Annex VIII

Analyses Using Multiple SI Decision Criteria

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1.0 Introduction

This annex provides analyses associated with using two decision criteria for classifying substances using the results from the LLNA: BrdU-ELISA: one criterion to classify substances as sensitizers and another criterion to classify substances as nonsensitizers. The data used for the analyses in this annex are the LLNA: BrdU-ELISA results for the 31 substances (22 traditional LLNA sensitizers and nine traditional LLNA nonsensitizers) that were reviewed by the Panel at the public meeting on April 28-29, 2009. **Section 2** of this annex discusses the accuracy produced by using the two decision criteria and includes an evaluation of discordant, or indeterminate, substances that produced SI values in between the sensitizer and nonsensitizer SI criteria. **Section 3** provides the reproducibility analysis using the decision criterion for sensitizers (**Sections 3.1** and **3.2**) and for tests yielding SI values in three categories: sensitizer, nonsensitizer, and indeterminate (i.e., in the range of uncertainty) (**Section 3.3**). The two SI values determined to be optimal were based on four animals per dose group and resulted in nine substances that could not be definitively classified because they produced SI values in the range of uncertainty. **Section 4** describes the impact of sample size on the range of the uncertainty between the sensitizer and nonsensitizer criteria. **Section 5** evaluates a number of physicochemical characteristics and other parameters to distinguish between traditional LLNA sensitizers and nonsensitizers in the LLNA: BrdU-ELISA when using multiple SI decision criteria for their potential use in providing additional information for use in classifying substances that produce SI values in the range of uncertainty.

2.0 Accuracy Analysis Using Multiple Stimulation Index Decision Criteria

The accuracy of the LLNA: BrdU-ELISA with respect to the traditional LLNA using a number of alternative decision criteria (and the most prevalent outcome for substances with multiple tests) was evaluated in **Section 6.5** of the BRD. This section evaluates the accuracy of the LLNA: BrdU-ELISA when two SI decision criteria are used to classify test substances: one criterion for sensitizers and another criterion for nonsensitizers. For the database of 31 substances, the lowest decision criterion with a 0% (0/9) false positive rate was SI $\geq$ 2.0, which was used by the JSAAE interlaboratory validation study. The accuracy at SI $\geq$ 2.0 was 84% (26/31) and the false negative rate was 23% (5/22). Higher SI values also produced false positive rates of 0% (0/9), but the false negative rate increased as the SI increased. The lowest false negative rate was produced at SI $\geq$ 1.3 (0% [0/22]), but the false positive rate at SI $\geq$ 1.3 was 44% (4/9).

The 0% false positive rate using SI $\geq$ 2.0 and the 0% false negative rate using SI $\geq$ 1.3 prompted the evaluation of using two decision criteria for LLNA: BrdU-ELISA results: one criterion to classify substances as sensitizers and another criterion to classify substances as nonsensitizers. Further examination of the LLNA: BrdU-ELISA results indicated that a lower SI criterion than SI $\geq$ 2.0, SI $\geq$ 1.9, also correctly identified traditional LLNA sensitizers with no false positives. Thus, SI $\geq$ 1.9 was proposed as the criterion to classify substances as sensitizers. The SI $\geq$ 1.3 criterion, when used to classify sensitizers, resulted in no false negative results with respect to the traditional LLNA results. Thus, SI $\leq$ 1.3 was proposed to classify substances as nonsensitizers because this criterion also resulted in no false negative results.

2.1 Indeterminate Results Using Multiple Alternative Decision Criteria

While optimum false positive and false negative rates can be achieved for the 31 substances evaluated in the LLNA: BrdU-ELISA accuracy analyses using these two different decision criteria, a range of SI values (i.e., 1.3 < SI < 1.9, the range of uncertainty) exists for which the correct classification is not definitive (i.e., there is a chance for false positive or false negative results for substances that produce SI values in this range). Chemical class, physical form, MW, peptide reactivity (see

Annex II for physicochemical properties), traditional LLNA EC3 range (**Table C-1**), and potential for skin irritation (**Annex III-1**) were examined to identify commonalities among the substances that produced SI values between 1.3 and 1.9 in an attempt to identify common characteristics among these substances that could be used to correctly classify such substances. **Section 5.0** of this annex provides a comprehensive evaluation of a number of physicochemical characteristics and other parameters, using the entire LLNA: BrdU-ELISA database, to distinguish between traditional LLNA sensitizers and nonsensitizers.

Of the nine substances that produced SI values in the range of uncertainty, between 1.3 and 1.9, five substances are nonsensitizers and four are sensitizers based on traditional LLNA results (**Table C-VIII-1**). The five substances classified by the traditional LLNA as nonsensitizers (hexane, isopropanol, lactic acid, methyl salicylate, and propylene glycol), represented four chemical classes (acyclic hydrocarbons, alcohols, carboxylic acids, and phenols).

- Two substances are classified as carboxylic acids (methyl salicylate, also a phenol, and lactic acid) and two were classified as alcohols (isopropanol and propylene glycol).
- Hexane is an acyclic hydrocarbon.

Other characteristics of the indeterminate substances that are traditional LLNA nonsensitizers include:

- All of the five substances are liquids and have minimal peptide reactivity.
- Four substances have MW < 100 g/mole. The other substance, methyl salicylate, has a MW of 152.15 g/mole.
- Four of the five substances were tested at irritating concentrations in the LLNA: BrdU-ELISA: hexane, lactic acid, methyl salicylate, and propylene glycol, based on skin irritation data from mice, rabbits, or humans. Isopropanol was tested at concentrations nonirritating to skin, based on skin irritation data from rabbits.
- Two of the five substances yielded SI < 2 in the traditional LLNA: isopropanol and propylene glycol. The other three substances yielded SI values between 2 and 3 (exclusive): hexane, lactic acid and methyl salicylate.

