substances tested in aqueous solutions
  - Six are pesticide ingredients.
  - The remaining 15 represent a variety of product classes.

Test Method Performance for Mixtures

- LLNA performance was compared to guinea pig data only; no human data was available for mixtures.
- The LLNA had less than 60% accuracy, sensitivity and specificity compared to guinea pig data. The false positive and false negative rates were 50% and 44%, respectively.
- There were improvements in accuracy (64%) and sensitivity (100%) when the performance evaluation was restricted to aqueous mixtures.

Test Method Performance for Substances in Aqueous Solutions

- The LLNA had 50% accuracy, 33% sensitivity and 100% specificity compared to human data. The false positive rate was 67%.
  - However, only 4 substances were available for the analysis of aqueous solutions.
  - By comparison, in the original analysis, LLNA performance compared to human data for all classes of substances (n=74) was 72%.
- The LLNA had 50% accuracy, sensitivity and specificity compared to guinea pig data. The false positive and false negative rates were high at 50% (n=6).
  - By comparison, in the original analysis, LLNA performance compared to guinea pig data for all classes of substances (n=126) was 86%.

Test Method Performance for Metal Compounds (Excluding Nickel)

- The LLNA had 86% accuracy, 100% sensitivity and 60% specificity compared to human data for all metal compounds (n=14). The false positive and false negative rates were 40% and 0%, respectively.
- The LLNA had similar accuracy and sensitivity when compared to guinea pig data (n=6). The false positive rate was 100%, albeit based on a single substance.

Draft ICCVAM Test Method Recommendations for LLNA Applicability Domain

- More data are needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures and aqueous solutions can be made.
- The LLNA appears useful for the testing of metal compounds, with the exception of nickel.
- However, the false positive rate of 40% (2/5) should be considered when evaluating positive results for metal compounds tested in the LLNA.
  - In this situation, LLNA results should always be subjected to a weight-of-evidence evaluation of supplemental information (e.g., peptide binding activity, other testing data).
  - If false positive results are suggested, confirmatory testing in another accepted skin sensitization test method should be considered.

3. LLNA:DA Test Method Protocol

LLNA Type	Days 1, 2, & 3	Days 4 & 5	Day 6	Day 7	Day 8
LLNA:DA	Pretreat with 1% SLS solution After one hour, apply 25 μL of test substance or vehicle to dorsum of each ear	———	———	Pretreat with 1% SLS solution After one hour, apply 25 μL of test substance or vehicle to dorsum of each ear	Excision of auricular lymph nodes Measurement of ATP content in lymph node cells
Traditional LLNA	Apply 25 μL of test substance or vehicle to dorsum of each ear	———	Administer ^{3}H-thymidine or ^{125}I via tail vein Excision of auricular lymph nodes Measurement of radioactivity incorporated into lymph node cells	———	———

Abbreviations: ATP=adenosine triphosphate; SLS=Sodium lauryl sulfate; Trad.=Traditional.

LLNA:DA Test Method Data

- Data from Daicel Chemical Industries, Ltd. on 31 substances tested in one laboratory.
  - *Received original individual animal data for these tests after the release of the BRD; provided to Peer Review Panel on January 30, 2008*
- Two of the 31 substances (isoeugenol and eugenol) were tested in the LLNA:DA at varying concentrations, in three different experiments, in order to assess intralaboratory reproducibility.
- Two-phased interlaboratory validation study evaluated the reliability and relevance of the LLNA:DA.
  - First phase: 10 laboratories, 12 coded substances
  - Second phase: 7 different laboratories, 5 coded substances
  - Combined: 17 laboratories, 14 different coded substances
  - Two substances not previously tested among the 31 original substances
  - *Individual animal data were not received by the time of the Peer Review Panel meeting (received on May 17, 2008)*

LLNA:DA Test Method Performance

- The LLNA:DA had at least 90% accuracy, sensitivity and specificity when compared to the traditional LLNA.
  - The false positive and false negative rates were 10% and 5%, respectively.
- Performance of the LLNA:DA was identical to the traditional LLNA when compared to human data (n=26).
- The LLNA:DA had a slightly lower performance than the traditional LLNA when compared to guinea pig data (i.e., 80% accuracy vs. 88% for the traditional LLNA, n=25).

Draft ICCVAM Test Method Recommendations for the LLNA:DA

- The LLNA:DA may be useful for identifying substances as potential skin sensitizers and non-sensitizers.
- If false results are suggested based on a weight-of-evidence evaluation, confirmatory testing in the traditional LLNA or another accepted skin sensitization test method should be considered.
- These recommendations are contingent upon receipt of additional data and information.
  - A discussion regarding the potential reason for the negative result for 2-mercaptobenzothiazole, which is a commonly used positive control substance for the traditional LLNA.
  - The original records for the interlaboratory validation studies.
  - A detailed protocol from Daicel Chemical Industries, Ltd.

