Appendix E

Supporting Documents: Development of the ICCVAM LLNA Performance Standards

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Appendix E1

Methods Applicable to the ICCVAM LLNA Performance Standards and Essential Test Method Components

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1.0 Introduction

In 2007, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) developed draft murine local lymph node assay (LLNA) Performance Standards, which were announced and released to the public for comment in a *Federal Register (FR)* notice on September 12, 2007 (72 FR 52130).[20] The European Centre for the Validation of Alternative Methods (ECVAM) also independently drafted LLNA performance standards in 2007, and the Japanese Center for the Validation of Alternative Methods (JaCVAM) initiated two validation studies of modified LLNA test methods using a list of proposed reference substances to evaluate their validity. With obvious international interest in developing LLNA performance standards, ICCVAM, JaCVAM, and ECVAM agreed that it would be useful to work together to attempt to develop internationally harmonized LLNA performance standards that could be proposed for inclusion in the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429, which describes the use of the LLNA for determining allergic contact dermatitis potential of chemicals and other substances.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and ICCVAM interacted closely with ECVAM and JaCVAM through the ECVAM and JaCVAM liaisons to the ICCVAM Immunotoxicity Working Group (IWG) and representatives of the ECVAM Task Force on Skin Sensitization. Additionally, at their October 2007 meeting, the ECVAM Scientific Advisory Committee considered both drafts of the LLNA performance standards (i.e., ICCVAM and ECVAM versions), along with the ICCVAM recommendations for a process to achieve harmonization of the two documents and subsequently deferred their evaluation of LLNA performance standards until their November 2008 meeting. They encouraged ECVAM and ICCVAM to continue working together to reach agreement on any outstanding differences.

After considering these comments, ICCVAM announced in an *FR* notice on January 8, 2008 (73 FR 1360),[21] the availability of a revised draft version of the LLNA Performance Standards. The ICCVAM Independent Scientific Peer Review Panel (Panel) considered the revised draft Performance Standards at a public meeting convened on March 4-6, 2008, at the Consumer Product Safety Commission Headquarters in Bethesda, MD. All comments received in response to the *FR* notice were provided to the Panel for their consideration. Subsequently, the Panel's conclusions and recommendations were announced in a May 2008 *FR* notice (73 FR 29136),[22] released to the public and to ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) for comment at their public meeting convened on June 18-19, 2008. The Panel Report and all comments by the public and SACATM were considered by the ICCVAM IWG and ICCVAM in preparing final LLNA performance standard recommendations for submittal to U.S. Federal agencies and for release to the public. Performance standards adopted by U.S. Federal regulatory authorities can be provided or referenced in test guidelines. Availability of these performance standards and ICCVAM test method evaluation reports, which provide ICCVAM recommendations

[20] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_18011.pdf
[21] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf
[22] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-11195.pdf

and a comprehensive evaluation of the usefulness and limitations of a test method, are announced in the *FR*, NTP newsletters, and by email to NICEATM-ICCVAM email list subscribers.

2.0 Revisions to the Methods Applicable to the ICCVAM LLNA Performance Standards

The original draft ICCVAM LLNA Performance Standards (i.e., the version released on September 12, 2007) stated that they were intended for LLNA test method protocols that incorporate modifications that use non-radioactive procedures (rather than radioactivity, which is used in the traditional LLNA) to evaluate lymphocyte proliferation in the draining auricular lymph nodes. After discussions with members of the ECVAM Skin Sensitization Task Force, the draft document was updated to reflect increased specificity with respect to the allowable modifications to the LLNA test method protocol. The performance standards released on January 8, 2008, were applicable only to LLNA test method protocols with "minor" modifications that vary only by using non-radioactive methods for assessing lymphocyte proliferation in the draining auricular lymph nodes. All other test method protocol modifications such as the strain of mice, the timing of exposures, the route and sites of exposure, and the measured endpoint (lymphocyte proliferation in the draining auricular lymph nodes) were considered "major" modifications. The performance standards stated that LLNA test method protocols with "major" modifications would be subjected to a more extensive validation effort.

However, the Panel considered the draft LLNA Performance Standards to be appropriate for evaluating modifications other than those defined as "minor." The Panel recommended that, instead of defining "minor" and "major" modifications, the performance standards should define criteria to ensure that a modified test method is mechanistically and functionally similar to the traditional LLNA. Thus, taking into consideration the Panel's comments, along with those of SACATM and the public, and relevant IWG discussions, the final ICCVAM LLNA Performance Standards indicate that they are to be applied to modified methods that are mechanistically and functionally similar to the traditional LLNA (see **Section 2.2** of the ICCVAM Recommended LLNA Performance Standards).