Table C-VIII-1 Indeterminate Results for LLNA: BrdU-ELISA When Multiple Decision Criteria Were Used [1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[5]	Skin Irritant?
Hexane	AOO	1.76, 100% 1.89, 50% (2/2 tests)	- (2.2, 100%)	Irritant at 100% (humans)
Isopropanol	AOO	1.57, 50% (1/7 tests)	- (1.7, 50%)[6]	No, up to 100% (rabbits)
Lactic acid	DMSO	1.80, 50% 1.89, 50% (2/3 tests)	- (2.2, 25%)	Slightly irritating at 10% (rabbits)
Methyl salicylate	AOO	1.40, 50% 1.43, 50% 1.44, 50% (3/3 tests)	- (2.9, 20%)	Irritant at 10% (mice)

continued

Table C-VIII-1 Indeterminate Results for LLNA: BrdU-ELISA When Multiple Decision Criteria Were Used [1] (continued)

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[5]	Skin Irritant?
Propylene glycol	AOO[7]	1.57, 50% (1/3 tests)	- (1.6, 100%)[8]	No, up to 25% (humans)
Aniline (47.5%)	AOO	1.50, 50%	+ (3.6, 100%)[7]	No, up to 100% (GP); Irritant at 20% (humans)
Hydroxycitronellal (24.0%)	AOO	1.34, 100%	+ 8.5, 100%)	No, up to 50% (GP)
Linalool (30.0%)	AOO	1.45, 100%[8]	+ (8.3, 100%)	Mild irritant at 100% (rabbits)
2-Mercaptobenzo-thiazole (1.7%)	DMF	1.62, 50%[9]	+ (8.6, 10%)	No, up to 10% (GP); No, up to 25% (humans)

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N*,*N*-dimethylformamide; DMSO = dimethyl sulfoxide; LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; LLNA = murine local lymph node assay; + = sensitizer; - = nonsensitizer.

[1] Data sources provided in **Annex III-1**.

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of 3) for substances that are sensitizers in the traditional LLNA; from **Table C-1**.

[3] Vehicles apply to tests for both the LLNA: BrdU-ELISA and the traditional LLNA unless otherwise noted.

[4] Numbers are highest SI values achieved and maximum concentration tested.

[5] Information in parentheses indicates the basis for the human outcome. Numbers indicate the incidence of positive human response and concentration tested.

[6] Highest SI occurred at 10%.

[7] The vehicle for the traditional LLNA was distilled water.

[8] Highest SI occurred at 50%.

[8] The solvent for the traditional LLNA was *N*,*N*-dimethylformamide.

[9] Highest SI occurred at 12.5%.

The four indeterminate substances classified by the traditional LLNA as sensitizers (aniline, hydroxycitronellal, linalool, and 2-mercaptobenzothiazole,) represent three chemical classes. Aniline is an amine, hydroxycitronellal and linalool are hydrocarbons (other), and 2-mercaptobenzothiazole is a heterocyclic compound. Other characteristics of the indeterminate substances that are classified as sensitizers by the traditional LLNA include:

- Three are liquids and one is a solid (2-mercaptobenzothiazole).

- All four substances have MW between 90 and 200 g/mole.
- Hydroxycitronellal exhibits low peptide reactivity, 2-mercaptobenzothiazole exhibits high peptide reactivity, and peptide reactivity information is not available for the other two substances.
- Aniline, linalool, and hydroxycitronellal were not strongly positive in the traditional LLNA (EC3 = 47.5%, 30%, and 24%, respectively), with maximum SI = 3.6, 8.3, and 8.5, respectively, when tested at concentrations up to 100%. 2-Mercaptobenzothiazole, however, was a strong positive (EC3 = 1.7%).
- All four substances were tested in the LLNA: BrdU-ELISA at concentrations that were irritating to skin, based on human, guinea pig, or rabbit data.

3.0 Test Method Reliability

An assessment of test method reliability (intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Intralaboratory reproducibility refers to the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Interlaboratory reproducibility refers to the extent to which different laboratories can replicate results using the same protocol and test substances and indicates the extent to which a test method can be transferred successfully among laboratories.

The available LLNA: BrdU-ELISA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. This section provides an assessment of reproducibility for the decision criterion of SI $\geq$ 1.9 to identify sensitizers. As described in **Section 2.0** of this annex, SI $\geq$ 1.9 was evaluated as the decision criterion for classifying substances as sensitizers with SI $\leq$ 1.3 as the criterion to identify nonsensitizers.

3.1 Intralaboratory Reproducibility

The test results for the LLNA: BrdU-ELISA were amenable to intralaboratory reproducibility analyses for three endpoints: sensitizer or nonsensitizer classification, SI values, and EC1.9 values. Analyses of intralaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 3.1.1** of this annex) and a coefficient of variation (CV) analysis for the quantitative results (SI values and EC3 values) (**Sections 3.1.2** and **3.1.3** of this annex, respectively).

3.1.1 Intralaboratory Reproducibility – Qualitative Results

The dataset available for an intralaboratory concordance analysis of the qualitative test results for the LLNA: BrdU-ELISA included nine substances that were tested multiple times and classified as sensitizers or nonsensitizers. Hexyl cinnamic aldehyde and eugenol were tested six times; isoeugenol was tested four times; diphenycyclopropenone and propylene glycol were tested three times; and 2,4-dinitrochlorobenzene, glutaraldehyde, hexane, and 4-phenylenendiamine were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a; unpublished data) (**Table C-VIII-2**). All substances were sensitizers in the traditional LLNA except for propylene glycol and hexane. The multiple test results for 9/9 substances were 100% concordant when SI $\geq$ 1.9 was used to classify substances as sensitizers.

By comparison, the qualitative intralaboratory concordance analysis for the traditional LLNA (ICCVAM 1999) was based on a dataset of six substances that included six results each for benzocaine and hexyl cinnamic aldehyde, five results for eugenol, four results each for isoeugenol and methyl salicylate, and three results for 2,4-dinitrochlorobenzene. Intralaboratory results for each substance were 100% concordant with the exception of benzocaine. One of the six benzocaine (5/6 or

83% concordance) results for the traditional LLNA was reported as equivocal because SI increased with dose, but did not reach the criterion of SI ≥ 3.0. Thus, the proportion of substances for which intralaboratory concordance of qualitative results was 100% was greater for LLNA: BrdU-ELISA (9/9) than for the traditional LLNA (5/6).