4. LLNA:BrdU-FC Test Method Protocol

- The LLNA:BrdU-FC protocol is identical to the traditional LLNA protocol except
  - IP injection of BrdU (instead of IV injection of ^{3}H-thymidine) on day 5 with harvest lymph nodes on day 6.
  - Lymph node cell proliferation is assessed using flow cytometry to detect individual cells containing BrdU-labeled DNA.
  - SI = Proportion of BrdU-labeled cells in the treatment group/Proportion of BrdU-labeled cells in the vehicle control group
- For substances with SI ≥3
  - Irritation is assessed (mouse ear swelling, >25% indicates irritation)
  - Optional Immunophenotyping Step (when mouse ear swelling >25%)

LLNA:BrdU-FC Test Method Data

- Data analyzed included:
  - Data submitted by MB Research Labs from testing 45 substances (3 additional substances had no comparative traditional LLNA data)
  - Original study records have not been obtained.
- Three of the 45 substances produced an "equivocal" result in the LLNA:BrdU-FC, one of which has been commonly used as a positive control in the traditional LLNA (2-mercaptobenzthiazole).
  - The rationale for the repeat testing of these substances and possible reasons for the discordant results have been requested, but have not been provided.
- There has not been an evaluation of interlaboratory reproducibility.

LLNA:BrdU-FC Test Method Performance

- The LLNA:BrdU-FC had at least 90% accuracy and sensitivity compared to the traditional LLNA (n=45). The false positive and false negative rates were 21% and 0%, respectively.
- The LLNA:BrdU-FC had slightly higher accuracy (69%) and a lower false negative rate (27%), but a higher false positive rate (44%) than the traditional LLNA when compared to human data (n=42).
- The LLNA:BrdU-FC had lower accuracy than the traditional LLNA (76% vs. 86%) when compared to guinea pig data (n=37).

Draft ICCVAM Test Method Recommendations for the LLNA:BrdU-FC

- The LLNA:BrdU-FC may be useful for identifying substances as potential skin sensitizers and non-sensitizers.
- However, at this time, more information and data are needed before a recommended use of the LLNA:BrdU-FC can be made.
  - The rationale for the repeat testing of the three (of 45) substances that produced "equivocal" results in the LLNA:BrdU-FC, one of which has been commonly used as a positive control in the traditional LLNA (2-mercaptobenzthiazole).
  - An evaluation of interlaboratory reproducibility is critical if this test method is to be accepted for use in laboratories other than that of the test method developer.
  - Original records (including original animal data) for the tests included in this evaluation.

5. LLNA:BrdU-ELISA Test Method Protocol

- The LLNA:BrdU-ELISA protocol is identical to the traditional LLNA protocol except
  - Lymph node cell proliferation is assessed by measuring the incorporation of BrdU (administered via intraperitoneal injection) into the cells using ELISA
  - SI values other than three as the threshold for a positive response have been considered.

LLNA:BrdU-ELISA Test Method Data

- Data were available for a total of 29 substances that were tested in one laboratory
  - 24/29 substances had been previously tested in the traditional LLNA.
- Intralaboratory data for five substances tested multiple times in one laboratory.
- Two-phased interlaboratory study has recently been completed (Data have not yet been provided).
  - Phase 1: 12 chemicals tested across 9 labs
  - Phase 2: 10 chemicals tested across 7 labs
  - All labs tested the same concentrations of the coded chemicals
    - *NOTE: Dosing solutions (already diluted to the requisite concentrations) provided to each laboratory by the Study Management Team*

LLNA:BrdU-ELISA Performance Using Various Decision Criteria to Identify Sensitizers

Criterion for Positive Results	N	Accuracy	Sensitivity	Specificity	False Positive	False Negative
Statistics[1]	23	87% (20/23)	88% (15/17)	83% (5/6)	17% (1/6)	12% (2/17)
≥ 95% CI	23	91% (21/23)	94% (16/17)	83% (5/6)	17% (1/6)	6% (1/17)
≥ 2 SD	23	91% (21/23)	94% (16/17)	83% (5/6)	17% (1/6)	6% (1/17)
≥ 3 SD	23	87% (20/23)	88% (15/17)	83% (5/6)	17% (1/6)	12% (2/17)
SI ≥ 3.0	23	74% (17/23)	71% (12/17)	83% (5/6)	17% (1/6)	29% (5/17)
SI ≥ 2.5	23	78% (18/23)	77% (13/17)	83% (5/6)	17% (1/6)	24% (4/17)
SI ≥ 2.0	23	78% (18/23)	77% (13/17)	83% (5/6)	17% (1/6)	24% (4/17)
SI ≥ 1.5[2]	23	91% (21/23)	94% (16/17)	83% (5/6)	17% (1/6)	6% (1/17)
SI ≥ 1.3[2]	23	96% (22/23)	100% (17/17)	83% (5/6)	17% (1/6)	0% (0/17)

Abbreviations: CI=Confidence interval; N = Number of substances included in this analysis; SD=Standard deviation; SI=Stimulation index

[1]Statistical test for difference of group means.