3.0 Revisions to the Essential Test Method Components of the ICCVAM LLNA Performance Standards

The original draft ICCVAM LLNA Performance Standards, released on September 12, 2007, stated that the essential test method components included all aspects of the traditional LLNA test method protocol as described by ICCVAM (1999) and Dean et al. (2001), upon which OECD TG 429 (OECD 2002) was based, with the exceptions being the method used to assess lymphocyte proliferation and the corresponding decision criteria for classifying a test substance as positive or negative. The original draft Performance Standards then described the information that should be provided to support the use of test method protocols that incorporate specific modifications, which were to focus specifically on incorporating non-radioactive procedures to assess to the measurement of lymphocyte proliferation. The essential test method components included as appendix to the document provided a list of the test method protocol elements such as animal species and housing, number of doses to test, selection of doses, etc.

The January 8, 2008, draft ICCVAM LLNA Performance Standards elaborated by noting that modified LLNA test method protocols with changes to any of the essential test method components were defined as "major" modifications to the traditional LLNA test method protocol and would therefore be subject to a more extensive evaluation and/or validation process than a comparison to the LLNA performance standards.

As noted above, the Panel recommended that, instead of defining "minor" and "major" modifications, the performance standards should define criteria to ensure that a modified test method is mechanistically and functionally similar to the traditional LLNA. In this regard, the final ICCVAM LLNA Performance Standards document now describes all of the essential test method components for the LLNA, detailed in **Appendix C**. This document indicates that modified LLNA test method protocols could include modifications that do not impact the functional and mechanistic basis of the method. Seven essential test method components are identified as the elements that determine whether a modified LLNA test method protocol is functionally and mechanistically similar to the traditional LLNA. If any of the criteria are not met, then these performance standards are not applicable to validation of the modified test method.

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 for testing 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 concentration needed to produce a stimulation index of 3) of the reference substances without producing 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.

Following are additional points to consider during the validation of modified LLNA test methods applicable to these performance standards, using the 18 required reference substances:

1. Consideration should be given to running concurrently a mix of negative, weakly, and strongly positive substances from the reference substance list so that the strongly positive substances 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 (i.e., in accordance with Good Laboratory Practice guidelines e.g., OECD 1998; EPA 2006a, 2006b; FDA 2006) are required.
4. The study should be conducted according to international validation principles (OECD Guidance Document 34 [OECD 2005]) and in compliance with other relevant regulatory requirements (e.g., animal care and use).

Thus, the final ICCVAM LLNA Performance Standards can be applied to a modified LLNA test method protocol provided that (1) the modified test method protocol incorporates the essential test method components, (2) test method protocol modifications are detailed and scientifically justified, and (3) the performance of the modified test method is equal to or better than that determined for the traditional LLNA.

Appendix E2

Selection of Proposed Performance Standards Reference Substances

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1.0 Revisions to the Draft ICCVAM List of Reference Substances for LLNA Performance Standards

Twenty substances were originally selected as proposed minimum reference substances for the murine local lymph node assay (LLNA) performance standards. The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) subsequently reviewed the original proposed list of minimum reference substances, and prepared revised draft LLNA Performance Standards and a revised draft proposed reference substances list (i.e., released to the public on January 8, 2008; 73 FR 1360[23]) As in the original draft ICCVAM LLNA Performance Standards (released to the public on September 12, 2007; announced in 72 FR 52130[24]), the criteria for consideration on any subsequent revisions to the reference substances list was that the substances:

- Are readily available commercially
- Have available LLNA data (including stimulation index [SI] and EC3, i.e., the estimated concentration needed to produce an SI of 3)
- Have available guinea pig data (i.e., Guinea Pig Maximization Test [GPMT] or Buehler Test [BT])
- Where possible, have available human data/experience (e.g., Human Maximization Test results, Human Repeat Insult Patch Test results, available as a patch test kit allergen, and/or clinical case studies/reports)

The criteria used to narrow this list to the draft reference substances were that the substances on the list also:

- Represent the full range of responses in the LLNA, from negative to highly positive/extreme sensitizer, based on EC3 and SI ranges
- Represent a relevant range of chemistry and chemical classes
- Have an approximately equal distribution of solids and liquids
- Include consideration of substances that were proposed in draft European Centre for the Validation of Alternative Methods (ECVAM) LLNA Performance Standards and/or included in Japanese Center for the Validation of Alternative Methods (JaCVAM) validation studies

The final list of reference substances includes 22 substances based on the revised design of the performance analysis, where 18 required substances must be tested and produce the same response as the traditional LLNA with the provision that a weak sensitizer may be missed. In addition, there are four optional substances that may be tested to demonstrate improved performance relative to the traditional LLNA. The revisions to the draft ICCVAM performance standards reference substance list for the LLNA were based on all comments received and on comparison to the proposed substances in the ECVAM draft LLNA

[23] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf
[24] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_18011.pdf

Performance Standards. Since during this period ECVAM also revised their draft LLNA Performance Standards and changed their list of reference substances, all 22 substances are included in both the final ICCVAM and ECVAM reference substances lists. In addition there are six substances in common between the final ICCVAM list and the list of substances used by JaCVAM in their recent validation efforts. **Table E-1** provides the final list of proposed ICCVAM LLNA performance standards reference substances.