Table C-VIII-2 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times

Substance Name	Highest Concentration Tested (%)	Highest SI	Outcome	Takeyoshi et al. Reference
2,4-Dinitro-chlorobenzene	2	17.86	+	2005
	2	6.84	+	2006, 2007b
Diphenylcyclopro-penone	2	19.10	+	2005; 2007b
	10	9.34	+	2005
	10	11.62	+	2007b
Eugenol	10	3.18	+	2003
	30	3.33	+	2004a
	30	3.83	+	2007a
	50	12.28	+	2005
	50	3.05	+	2006
	50	17.69	+	2007b
Glutaraldehyde	2	14.60	+	2005, 2007b
	10	15.50	+	2005, 2007b
Hexane	50	1.89	-	2005
	100	1.76	-	unpublished data
Hexyl cinnamic aldehyde	25	2.41	+	2003
	50	3.64	+	2003
	50	5.90	+	2005
	50	3.64	+	2006
	50	2.72	+	2006
	50	3.02	+	2007b
Isoeugenol	10	8.36	+	2005
	10	2.36	+	2006, 2007b
	10	7.20	+	2005
	30	6.73	+	2007a
4-Phenylenediamine	2	11.70	+	2005, 2007b
	10	14.70	+	2005, 2007b
Propylene glycol	10	1.20	-	2005
	50	1.57	-	2005
	50	0.91	-	2006, 2007b

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

+ = sensitizer; - = nonsensitizer.

3.1.2 Intralaboratory Reproducibility – SI

There were seven substances that were tested multiple times at the same concentrations by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished data). Because two substances had multiple tests for more than one concentration, there were 10 substance/concentration combinations that were tested two to five times in separate experiments. The multiple SI values for each substance/concentration were used to calculate a CV for the assessment of intralaboratory variability. As shown by **Table C-VIII-3**, the CVs ranged from 1% (25% hexyl cinnamic aldehyde) to 80% (10% isoeugenol). The intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

3.1.3 Intralaboratory Reproducibility – EC1.9

CV values were also calculated for the EC1.9 values for the three sensitizers that were tested more than once using multiple doses by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b). The individual animal data for eugenol, hexyl cinnamic aldehyde, and isoeugenol were used to calculate EC1.9 values for the LLNA: BrdU-ELISA. The methods for calculating EC1.9 values for each sensitizer were modified from those used by Ryan et al. (2007) to calculate EC3 values. Linear interpolation was used to calculate EC1.9 values for each test with SI values higher or lower than 1.9, and extrapolation was used to calculate EC1.9 values for tests with no SI values below 1.9. The equation for linear interpolation was:

$$EC1.9 = c + \left[\frac{(1.9 - d)}{(b - d)}\right] \times (a - c)$$

The linear interpolation equation uses the points immediately above and below SI = 1.9, with the (dose, SI) coordinates of (a, b) immediately above SI = 1.9 and (c, d) immediately below SI = 1.9. The equation for extrapolation was:

$$EC1.9_{ex} = 2^{\left\{\log_2(c) + \frac{(1.9-d)}{(b-d)} \times [\log_2(a) - \log_2(c)]\right\}}$$

Table C-VIII-3 Intralaboratory Reproducibility for the SI of Tested Substances in LLNA: BrdU-ELISA – Coefficient of Variation

Substance Name	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
2,4-Dinitrochlorobenzene	2	17.86	12.35	7.79	63	2005
		6.84				2006, 2007b
Diphenylcyclopropenone	10	9.34	10.48	1.61	15	2005; 2007b
		11.62				2007b
Eugenol	30	3.33	3.58	0.35	10	2004a
		3.83				2007a
Eugenol	50	12.28	11.01	7.40	67	2005
		3.05				2006
		17.69				2007b
Hexane	50	1.89	1.64	0.36	22	2005
		1.38				Unpublished

continued

Table C-VIII-3 Intralaboratory Reproducibility for the SI of Tested Substances in LLNA: BrdU-ELISA – Coefficient of Variation (continued)

Substance Name	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
Hexyl cinnamic aldehyde	12.5	1.88	1.74	0.21	12	2003
		1.59				2003
Hexyl cinnamic aldehyde	25	2.44	2.42	0.02	1	2003
		2.41				2003
Hexyl cinnamic aldehyde	50	3.64	3.78	1.25	33	2003
		5.90				2005
		3.64				2006
		2.72				2006
		3.02				2007b
Isoeugenol	10	8.36	5.09	3.15	80	2005
		7.20				2005
		2.36				2006, 2007b
		2.43				2007a
Propylene glycol	50	1.57	1.14	0.62	54	2005
		0.70				2006, 2007b

Abbreviations: CV = coefficient of variation; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation, SI = stimulation index.

The extrapolation equation uses the two points immediately above SI = 1.9, with the coordinates of (a, b) for the point closest to SI = 1.9, and (c, d) for the higher point. As shown in **Table C-VIII-4**, there were five EC1.9 values for hexyl cinnamic aldehyde, four EC1.9 values for eugenol, and two EC1.9 values for isoeugenol. The CV values were 72% for eugenol, 27% for hexyl cinnamic aldehyde, and 21% for isoeugenol. The ICCVAM LLNA *Performance Standards* criteria for demonstrating adequate intralaboratory reproducibility are based on results from at least four independent tests of hexyl cinnamic aldehyde (ICCVAM 2009). Intralaboratory reproducibility is considered adequate when each test yields an ECt value (i.e., the estimated concentration needed to produce an SI of a specific threshold value; in this case, SI = 1.9) within 5% to 20% (ICCVAM 2009). Two of the five EC1.9 values for hexyl cinnamic aldehyde were within the acceptable range for intralaboratory reproducibility.

Table C-VIII-4 Intralaboratory Reproducibility for the EC1.9 of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance Name	EC1.9	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	10.1	11.5	8.3	72	2004a
	21.1				2006
	1.2				2007b
	13.7				2007a
Hexyl cinnamic aldehyde	12.9	20.5	5.6	27	2003
	17.2				2003
	27.1				2006
	24.0				2006
	21.4				2007b
Isoeugenol	8.0	7.0	1.5	21	2006; 2007b
	5.9				2007a

Abbreviations: CV = coefficient of variation; EC1.9 = estimated concentration needed to produce a stimulation index of 1.9; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation.

The intralaboratory reproducibility of the traditional LLNA was assessed by CV analysis of EC3 values using a larger dataset (ICCVAM 1999) than that available for the LLNA: BrdU-ELISA analysis. Two EC3 values were reported by each of five laboratories for 2,4-dinitrochlorobenzene, five EC3 values were reported by one laboratory for isoeugenol, six EC3 values were reported for hexyl cinnamic aldehyde by two laboratories, and five EC3 values were reported for eugenol by one laboratory (**Table C-VIII-5**).