[2]More than five animals per group would be necessary to achieve 95% power for detecting a positive response using this criterion.

Draft ICCVAM Test Method Recommendations for LLNA:BrdU-ELISA

- The LLNA:BrdU-ELISA may be useful for identifying substances as potential skin sensitizers and nonsensitizers.
- However, at this time, more information and data are needed before a recommended use of the LLNA:BrdU-ELISA can be made.
  - A detailed protocol, including a defined and adequately justified decision criteria for distinguishing between sensitizers and non-sensitizers.
  - Quantitative results for all of the studies included in this evaluation (provided on February 25, 2008)
  - A formal evaluation of interlaboratory reproducibility. Two interlaboratory validation studies have been completed for the LLNA:BrdU-ELISA, but information about the study designs, the protocol, and the results are not yet available.
    - *Study design and protocol were provided February 27, 2008.*

6. Draft LLNA Performance Standards

- Proposed for the assessment of versions of the LLNA that vary **only** from the ICCVAM recommended LLNA (ICCVAM 1999, Dean et al. 2001) by using non-radioactive versus radioactive methods for assessing lymphocyte proliferation in the draining auricular lymph nodes.
- The modified LLNA procedure should adhere to the ICCVAM recommended LLNA procedures in all other aspects
  - Examples: strain of mice, timing of exposures, route and sites of exposure, measured endpoint (lymphocyte proliferation in the draining auricular lymph nodes).
  - All procedural modifications should be accompanied by a scientific rationale.
    - Other, more significant changes to the traditional LLNA would necessarily be subject to a more extensive evaluation and/or validation process.

Essential Test Method Components

- Should follow the LLNA procedure described by ICCVAM (1999; Dean et al. 2001) and the EPA Health Effects Test Guidelines (EPA 2003), which requires:
  - A concurrent positive control
  - Five animals per dose group
  - Collection and analysis of individual animal data
- Only change would be the method used to evaluate lymphocyte proliferation.

Proposed Minimum List of Reference Substances

- Includes a total of 22 substances
  - 18 “required” substances
    - 13 sensitizers
    - 5 non-sensitizers
  - 4 “optional” substances for demonstrating improved performance
- Representative of the full range of responses in the LLNA, from negative to strongly positive
- Available LLNA, guinea pig, and/or human data

Proposed Accuracy Standards

- Based on a chemical-by-chemical match
  - An alternative protocol must obtain the correct call for all of the “required” reference substances (n = 18) on the list
  - For the sensitizing reference substances (n = 13) must also obtain an EC “threshold” (ECt, the estimated concentration required to produce a “threshold” response; e.g., EC3) that falls within the 0.5x to 2.0x EC3 included in the reference substances list.
- The set of “optional” substances (n = 4) could be tested to demonstrate improved accuracy vs. the traditional LLNA.

Proposed Intralaboratory Reproducibility Standards

- ECt values for hexyl cinnamic aldehyde (HCA) should be derived on four separate occasions and at least one week between tests to ensure that the tests are independent.
- Acceptable reproducibility = ECt values for HCA that are within 0.5x to 2.0x the EC3 concentration (5% to 20%).

Proposed Interlaboratory Reproducibility Standards

- Two specified chemicals with known skin sensitizing potential (2,4-dinitrochlorobenzene [DNCB] and HCA) are to be tested
- ECt values for DNCB and HCA should be derived at least once in at least three separate laboratories.
- Acceptable reproducibility = ECt values for HCA and DNCB that are within 0.5x to 2.0x the EC3 concentration (5% to 20% and 0.025 to 0.1%, respectively) for all three laboratories.

7. LLNA for Potency Categorization

- Evaluation of the usefulness and limitations of the murine local lymph node assay (LLNA) as a stand-alone assay for the hazard categorization of skin sensitization potency.
- The LLNA was evaluated for its ability to categorize substances for skin sensitization potency based on the EC3 (i.e., estimated concentration that produces a 3-fold increase in lymphocyte proliferation over a vehicle control).