Table E-1 ICCVAM-Recommended Performance Standards Reference Substances for the LLNA

Number	Substance	CASRN	Form	Veh	EC3 (%)[1]	N[2]	0.5x - 2.0x EC3	Actual Range	LLNA vs. GP	LLNA vs. Human
1	5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	Liq	DMF	0.009	1	0.0045-0.018	NC	+/+	+/+
2	DNCB	97-00-7	Sol	AOO	0.049	15	0.025-0.099	0.02-0.094	+/+	+/+
3	4-Phenylenediamine	106-50-3	Sol	AOO	0.11	6	0.055-0.22	0.07-0.16	+/+	+/+
4	Cobalt chloride	7646-79-9	Sol	DMSO	0.6	2	0.3-1.2	0.4-0.8	+/+	+/+
5	Isoeugenol	97-54-1	Liq	AOO	1.5	47	0.77-3.1	0.5-3.3	+/+	+/+
6	2-Mercaptobenzothiazole	149-30-4	Sol	DMF	1.7	1	0.85-3.4	NC	+/+	+/+
7	Citral	5392-40-5	Liq	AOO	9.2	6	4.6-18.3	5.1-13	+/+	+/+
8	HCA	101-86-0	Liq	AOO	9.7	21	4.8-19.5	4.4-14.7	+/+	+/+
9	Eugenol	97-53-0	Liq	AOO	10.1	11	5.05-20.2	4.9-15	+/+	+/+
10	Phenyl benzoate	93-99-2	Sol	AOO	13.6	3	6.8-27.2	1.2-20	+/+	+/+
11	Cinnamic alcohol	104-54-1	Sol	AOO	21	1	10.5-42	NC	+/+	+/+
12	Imidazolidinyl urea	39236-46-9	Sol	DMF	24	1	12-48	NC	+/+	+/+
13	Methyl methacrylate	80-62-6	Liq	AOO	90	1	45-100	NC	+/+	+/+
14	Chlorobenzene	108-90-7	Liq	AOO	NA	1	NA	NA	-/-	-/*
15	Isopropanol	67-63-0	Liq	AOO	NA	1	NA	NA	-/-	-/+
16	Lactic acid	50-21-5	Liq	DMSO	NA	1	NA	NA	-/-	-/*
17	Methyl salicylate	119-36-8	Liq	AOO	NA	9	NA	NA	-/-	-/-
18	Salicylic acid	69-72-7	Sol	AOO	NA	1	NA	NA	-/-	-/-

Number	Substance	CASRN	Form	Veh	EC3 (%)[1]	N[2]	0.5x - 2.0x EC3	Actual Range	LLNA vs. GP	LLNA vs. Human
Optional Substances to Demonstrate Improved Performance Relative to the Traditional LLNA										
19	Sodium lauryl sulfate	151-21-3	Sol	DMF	8.1	5	4.05-16.2	1.5-17.1	+/-	+/-
20	Ethylene glycol dimethacrylate	97-90-5	Liq	MEK	28	1	14-56	NC	+/-	+/+
21	Xylene	1330-20-7	Liq	AOO	95.8	1	47.9-100	NC	+/**	+/-
22	Nickel chloride	7718-54-9	Sol	DMSO	NA	2	NA	NA	-/+	-/+

Abbreviations: AOO = acetone: olive oil (4:1); CASRN = Chemical Abstracts Service Registry Number; 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; Sol = solid; Veh = vehicle

[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

2.0 Rationale for Exclusion of Substances from the Revised ECVAM List or Removal of Substances from the Original Draft ICCVAM List

Table E-2 details the current revisions to the draft ICCVAM-recommended performance standards reference substances for the LLNA based on the LLNA Peer Review Panel meeting, public comments, and comparison with the revised draft ECVAM LLNA Performance Standards. The original ICCVAM list represents the draft version released for public comment on September 12, 2007, and initial revisions to the original ICCVAM list were provided to the Peer Review Panel and released to the public on January 8, 2008. The revised ECVAM list represents the version distributed to the ECVAM Scientific Advisory Committee (ESAC) members for discussion at its 28th ESAC meeting on May 7-8, 2008.