Table C-VIII-5 Intralaboratory Reproducibility for the EC3 of Tested Substances in the Traditional LLNA[1]

Substance Name	Number of Laboratories	Number of Tests per Laboratory	CV (%)
2, 4-Dinitrochlorobenzene	5	2	13-47
Isoeugenol	1	5	26
Hexyl cinnamic aldehyde	2	6	19-27
Eugenol	1	5	18

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay;

[1] From ICCVAM (1999).

For one of three substances, the intralaboratory CV values for the EC1.9 values from LLNA: BrdU-ELISA tests were higher than EC3 values for the same substances from the traditional LLNA reported in ICCVAM (1999). The intralaboratory EC1.9 CV from the LLNA: BrdU-ELISA tests of eugenol was higher that that reported by ICCVAM (1999) for EC3 values (72% vs. 18%). However, the intralaboratory EC1.9 CV from the LLNA: BrdU-ELISA tests of isoeugenol was less than that for EC3 values from ICCVAM (1999) (21% vs. 26%). The intralaboratory EC1.9 CV from the

LLNA: BrdU-ELISA tests of hexyl cinnamic aldehyde was within the range reported by ICCVAM (1999) for EC3 values (27% vs. 19% to 27%).

3.2 Interlaboratory Reproducibility

The interlaboratory reproducibility of the LLNA: BrdU-ELISA was assessed using the individual animal data from the multilaboratory validation study organized by the JSAAE (Kojima et al. 2008). Phase I of the study evaluated the reliability and transferability of the test method protocol by testing 12 substances in three to nine laboratories. With the exception of the positive control data, neither the summary results nor the individual animal data from phase I of the validation study have been released. Phase II of the study tested 10 substances in three to seven laboratories as shown in **Table C-VIII-6**. All the laboratories that participated in the validation study used the same experimental protocol (**Annex I** of the BRD) and participated in a one-day seminar that explained the protocol and execution of the test method. The same commercial ELISA kit, test materials, and the same doses of the test substances were used in all of the laboratories. The Validation Management Team determined the doses and vehicles for testing and coded the identity of the test substances prior to distribution to the test laboratories. Seven substances were sensitizers and three substances were nonsensitizers according to the traditional LLNA. Six substances were ICCVAM *Recommended Performance Standards* reference substances: 2,4-dinitrochlorobenzene, eugenol, hexyl cinnamic aldehyde, lactic acid, isopropanol, and methyl salicylate (ICCVAM 2009).

Table C-VIII-6 Substances and Test Allocation for the Phase II Interlaboratory Validation Study of the LLNA: BrdU-ELISA

Substance Name[1]	Vehicle	Concentrations Tested			Laboratory[2]						
					1	2	3	4	5	6	7
Nickel sulfate (+)	DMSO	1%	3%	10%			X	X			X
Isopropanol (-)	AOO	10%	25%	50%	X	X	X	X	X	X	X
Eugenol (+)	AOO	10%	25%	50%		X				X	X
Cinnamic aldehyde (+)	AOO	1%	3%	10%		X		X	X		
2,4-Dinitrochlorobenzene (+)	AOO	0.1%	0.3%	1%	X	X	X	X	X	X	X
Glutaraldehyde (+)	ACE	0.1%	0.3%	1%	X				X	X	
Methyl salicylate (-)	AOO	10%	25%	50%	X	X	X				
Hexyl cinnamic aldehyde (+)	AOO	10%	25%	50%	X	X	X	X	X	X	X
Lactic acid (-)	DMSO	10%	25%	50%			X	X			X
Formaldehyde (+)	ACE	1%	3%	10%	X				X	X	

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] X indicates that a substance was tested in a particular laboratory. 1 = Daicel Chemical Industries Ltd.; 2 = Food and Drug Safety Center; 3 = Otsuka Pharmaceutical Co. Ltd.; 4 = Taisho Pharmaceutical Co. Ltd.;

5 = Fuji Film Co. Ltd.; 6 = Biosafety Research Center, Foods, Drugs and Pesticides; 7 = National Institute of Health Sciences.

The LLNA: BrdU-ELISA test results from the JSAAE validation study were used for interlaboratory reproducibility analyses for both qualitative and quantitative endpoints. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 3.2.1** of this annex) and a CV analysis for the quantitative results (EC1.9 values) (**Sections 3.2.2** of this annex).

3.2.1 Interlaboratory Reproducibility – Qualitative Results

The available quantitative absorbance data for interlaboratory reproducibility analysis were used to calculate SI values for each substance and dose tested. Substances with SI $\geq$ 1.9 at any dose were classified as sensitizers. The qualitative (sensitizer/nonsensitizer) interlaboratory concordance analysis for the 10 substances tested during Phase II of the JSAAE interlaboratory validation study is shown in **Table C-VIII-7**. The qualitative comparison evaluated the consistency of LLNA: BrdU-ELISA results (i.e., positive vs. negative) for 10 substances tested among up to 7 laboratories. The results show that interlaboratory concordance was 100% (3/3, 6/6, or 7/7) for eight substances. There were two discordant substances (isopropanol and lactic acid) for which interlaboratory concordance was 67% (2/3 or 4/6). Two of the six tests of isopropanol yielded SI $\geq$ 1.9 (SI = 2.04 and SI = 2.22), while the others yielded SI < 1.9. One of the three tests for lactic acid produced SI $\geq$ 1.9 (i.e., SI = 2.53), while the others yielded SI < 1.9. The Validation Management Team, which used SI $\geq$ 2.0 as the decision criterion, considered the interlaboratory reproducibility to be acceptable (Kojima et al. 2008). Because the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional concordance data for comparison with the LLNA: BrdU-ELISA concordance.

Table C-VIII-7 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance Name	Laboratory							Concordance
	1	2	3	4	5	6	7	
2,4-Dinitrochlorobenzene	+ (4.30)	+ (8.37)	+ (6.26)	+ (5.50)	+ (18.80)	+ (4.83)	+ (12.98)	7/7
Glutaraldehyde	+ (3.72)				+ (28.64)	+ (2.25)		3/3
Nickel sulfate			+ (2.58)	+ (4.53)			+ (2.66)	3/3
trans-Cinnamic aldehyde		+ (3.37)		+ (3.50)	+ (4.11)			3/3
Formaldehyde	+ (4.40)				+ (16.59)	+ (1.97)		3/3
Eugenol		+ (3.17)				+ (3.18)	+ (7.09)	3/3
Hexyl cinnamic aldehyde	+ (3.40)	-[3]	+ (2.87)	+ (3.34)	+ (13.50)	+[4] (3.27)	+ (3.84)	6/6
Isopropanol	+[2] (2.22)	-[3]	- (0.98)	- (1.57)	- (0.94)	+[2,5] (2.04)	- (1.01)	4/6
Lactic acid			- (1.80)	- (1.89)			+ (2.53)	2/3
Methyl salicylate	- (1.43)	- (1.44)	- (1.40)					3/3

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine.