Proposed Classification Categories for Skin Sensitizers

Category	LLNA EC3	Human Threshold[1]	GPMT Response	BT Response
1 (Strong sensitizer)	Option A ≤1% Option B ≤2%	Option A <250 μg/cm^2 Option B <500 μ g/cm^2	≥60% responders at >0.1% to ≤1.0% intradermal induction dose OR ≥30% responders at ≤0.1% intradermal induction dose	≥60% re0sponders at >0.2% to ≤20% topical induction dose OR ≥15% responders at ≤0.2% topical induction dose

[1]Human threshold for this purpose is no observed effect level (NOEL), lowest observed effect level (LOEL) or LOEL/10 from human maximization tests (HMT) or human repeat patch insult tests (HRIPT).

LLNA, Guinea Pig, and Human Test Method Data

- Data analyzed included:
  - 170 substances with LLNA, human, and/or guinea pig data
    - 112 substances with LLNA and human data (97 with human NOELs and/or LOELs, 15 non-sensitizers)
    - 105 substances with LLNA and guinea pig data (52 sensitizers, 53 non-sensitizers)
    - 47 substances with LLNA, human, and guinea pig data (34 sensitizers, 13 non-sensitizers)

LOEL = lowest observed effect level; NOEL = no observed effect level

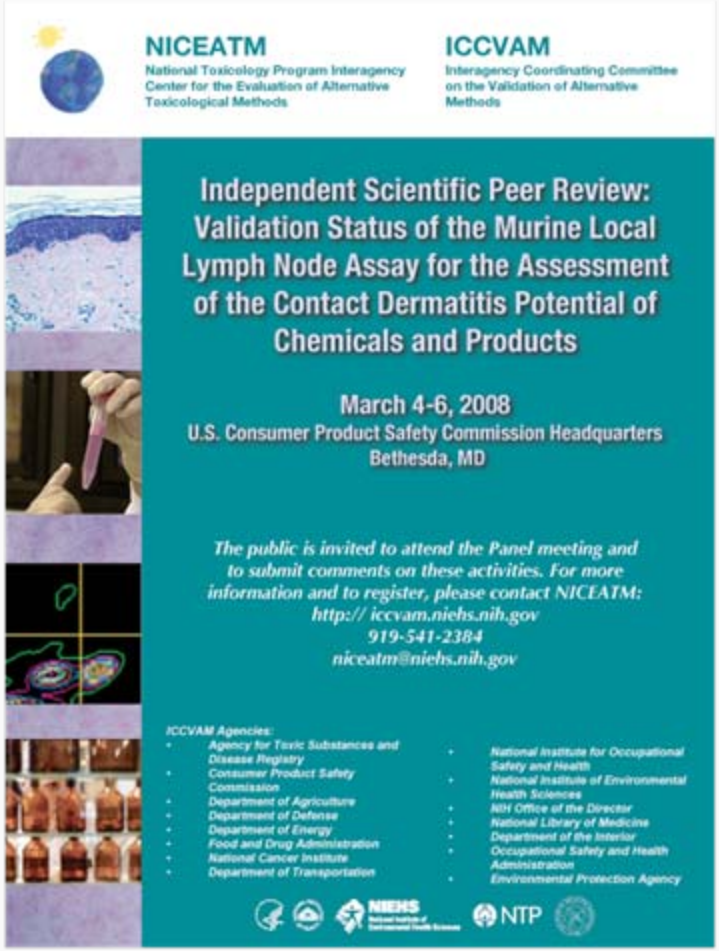
Rates for Prediction of Human Potency Category: LLNA EC3 vs. Guinea Pig Data

- At the optimized calculated LLNA EC3 value (9.4%), there is approximately a 60% correct call for the two human cut-off values, with a better performance for the human cut-off value of 250 µg/cm².
- Categorization for strong sensitizers category is better than for weak sensitizers (75% correct for strong vs. 53% correct for weak).
- The guinea pig classification cut-offs have a poorer predictive performance (~ 50% correct) than the LLNA EC3 for strong sensitizers but a better predictive performance for non-sensitizers (75% correct).

Draft ICCVAM Recommendations for the Use of LLNA for Skin Sensitization Potency Categorization

- Although there is a significant positive correlation between LLNA EC3 values and human sensitization threshold doses, this correlation is not strong (R^2=0.405).
- Therefore, the LLNA should not be considered as stand-alone test method for predicting skin sensitization potency category, but should instead be used as part of a weight-of-evidence evaluation to discriminate between strong and weak sensitizers.

Independent Scientific Peer Panel



- Held March 4-6, 2008 at CPSC Headquarters, Bethesda, MD
- Evaluated Modifications and New Applications of the Murine Local Lymph Node Assay
- Panel included international experts in dermatology, toxicology, biostatistics, regulatory policy, immunology and veterinary medicine
- Over 50 people from five countries attended.