Initially, based on comments received from ECVAM and additional searches by NICEATM for reference data, six substances from the original ICCVAM list (i.e., the September 12, 2007 version) were not included on the revised list of ICCVAM reference substances (i.e., the January 8, 2008 draft). These substances and the rationale for their exclusion are as follows:

- Benzoquinone was removed because no human data were located. Another substance, 5-chloro-2-methyl-4-isothiazolin-3-one, was identified as an adequate replacement based the availability of concordant guinea pig and human data for this substance and its associated history of demonstrated results in the guinea pig and human as an extreme sensitizer.
- Cinnamic aldehyde was removed in response to an ECVAM comment noting that another aldehyde (hexyl cinnamic aldehyde [HCA]) was already on the list, which is also a positive control substance used in the traditional LLNA.
- Formaldehyde was removed in response to an ECVAM comment noting that another aldehyde (HCA) was already on the list. HCA has also been extensively studied as a sensitizing substance and is a positive control substance used in the traditional LLNA.
- 2-Hydroxyethyl acrylate was removed in response to an ECVAM comment that suggested this substance is unstable and is therefore susceptible to variable results.
- Nickel sulfate was removed in response to the ECVAM comment that inclusion of two nickel salts is unnecessary.
- Tween 80 was removed in response to an ECVAM comment that commercially available batches of Tween 80 may vary and the substance is therefore susceptible to variable results.

One substance (i.e., ethyl acrylate) included on the revised draft ECVAM reference substances list but not on the original draft ICCVAM list (i.e., the September 12, 2007 draft) is still not included in the final ICCVAM LLNA Performance Standards because no guinea pig test reference data were located.

Table E-2 Current Revisions to the Draft ICCVAM-Recommended Performance Standards Reference Substances for the LLNA Based on Public Comments and Comparison to the Revised Draft ECVAM LLNA Performance Standards

Substance[1]	CASRN	Form	Veh	EC3 (%)[2]	N[3]	Orig I	Rev I	Curr I	E	J	Rationale for Exclusion/Inclusion or Current Data Gap
5-Chloro-2-methyl-4-isothiazolin-3-one	**26172-55-4**	**Liq**	**DMF**	**0.009**	**1**		**X**	**X**	**X**		**Concordant GP and human data**
Benzoquinone	106-51-4	Sol	AOO	0.01	1	X					No available human data
DNCB	**97-00-7**	**Sol**	**AOO**	**0.049**	**15**	**X**	**X**	**X**	**X**	**X**	
4-Phenylenediamine	**106-50-3**	**Sol**	**AOO**	**0.11**	**6**	**X**	**X**	**X**	**X**		
Cobalt chloride	**7646-79-9**	**Sol**	**DMSO**	**0.6**	**2**		**X**	**X**	**X**	**X**	**Concordant GP and human data and also on JaCVAM list**
Formaldehyde	50-00-0	Liq	ACE	0.61	1	X				X	Another aldehyde (HCA) already on the list
4-Methylaminophenol sulfate	55-55-0	Sol	DMF	0.8	1		X				Replaced with an acrylate that is a "weak" sensitizer with available GP and human data (methyl methacrylate)
2-Hydroxyethyl acrylate	818-61-1	Liq	AOO	1.4	1	X					Unstable compound
Isoeugenol	**97-54-1**	**Liq**	**AOO**	**1.5**	**47**	**X**	**X**	**X**	**X**	**X**	
2-Mercaptobenzothiazole	**149-30-4**	**Sol**	**DMF**	**1.7**	**1**	**X**	**X**	**X**	**X**		
Cinnamic aldehyde	104-55-2	Liq	AOO	3.0	1	X					Only need HCA (since it is an OECD positive control, and also because it has been tested extensively in the standard LLNA)
Citral	**5392-40-5**	**Liq**	**AOO**	**9.2**	**6**	**X**	**X**	**X**	**X**		
HCA	**101-86-0**	**Liq**	**AOO**	**9.7**	**21**	**X**	**X**	**X**	**X**	**X**	
Eugenol	**97-53-0**	**Liq**	**AOO**	**10.1**	**11**		**X**	**X**	**X**		
Phenyl benzoate	**93-99-2**	**Sol**	**AOO**	**13.6**	**3**		**X**	**X**	**X**		
Cinnamic alcohol	**104-54-1**	**Sol**	**AOO**	**21**	**1**		**X**	**X**	**X**		
Imidazolidinyl urea	**39236-45-9**	**Sol**	**DMF**	**24**	**1**	**X**	**X**	**X**	**X**		
Ethyl acrylate	140-88-5	Liq	AOO	32.4	2						No available GP data. ECVAM agreed to replace with methyl methacrylate in September 2008.
Methyl methacrylate	**80-62-6**	**Liq**	**AOO**	**90**	**1**			**X**	**X**		**Acrylate with concordant GP and human data**
Chlorobenzene	**108-90-7**	**Liq**	**AOO**	**NA**	**1**		**X**	**X**	**X**		**Concordant GP data***
Isopropanol	**67-63-0**	**Liq**	**AOO**	**NA**	**1**	**X**	**X**	**X**	**X**	**X**	**Case report of human sensitizer**
Lactic acid	**50-21-5**	**Liq**	**DMSO**	**NA**	**1**		**X**	**X**	**X**		**Concordant GP data***
Methyl salicylate	**119-36-8**	**Liq**	**AOO**	**NA**	**9**	**X**	**X**	**X**	**X**	**X**	
Salicylic acid	**69-72-7**	**Sol**	**AOO**	**NA**	**1**	**X**	**X**	**X**	**X**		**Concordant human and GP data**
Tween 80	9005-65-6	Liq	AOO	NA	1	X					This is a mixture and commercially available batches may vary
Optional Substances to Demonstrate Improved Performance Relative to the Traditional LLNA											
Sodium lauryl sulfate	**151-21-3**	**Sol**	**DMF**	**8.1**	**5**	**X**	**X**	**X**	**X**		**Included as a false positive**