[1] + indicates sensitizer result; - indicates nonsensitizer result.

[2] Stimulation index (SI) ≥ 1.9 at lowest dose tested, but <1.9 at the higher doses. The Validation Management Team considered these to be nonsensitizer results (Kojima et al. 2008).

[3] Test failed because concurrent positive control failed (i.e., SI < 1.9). Result not included in the concordance analysis.

[4] Three mice tested at highest dose.

[5] Three mice per dose group.

3.2.2 Interlaboratory Reproducibility – EC1.9 Values

The SI values from the interlaboratory validation study were used to calculate EC1.9 values for each sensitizer according to the methods reported in **Section 3.1.3** of this annex. The EC1.9 values from each laboratory were then used to calculate CV values for each substance. The resulting values are shown in **Table C-VIII-8**. CV values ranged from 27% (*trans*-cinnamic aldehyde) to 87% (glutaraldehyde). The mean CV was 62%.

The ICCVAM LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA (ICCVAM 2009). Acceptable reproducibility is attained when each laboratory obtains ECt values within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). EC1.9 values from three laboratories were outside the range for 2,4-dinitrochlorobenzene, and the EC1.9 values from two laboratories were outside the range for hexyl cinnamic aldehyde. Laboratories 2, 5, and 6 reported EC1.9 values that were lower than the specified acceptance range for 2,4-dinitrochlorobenzene (0.017%, 0.0024%, and 0.023%, respectively). For hexyl cinnamic aldehyde, Laboratory 3 obtained an EC1.9 value of 22.21%, which was higher than the acceptance range. Laboratory 5 obtained an EC1.9 value of 3.96%, which was lower than the acceptance range.

The interlaboratory CV values for the LLNA: BrdU-ELISA EC1.9 values were higher than those for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 7 to 84% for five substances tested in five laboratories (**Table C-VIII-9**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: BrdU-ELISA. All interlaboratory CV values for LLNA: BrdU-ELISA were greater than those for the traditional LLNA. The CV of 77% for the EC1.9 of 2,4-dinitrochlorobenzene was greater than the two CV values of 37% and 27% calculated from five EC3 values each, reported by ICCVAM (1999). The CV of 51% for the EC1.9 of hexyl cinnamic aldehyde tested in the LLNA: BrdU-ELISA was greater than the 7% for the EC3 reported by ICCVAM (1999). The CV of 55% for the EC1.9 of eugenol tested in the LLNA: BrdU-ELISA was greater than the 42% reported by ICCVAM (1999) for the EC3.

Table C-VIII-8 EC1.9 Values from the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance Name	Laboratory							Mean ± SD	% CV
	1	2	3	4	5	6	7		
2,4-Dinitro-chlorobenzene	0.078 (4.3 @ 1%)	***0.017 (8.37 @ 1%)***	0.027 (5.99 @ 0.3%)	0.028 (5.50 @ 1%)	***0.0024 (18.80 @ 0.3%)***	***0.023 (4.83 @ 0.3%)***	0.053 (12.18 @ 1%)	0.033 ± 0.025	77
Hexyl cinnamic aldehyde	14.76 (3.4 @ 50%)	-[1] (1.83 @ 50%)	***22.21 (2.87 @ 50%)***	7.92 (3.34 @ 50%)	***3.96 (13.5 @ 50%)***	11.65[2] (3.27 @ 50%)	13.18 (3.84 @ 50%)	12.28 ± 6.23	51
Glutaraldehyde	0.14	NT	NT	NT	0.033	0.32	NT	0.17 ± 0.14	87
Nickel sulfate	NT	NT	2.93	0.86	NT	NT	1.05	1.61 ± 1.14	71
trans-Cinnamic aldehyde	NT	2.42	NT	1.48	2.58	NT	NT	2.16 ± 0.59	27
Formaldehyde	0.37	NT	NT	NT	0.28	0.071	NT	0.24 ± 0.16	64
Eugenol	NT	17.76	NT	NT	NT	15.20	4.70	12.55 ± 6.92	55

Note: Boldface indicates substances recommended for assessing interlaboratory reproducibility in *Recommended Performance Standards* (ICCVAM 2009). Boldface italic EC1.9 values are outside of the acceptable range from the ICCVAM LLNA performance standards: 5%-20% for hexyl cinnamic aldehyde and 0.025%-0.1% for 2,4-dinitrochlorobenzene. Values in parentheses are the highest SI values achieved.

Abbreviations: CV = coefficient of variation; EC1.9 = estimated concentration needed to produce a stimulation index of 1.9; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NT = not tested; SD = standard deviation; SI = stimulation index.

[1] Test failed because associated positive control failed (i.e., SI < 1.9; vehicle control absorbance was unusually high). Result not included in the mean EC1.9 and CV.

[2] Three mice tested at highest dose.

Table C-VIII-9 Interlaboratory Reproducibility of the EC3 for Substances Tested in the Traditional LLNA[1]

Substance Name	Laboratory					CV (%)
	1	2	3	4	5	
2, 4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37
	0.5	0.6	0.4	0.6	0.3	27
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	7
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41
Eugenol	5.8	14.5	8.9	13.8	6.0	42
Sodium lauryl sulfate	13.4	4.4	1.5	17.1	4.0	84

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay.

[1] From ICCVAM (1999).

3.3 Reproducibility for the LLNA: BrdU-ELISA Using Multiple Alternative Decision Criteria

Section 2.0 of this annex discusses the accuracy for the LLNA: BrdU-ELISA when using two decision criteria for LLNA: BrdU-ELISA results: one criterion to classify substances as sensitizers (SI ≥ 1.9) and another criterion to classify substances as nonsensitizers (i.e., SI ≤ 1.3). SI ≥ 1.9 was evaluated for classifying sensitizers because it resulted in no false positives with respect to the traditional LLNA. SI ≤ 1.3 was evaluated for classifying substances as nonsensitizers because it resulted in no false negatives. This section evaluates reproducibility of the concordance with the traditional LLNA results by examining the frequency with which SI values in the validation database of 31 substances occurred in one of three SI categories, regardless of whether the tests were performed in one or multiple laboratories (i.e., intra- and inter-laboratory data have been combined for this analysis). The three SI categories were:

- SI ≤ 1.3 for classifying nonsensitizers
- SI > 1.3 and <1.9, the range of uncertainty with respect to classification by the traditional LLNA (i.e., indeterminate results)
- SI ≥ 1.9 to classify substances as sensitizers

The database for this analysis consisted of 106 LLNA: BrdU-ELISA tests of 31 substances. The maximum SI achieved by each test and the traditional LLNA outcome (sensitizer vs. nonsensitizer) were used to determine the frequency of the maximum SI by category. **Table C-VIII-10** shows the proportion of sensitizers and nonsensitizers, according to the traditional LLNA, for each of three SI categories: SI ≤ 1.3, 1.3 < SI < 1.9, and SI ≥ 1.9. All of the tests (10/10 [100%]) that yielded SI < 1.3 were for substances that were classified as nonsensitizers by the traditional LLNA. Thirty-one percent (4/13) of the tests that yielded SI values in the range of uncertainty; 1.3 < SI < 1.9, were for substances that were classified as sensitizers by the traditional LLNA. The remainder of the tests in the 1.3 < SI < 1.9 category, 69% (9/13), were classified as nonsensitizers by the traditional LLNA. Ninety-six percent (80/83) of the tests that yielded SI ≥ 1.9 were for substances that were classified as sensitizers by the traditional LLNA, and only 4% (3/83) were classified as nonsensitizers. The three

nonsensitizer tests were two tests of isopropanol, which yielded SI = 2.02 and 2.22 in the LLNA: BrdU-ELISA, and one test of lactic acid, which produced SI = 2.53.

Table C-VIII-10 Frequency of Maximum SI for LLNA: BrdU-ELISA Tests by Category and Traditional LLNA Outcome

Classification Based on Traditional LLNA	Classification Concordance with Traditional LLNA[1]			
	Maximum SI ≤ 1.3	1.3 < Maximum SI < 1.9	Maximum SI ≥ 1.9	Total
Sensitizer	0 (0%)	4 (31%)	80 (96%)	84
Nonsensitizer	10 (100%)	9 (69%)	3 (4%)	22
Total	10	13	83	106

Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

[1] Numbers shown reflect number of tests. Includes all tests of substances that were tested multiple times. Percentage in parentheses reflects percentage of the total number of tests for each SI category.

The 106 tests evaluated in **Table C-VIII-10** include multiple tests for 15 substances. For the 15 substances, two to 12 tests were available. **Table C-VIII-11** shows the proportion of the tests for each substance that produced SI values in each category. For the 10 sensitizers with multiple test results, there were no tests that produced SI ≤ 1.3 or 1.3 < SI < 1.9. However, the LLNA: BrdU-ELISA tests for traditional LLNA nonsensitizers were more variable. The results for isopropanol were particularly variable: 57% (4/7) of the tests produced SI ≤ 1.3 (SI = 0.92, 0.94, 0.98, and 1.01), 14% (1/7) produced 1.3 < SI < 1.9 (SI = 1.57), and 29% (2/7) produced SI ≥ 1.9 (SI = 2.04 and 2.22). Lactic acid tests produced SI values in two categories: 67% (2/3) of the tests had 1.3 < SI < 1.9 (SI = 1.80 and 1.89), and 33% (1/3) of the tests had SI ≥ 1.9 (SI = 2.53). Propylene glycol tests produced SI values in two categories: 67% (2/3) of the tests had SI < 1.3 (SI = 0.91 and 1.20) and one test produced 1.3 < SI < 1.9 (SI = 1.57). The multiple test results for hexane and methyl salicylate were 100% concordant, with all results in the 1.3 < SI < 1.9 category. The two hexane tests produced SI values of 1.76 and 1.89, and the three methyl salicylate tests also produced SI values of 1.40, 1.43, and 1.44.

Table C-VIII-11 Concordance of LLNA: BrdU-ELISA Tests for Substances with Multiple Tests by Maximum SI Category

Substance Name	Concordance Among Multiple Tests[1]			Total
	Maximum SI ≤ 1.3	1.3 < Maximum SI < 1.9	Maximum SI ≥ 1.9	
Sensitizers[2]				
2,4-Dinitrochloro-benzene	0 (0%)	0 (0%)	9 (100%)	9
Diphenylcyclopro-penone	0 (0%)	0 (0%)	3 (100%)	3
Eugenol	0 (0%)	0 (0%)	9 (100%)	9
Formaldehyde	0 (0%)	0 (0%)	3 (100%)	3
Glutaraldehyde	0 (0%)	0 (0%)	5 (100%)	5
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	12 (100%)	12
Isoeugenol	0 (0%)	0 (0%)	3 (100%)	3
Nickel sulfate	0 (0%)	0 (0%)	3 (100%)	3
1,4-Phenylenediamine	0 (0%)	0 (0%)	2 (100%)	2
trans-Cinnamaldehyde	0 (0%)	0 (0%)	4 (100%)	4

continued

Table C-VIII-11 Concordance of LLNA: BrdU-ELISA Tests for Substances with Multiple Tests by Maximum SI Category (continued)

Substance Name	Concordance Among Multiple Tests[1]			Total
	Maximum SI ≤ 1.3	1.3 < Maximum SI < 1.9	Maximum SI ≥ 1.9	
Nonsensitizers[2]				
Hexane	0 (0%)	2 (100%)	0 (%)	2
Isopropanol	4 (57%)	1 (14%)	2 (29%)	7
Lactic acid	0 (0%)	2 (67%)	1 (33%)	3
Methyl salicylate	0 (0%)	3 (100%)	0 (0%)	3
Propylene glycol	2 (67%)	1 (33%)	0 (0%)	3

Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

[1] Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of tests for each substance.

[2] According to traditional LLNA results.

4.0 The Impact of Increasing the LLNA: BrdU-ELISA Sample Size on the Substances in the Range of Uncertainty

This section examines the impact of increasing the number of animals used in each LLNA: BrdU-ELISA control and treatment group (i.e., sample size) on the size of the range of uncertainty (i.e., 1.3 < SI < 1.9) and on the number of substances in the range of uncertainty.