Substance[1]	CASRN	Form	Veh	EC3 (%)[2]	N[3]	Orig I	Rev I	Curr I	E	J	Rationale for Exclusion/Inclusion or Current Data Gap
Ethylene glycol dimethacrylate	**97-90-5**	**Liq**	**MEK**	**28**	**1**	**X**	**X**	**X**	**X**		**Included as 1 of 3 false positives (with respect to GP only) on ICCVAM list**
Xylene	**1330-20-7**	**Liq**	**AOO**	**95.8**	**1**				**X**		**Substituted for sulfanilamide as a false positive (with respect to human only)**
Nickel chloride	**7718-54-9**	**Sol**	**DMSO**	**NA**	**2**	**X**			**X**		**Included as a false negative**
Nickel sulfate	7786-81-4	Sol	DMF	NA	2	X	X	X		X	Don't need two nickel salts
Sulfanilamide	63-74-1	Sol	DMF	NA	1	X	X	X			Excluded as a false negative because the human results were equivocal (i.e., usually negative rather than positive)

ACE = acetone; AOO = acetone: olive oil (4:1); CASRN = Chemical Abstracts Service Registry Number; Curr I = final ICCVAM LLNA Performance Standards list; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; E = draft ECVAM LLNA Performance Standards list; EC3 = estimated concentration needed to produce a stimulation index of 3; GP = guinea pig test result; HCA = hexyl cinnamic aldehyde; J = JaCVAM list of substances used in non-radiolabeled LLNA validation studies; Liq = liquid; LLNA = murine local lymph node assay results; MEK = methyl ethyl ketone; NA = not applicable since stimulation index <3; NC = not calculated since data was obtained from a single study; NP = not provided in draft ECVAM LLNA Performance Standards; Orig I = September 12, 2007, ICCVAM LLNA Performance Standards list; Rev I = January 8, 2008, ICCVAM LLNA Performance Standards list; Sol = solid; Veh = vehicle

[1] Substances are listed by EC3 value in ascending order. Substances for which no EC3 value was available are listed after those with the highest EC3 values. Substances that are on the final ICCVAM list are indicated in boldface (see also **Table E-1**).

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

[3] 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.

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3.0 Rationale for Inclusion of Substances on the Revised Draft ICCVAM List

Four of the substances included in the draft ECVAM reference substances list but not on the original draft ICCVAM list (i.e., the September 12, 2007, draft) were included in the revised draft ICCVAM list (i.e., the January 8, 2008, draft):

- Cinnamic alcohol was included in the revised list to help achieve the goal of a reference list with a range of sensitizing potency and a variety of different chemical classes. It also has available concordant reference data for the guinea pig and human.
- Eugenol was included in the revised list to help achieve the goal of a reference list with a range of sensitizing potency and a variety of different chemical classes. It also has available concordant reference data for the guinea pig and human, and it has been extensively evaluated in the traditional LLNA.
- Lactic acid was included in the revised list as a non-sensitizer based on available concordant guinea pig data, although human data were not located. It was 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.
- Phenyl benzoate was included in the revised list to help achieve the goal of a reference list with a range of sensitizing potency and a variety of different chemical classes. It also has available concordant reference data for the guinea pig and human.