Since the LLNA: BrdU-ELISA accuracy analyses were based on studies with four animals per dose group, additional analyses were performed in order to determine if the sample size per dose group contributed to these indeterminate classifications. As detailed below, increasing the sample size for each dose group is unlikely to impact either the number of substances classified as uncertain or the SI values that define the range.

Table C-VIII-12 shows the 31 substances evaluated, along with their LLNA: BrdU-ELISA SI values and corresponding traditional LLNA results. Based on the LLNA: BrdU-ELISA SI values, 18 substances were sensitizers (SI ≥ 1.9), four were nonsensitizers (SI ≤ 1.3), and nine were in the range of uncertainty (1.3 < SI < 1.9). Of the nine substances in the range of uncertainty, four were sensitizers and five were nonsensitizers in the traditional LLNA.

Increasing the sample size could effectively move any of the borderline substances into or out of the range of uncertainty. Also, changing the sample size could widen or narrow the range of the uncertainty interval and thus either increase or decrease the number of substances in the range of uncertainty.

Table C-VIII-12 Distribution of LLNA: BrdU-ELISA Maximum SI Data for 31 Substances

Substance Name	LLNA: BrdU-ELISA Maximum SI Values[1]	Traditional LLNA Result
LLNA: BrdU-ELISA Positive; SI ≥ 1.9 (N = 18)		
2,4-Dinitrochlorobenzene	4.30, 4.83, 5.50, 6.26, 6.84, 8.37, 12.98, 17.90, 18.80	+
3-Aminophenol	3.06	+
4-Chloroaniline	2.53	+
Benzoquinone	6.90	+
Cinnamic aldehyde	3.97	+
Citral	1.84, 16.35	+
Cyclamen aldehyde	1.97	+
Diphenylcyclopropenone	9.34, 19.10	+
Eugenol	3.10, 3.17, 3.18, 3.18, 3.30, 3.83, 7.09, 12.30, 17.70	+
Formaldehyde	1.97, 4.40, 16.59	+
Glutaraldehyde	2.25, 3.72, 14.60, 15.50, 28.64	+
Hexyl cinnamic aldehyde	2.44, 2.72, 2.87, 3.02, 3.27, 3.34, 3.40, 3.64, 3.64, 3.84, 5.90, 13.50	+
Isoeugenol	2.40, 6.73, 8.40	+
Isopropyl myristate	1.10, 4.20	+
Nickel sulfate	2.58, 2.66, 4.53	+
4-Phenylenediamine	11.70, 14.70	+
trans-Cinnamaldehyde	3.37, 3.50, 4.11	+
Trimellitic anhydride	7.85	+
LLNA: BrdU-ELISA Negative; SI ≤ 1.3 (N = 4)		
2-Hydroxypropyl methacrylate	1.13	-
Diethyl phthalate	0.88	-
Dimethyl isophthalate	1.26	-
Glycerol	1.29	-
LLNA: BrdU-ELISA Range of Uncertainty; 1.3 < SI < 1.9 (N = 9)		
2-Mercaptobenzothiazole	**1.62**	+
Aniline	**1.50**	+
Hexane	0.73, **1.76, 1.89**	-
Hydroxycitronellal	**1.34**	+
Isopropanol	0.92, 0.94, 0.98, 1.01, **1.57**, 2.04, 2.22	-
Lactic acid	**1.80, 1.89,** 2.53	-
Linalool	**1.45**	+
Methyl salicylate	**1.40, 1.43, 1.44**	-
Propylene glycol	0.87, 1.20, **1.57**	-

Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; N = number of substances; SI = stimulation index; + = sensitizer; - = nonsensitizer.

[1] Multiple values indicate multiple test results. The bold text indicates LLNA: BrdU-ELISA tests with maximum SI values between 1.3 and 1.9.

4.1 Impact of Sample Size on the Size of the Range of Uncertainty

There are two substances that determine the limits of the range of uncertainty: hydroxycitronellal (the sensitizer, based on traditional LLNA data, with the lowest SI value in the range of uncertainty, 1.34) and lactic acid (the nonsensitizer, based on traditional LLNA data, with the highest SI value in the range of uncertainty, 1.89).

To illustrate the impact of additional animals, consider hydroxycitronellal. Based on the individual animal data, the four animals had SI values of 1.38, 1.25, 1.57, and 1.17 (Annex IV-1). The mean SI value for these four animals is 1.34, which is effectively the lower limit of the range of uncertainty. The standard deviation (SD) is 0.18. Assume an underlying normal distribution with a mean of 1.34 and an SD of 0.18 (range of 1.16 to 1.52) and consider how the range might change if, for example, 10 animals are used rather than four. A mean will be contained in a range of the mean plus or minus 1.28 times the standard error (SE) for 80% of the time. For a sample of size 10, the SE is 0.055. There is 80% confidence that if a mean SI had been calculated based on 10 animals, it would fall between 1.27 and 1.41, which does not have any appreciable impact on the size of the range of uncertainty.

4.2 Impact of Sample Size on the Number of Substances in the Range of Uncertainty

Regarding the number of substances within the range, if the revised mean SI were as low as 1.27, then it is possible that glycerol (which had an overall mean SI of 1.29) could be added to the range of uncertainty. The most likely outcome is no change at all and only a minor shift in the lower end of the range (either slightly upward or slightly downward).

The upper limit is somewhat different, since the SI data for lactic acid are more variable, and, importantly, there are three tests rather than one. The individual animal SI values for one test were 1.83, 2.84, 0.97, 1.56 (Annex IV-2), producing a mean SI of 1.80 and an SE (for N = 10) of 0.25. Thus, the upper limit (with 80% confidence) could shift as low as SI = 1.48 or as high as SI = 2.12. If this were the only study, then raising the upper limit would potentially add three substances to the range of uncertainty.

However, the lower limit for the range of uncertainty could not be reduced to SI = 1.48, because of hexane (negative, despite SI = 1.76 and SI = 1.89). Reducing the lower limit below an SI of 1.76 would make hexane a false positive. Lactic acid had three studies, not one, and in order to lower the range of uncertainty, two of the three would have to be revised downward. The likelihood of both the SI = 1.80 and the SI = 1.89 lactic acid studies being revised downward to SI = 1.48 based on additional animals is quite small (less than 5%). So, because of the multiple studies for lactic acid (and the results for hexane) additional animals would have little appreciable effect on the upper limit of the range of uncertainty.