At the time, there were also six substances that were included on the revised draft ICCVAM list (i.e., the January 8, 2008, draft) that were not included on the ECVAM list. These substances and their rationale for inclusion are as follows:

- 5-Chloro-2-methyl-4-isothiazolin-3-one was identified, as indicated above, as an adequate replacement for benzoquinone based on the availability of concordant guinea pig and human data. It has a history of demonstrated results in the guinea pig and human as an extreme sensitizer.
- Chlorobenzene was included as a non-sensitizer based on available concordant guinea pig data, although no human data were located. It was also 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.
- Cobalt chloride was included as a moderate sensitizer based on LLNA results with concordant guinea pig and human data. It was also included on the JaCVAM list of substances used for validation.
- Ethylene glycol dimethacrylate was not included by ECVAM, as their list only includes one false positive substance. The revised ICCVAM list included two false positive substances that may be tested if improved performance relative to the traditional LLNA is the goal of a validation study.

- 4-Methylaminosulfate was included as a strong sensitizer based on LLNA results with available concordant guinea pig and human data.
- Sulfanilamide was not included by ECVAM, as their list only included one false negative substance. The revised ICCVAM list included two false negative substances that may be tested if improved performance relative to the traditional LLNA is the goal of a validation study.

For the May 7-8, 2008, ESAC meeting, ECVAM revised their list and cited the rationale for their revisions as follows:

- Benzoquinone was replaced with 5-chloro-2-methyl-4-isothiazolin-3-one for reasons mentioned above.
- Diethyl maleate was replaced with cobalt chloride to aid the process of harmonization, despite it being unnecessary to have another metal on the list.
- Hexane was replaced with chlorobenzene as there are no guinea pig data for hexane.
- A proposal to substitute ethyl acrylate with 4-methlyaminophenol sulfate was rejected. Ethyl acrylate represents the acrylates and is a weak sensitizer, and therefore substituting that compound with 4-methylaminophenol, which is not an acrylate and a strong sensitizer, is not acceptable. ECVAM would consider substituting ethyl acrylate with another weak sensitizer for which guinea pig and human data are available.

Subsequently, ICCVAM replaced 4-methylaminosulfate with methyl methacrylate, to represent an acrylate and a weak sensitizer with available guinea pig and human data.

Finally, at the September 23-24, 2008 meeting for the Harmonization of Performance Standards for the LLNA, ECVAM and ICCVAM agreed upon a list of 18 required reference substances and four optional substances. At this meeting, there was agreement to:

- Accept methyl methacrylate as a replacement for ethyl acrylate as a weak sensitizer
- Replace the nickel sulfate with nickel chloride as an optional test substance because the available LLNA results for nickel sulfate were equivocal (i.e., both positive and negative), while the results for nickel chloride were consistently negative
- Include a total of four optional test substances. This included replacement of sulfanilamide with xylene because the reliability of the positive human result with sulfanilamide was questioned. Thus, the four optional substances are ethylene glycol dimethacrylate, sodium lauryl sulfate, nickel chloride, and xylene.

4.0 Database Used to Select Reference Substances

The candidate list used to select proposed minimum reference substances ("reference list") for the draft proposed LLNA Performance Standards was initially generated from the database originally submitted to ICCVAM for the 1998 evaluation of the LLNA. This database of 209 substances was reduced to 97 candidate substances by identifying those substances for which comparative GPMT or BT data that were collected using a standard test method protocol (e.g., U.S. Environmental Protection Agency [EPA] Health Effects Test Guideline OPPTS 870.2600 [EPA 2003]) were available. The availability of such data is important because any accuracy comparisons of new or revised methods must include the currently accepted regulatory test methods (i.e., in this case, the LLNA, and the GPMT and/or BT), as well as comparison to available human data and/or experience. Substances must also be readily available from commercial sources. Further limiting the list of substances to those that are readily available commercially reduced the list from 97 to 81 candidate substances. **Table E-3** provides a breakdown of the impact that specific criteria had on the list of candidate substances.

Table E-3 Impact of Selection Criteria on Candidate List

Criteria for Substance Selection	Number of Substances
Original 1998 LLNA Database	209
Substances with LLNA and GPMT/BT data	127
Substances where GPMT/BT data collected using standard test method protocol	98
Substances where LLNA result was not equivocal	97
Commercially available substances	**81**

Abbreviations: BT = Buehler Test; GPMT = Guinea Pig Maximization Test; LLNA = murine local lymph node assay

The candidate list was then reduced to a draft list of 22 reference substances taking into consideration, where feasible, the following criteria:

- Availability of human data
- Approximately equal distribution of solids and liquids
- Have produced consistent results and an adequate range of responses in the LLNA based on EC3 and SI values
- Consideration of substances used in the JaCVAM validation studies (6 substances) and in the draft LLNA Performance Standards proposed by ECVAM (22 substances)

Table E-4 provides the distribution of responses for the substances in the proposed reference list. The number of substances that have concurrent human data (i.e., human maximization test data; included as part of a human patch test allergen kit; clinical case studies) also is provided. While the selection criteria included the availability of human data whenever possible, two substances without such data was included in order to maintain the desired dynamic range of responses, and range of physical and chemical characteristics.

Table E-4 Distribution of Substances and Available Human Data for the 22 Proposed Reference Substances

LLNA	GPMT/BT	No.	No. w/ HMT, HPTA, or Other Human Data[1]	HMT only	HPTA only	Both HMT and HPTA	Other Human Data[1]
+	+	13	13	2	4	3	4
+	-	2	2	0	1	1	0
-	+	1	1	0	0	0	1
-	-	5	3[2]	0	0	2	1
+	NA	1	1	1	0	0	0
Totals		**22**	**20**	**3**	**5**	**6**	**6**

Abbreviations: BT = Buehler Test; GPMT = Guinea Pig Maximization Test; HMT = Human Maximization Test; HPTA = Human Patch Test Allergen; LLNA = murine local lymph node assay; NA = not available; No. = number

[1] Other human data include published reports of patch tests or case studies with the substance in question.

[2] 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.

Table E-5 provides a breakdown of the various characteristics of the proposed list of 22 substances, including EC3 ranges, physical form information, and peptide reactivity.

Table E-5 Characteristics of the Proposed List of Reference Chemicals

No. Chems	Solid/ Liquid	EC3 Range	Maximum SI Range	Human Data	Peptide Reactivity (High/Mod/Min/Unk)[1]	Included on lists: ECVAM/JaCVAM/ Both
2	1/1	0.009 - 0.05	22.7 - 43.9	2	2/0/0/0	2/1/1
2	2/0	0.11 - 0.6	7.2 - 26.4	2	0/0/0/2	2/1/1
4	1/3	1.5 - 9.7	8.6 - 25.3	4	1/0/1/2	4/2/2
5	3/2	10.1 - 90.1	3.6 - 17.0	5	0/1/0/4	5/0/0
5	1/4	NA	1.7 - 2.7	3	0/0/4/1	5/2/2
Optional Substances to Demonstrate Improved Performance Relative to the Traditional LLNA						
3	1/2	8.1 - 95.8	3.1 - 8.9	3	1/0/0/2	3/0/0
1	1/0	NA	2.4	1	0/0/0/1	1/0/0
Totals						
22	**10/12**	**0.009 - 95.8**	**1.7 - 43.9**	**20**	**4/1/5/12**	**22/6/6**

Abbreviations: Chems = chemicals; EC3 = estimated concentration needed to produce a stimulation index of 3; ECVAM = European Centre for the Validation of Alternative Methods; JaCVAM = Japanese Center for the Validation of Alternative Methods; LLNA = murine local lymph node assay; NA = not applicable; No. = number; Min = minimal; mod = Moderate; SI = stimulation index; Unk = unknown

[1] Data obtained from: Gerberick et al. 2007.

The proposed list of substances includes an adequate number of correctly identified sensitizers, non-sensitizers, false positives, and false negatives, as well as a range of physicochemical properties (e.g., distribution of solids and liquids) to provide meaningful data relevant to the wide range of substances associated with this type of testing. Some of the 22 substances in the proposed reference list lacked data on peptide reactivity and/or from human testing in order to satisfy other criteria for selection or meet specific goals. For example, nickel chloride is included on the reduced list of 22 chemicals because it belongs to a chemical class (metal salts) that is not correctly identified by the traditional LLNA. This provides the opportunity for superior performance to be demonstrated by a modified LLNA.

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Appendix E3

Rationale for the Required Accuracy and Reliability Statistics Included in the Test Method Performance Evaluation

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1.0 Introduction

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Murine Local Lymph Node Assay (LLNA) performance standards describe performance statistics (**Section 2.4**) to be used in the development of new functionally and mechanistically similar test methods. The following text provides an overview of how the performance statistics (i.e., accuracy and reliability values) were selected. Similar to the list of reference substances (**Appendix F**), these recommended statistics represent the culmination of interactions between the ICCVAM Immunotoxicity Working Group (IWG) and liaisons from the Japanese Center for Validation of Alternative Methods (JaCVAM) and the European Centre for the Validation of Alternative Methods (ECVAM), and with members of the ECVAM Sensitization Task Force.

2.0 Test Method Accuracy

Accuracy is defined as the closeness of agreement between a test method result and an accepted reference value (ICCVAM 2003). In the draft LLNA Performance Standards released to the public for comment on September 12, 2007 (announced in *Federal Register* [*FR*] notice 72 FR 52130),[25] the accuracy evaluation was based on meeting or exceeding the performance to the traditional LLNA based on calculated accuracy, sensitivity, specificity, and false negative and false positive rates when using the minimum list of recommended reference substances.

After consideration and discussions with ECVAM, an *FR* notice released on January 8, 2008 (73 FR 1360),[26] announced the availability of a draft version that required a "chemical by chemical" match which required 100% concordance with the traditional LLNA results for the 18 required substances. An optional list of four substances (two false positive/two false negative with respect to guinea pig data) was provided to allow for a modified LLNA test method protocol to demonstrate that its performance exceeded that of the traditional LLNA.

As an additional measure of test method accuracy, the January 8, 2008, draft included a range of ECt values (i.e., the concentration required to achieve the defined threshold stimulation index used to distinguish between sensitizers and non-sensitizers) for the sensitizing substances on the reference list (these values are based on the EC3 values, i.e., the estimated concentrations needed to produce a stimulation index of 3, for each sensitizer). This provided assurance that, not only does a modified LLNA test method protocol achieve the correct call (i.e., sensitizer versus non-sensitizer), but that it does so at a substance dose level similar to that observed in the traditional LLNA. This range was originally proposed by ECVAM based on the personal experience of members of the ECVAM Sensitization Task Force.

In their review of the January 8, 2008, draft ICCVAM LLNA Performance Standards, an international independent scientific peer review panel (hereafter, “Panel”) concluded that the acceptability range of 0.5x to 2.0x was too restrictive. They also emphasized that it was not appropriate to define an acceptability range for which there was only one or two EC3 values available to calculate the range. The Panel also recommended that modified LLNA test

[25] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_18011.pdf
[26] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf

methods should be evaluated with all 22 substances (including false negatives and false positives) and accuracy statistics calculated. To the extent possible, rationale for discordant results should be provided. However, the most potent sensitizers (e.g., 2,4-dinitrochlorobenzene [DNCB]) should always be identified correctly.

Considering comments from the Panel, the Scientific Advisory Committee on Alternative Toxicological Methods and the public, IWG discussions, and discussions with ECVAM, the final ICCVAM LLNA Performance Standards state that the proposed test method should result in the correct sensitizer/non-sensitizer classification for each of the 18 required reference substances, but that a misclassification of one weak sensitizer could be allowed. The rationale for the discrepancy must be provided and would be assessed on a case-by-case basis to determine acceptability. In addition, to demonstrate equivalent or improved performance relative to the traditional LLNA, any of the four optional substances may be tested in addition to the required 18 substances.

3.0 Test Method Reliability

The original draft ICCVAM LLNA Performance Standards (September 12, 2007) stated that the modified LLNA test method should have an intralaboratory reproducibility that is equivalent to or better than the intralaboratory reproducibility of hexyl cinnamic aldehyde (HCA), or other comparable positive control substance in the traditional LLNA. ECt values should be derived on four separate occasions with at least one week between tests to ensure that there is no overlap between tests. However, this evaluation did not take into consideration the importance of producing an ECt that is within an acceptable range of the historical EC3 concentration for HCA, based on traditional LLNA studies. Instead, the test method could achieve an acceptable coefficient of variation that is based on EC3 concentrations that differ significantly from the historical range (i.e., the method could produce reproducible, but inaccurate results).

For this reason, the January 8, 2008, draft of the ICCVAM LLNA Performance Standards criteria for intralaboratory reproducibility was revised to reflect that acceptable reproducibility is indicated when each of at least three laboratories obtain ECt values for HCA and DNCB that are generally within 0.5x to 2.0x the historical mean EC3 concentration (5% to 20% and 0.025 to 0.1%, respectively) for these substances when tested in the traditional LLNA. The Panel agreed with the proposed intralaboratory reproducibility standard. This section remains unchanged from the January 8, 2008, draft.

3.1 Interlaboratory Reproducibility

The original draft ICCVAM LLNA Performance Standards (September 12, 2007) stated that a modified LLNA test method should be equally (or more) reproducible than the traditional LLNA, based on DNCB and HCA test results in the traditional LLNA, which would be based on coefficients of variations. However, similar to the assessment of intralaboratory reproducibility, this evaluation also did not take into account the acceptable range of the historical EC3 values for HCA and DNCB, based on traditional LLNA studies. For this reason, the evaluation of interlaboratory reproducibility was revised to reflect the same range of acceptable EC3 values that is being applied the assessment of test method accuracy (i.e., 0.5x to 2.0x ECt). Acceptable reproducibility will now be indicated by each of at least three laboratories obtaining ECt values for HCA and DNCB that are generally within 0.5x to 2.0x the EC3 concentration (5% to 20%

and 0.025 to 0.1%, respectively) as specified for these substances when tested in the traditional LLNA. The Panel agreed with the proposed interlaboratory reproducibility standard. This section remains unchanged from the January 8, 2008, draft.