There is not a single SI value that would produce accurate classifications for all the substances in the range of uncertainty. For example, if the range of uncertainty is eliminated, and an SI = 1.50 is proposed as the cutoff point, even with more animals, there is a strong likelihood that lactic acid (traditional LLNA negative, despite LLNA: BrdU-ELISA SI of 1.80, 1.89, and 2.53) and hexane (traditional LLNA negative, despite LLNA: BrdU-ELISA SI of 1.76 and 1.89) would be still be misclassified, as likely would hydroxycitronellal (traditional LLNA positive, despite LLNA: BrdU-ELISA SI of 1.34). Different proposed SI cutoff points (higher or lower than 1.50) would still produce misclassifications. As the number of animals approaches infinity, the means converge to the observed mean, so in the limit, there would be no change at all in the range of uncertainty by increasing the sample size (assuming that the means observed are essentially correct).

The SI values determined for these 31 substances were based on four animals per dose. The analyses described above indicates that additional animals would likely not have had an appreciable impact on either the number of substances in the range of uncertainty or on the range of the uncertainty interval.

5.0 Analysis of Physicochemical Characteristics of Substances in the Range of Uncertainty

5.1 Introduction

The following information is presented to evaluate the use of physicochemical characteristics and other parameters to distinguish between traditional LLNA sensitizers and nonsensitizers in the LLNA: BrdU-ELISA when using multiple SI decision criteria to identify sensitizers and nonsensitizers (SI ≥ 1.9 and SI ≤1.3 for sensitizers and nonsensitizers, respectively). Characteristics that distinguish between sensitizers and nonsensitizers may aid in the interpretation of LLNA: BrdU-ELISA SI values that fall within the range of uncertainty, 1.3 < SI < 1.9.

The physicochemical information evaluated included peptide reactivity, molecular weight, octanol/water partition coefficient, physical form, and chemical class. The other parameters evaluated were vehicle control substance and potential local skin irritation at the highest concentration tested. The "potentially irritating" concentration is based on either (1) skin irritation at the concentration tested based on hazard identification (e.g., ECETOC skin irritation database; published traditional LLNA studies that provided skin irritation data), (2) the concentration tested in the LLNA exceeded the challenge concentration used in the GPMT (i.e., the maximum nonirritating concentration is used in the GPMT), (3) human skin irritation data from predictive skin sensitization patch testing, or (4) mouse skin irritation data. The information used for this analysis is provided in **Annexes II** and **III** unless otherwise noted.

The nine substances in the range of uncertainty, 1.3 < SI < 1.9, for the LLNA: BrdU-ELISA test method along with the LLNA: BrdU-ELISA SI values and the traditional LLNA SI values are listed in **Table C-VIII-13**. Based on the traditional LLNA, four substances were sensitizers and five substances were nonsensitizers.

Table C-VIII-13 Substances with Tests in the Range of Uncertainty: 1.3 < SI < 1.9

Substance	Maximum SI LLNA: BrdU-ELISA[1]	Maximum SI Traditional LLNA	Traditional LLNA Result
Aniline	1.50	3.6	+
Hexane	1.76, 0.73, 1.89	2.2	-
Hydroxycitronellal	1.34	8.5	+
Isopropanol	2.22, 0.98, 1.57, 0.94, 2.04, 1.01, 0.92	1.7	-
Lactic acid	2.53, 1.89, 1.80	2.2	-
Linalool	1.45	8.3	+
2-Mercaptobenzothiazole	1.62	8.6	+
Methyl salicylate	1.44, 1.43, 1.40	2.9	-
Propylene glycol	1.2, 1.57, 0.91	1.6	-

Abbreviations: + = sensitizer; - = nonsensitizer; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; SI = stimulation index.

[1] Multiple values indicate multiple test results.

5.2 Peptide Reactivity

Because the ability to form stable conjugates with protein is a key requirement for a substance to produce skin sensitization, peptide reactivity information may assist in determining skin sensitization potential (Jowsey et al. 2006).

5.2.1 Categorical Analysis

Gerberick et al. (2007) classified peptide reactivity as high, moderate, low, and minimal based on a classification tree model used to relate the depletion of cysteine- and lysine-containing peptides to relative skin sensitization potency categories from Kimber et al. (2003) that were based on LLNA EC3 values. The preferred model, which was based on the average of two peptide depletion measurements (i.e., one using a cysteine-containing peptide at a 1:10 molar ratio with the test substance and one using a lysine-containing peptide at a 1:50 molar ratio with the test substance), accurately predicted the sensitizer or nonsensitizer outcomes of 89% (72/81) of the substances evaluated (Gerberick et al. 2007). The peptide reactivity categories for 20/31 substances tested in the LLNA: BrdU-ELISA were available from Gerberick et al. (2007). These data were used to analyze the association of the high, moderate, low, and minimal peptide reactivity categories with the traditional LLNA sensitizer and nonsensitizer status of the 20 test substances (12 traditional LLNA sensitizers and eight traditional LLNA nonsensitizers).

Table C-VIII-14 lists the nine substances in the range of uncertainty and the corresponding peptide reactivity categories available from Gerberick et al. (2007). Peptide reactivity categories were available for 7/9 substances. **Annex VIIIa** shows the peptide reactivity information for all 20 substances available from Gerberick et al. (2007).

Table C-VIII-14 Peptide Reactivity Data for Substances in the Range of Uncertainty

Substance	Traditional LLNA Result	Peptide Reactivity Category[1]	% Cysteine Depletion[2]
Aniline	+	NA	NA
Hexane	-	Minimal	-0.4
Hydroxycitronellal	+	Low	46.7
Isopropanol	-	Minimal	0.3
Lactic acid	-	Minimal	2.5
Linalool	+	NA	2.0
2-Mercaptobenzothiazole	+	High	100
Methyl salicylate	-	Minimal	0.3
Propylene glycol	-	Minimal	-0.9

Abbreviations: LLNA = murine local lymph node assay.

+ = sensitizer; - = nonsensitizer.

[1] Categories from Gerberick et al. (2007).

[2] Values from Natsch et al. (2009).

Table C-VIII-15 shows the proportions of the 12 sensitizers and eight nonsensitizers in each category of peptide reactivity. Traditional LLNA nonsensitizers, across all relevant LLNA: BrdU-ELISA SI categories (i.e., whether SI ≤ 1.3 or 1.3 < SI < 1.9) were associated with minimal to low peptide reactivity; 100% (8/8) of the nonsensitizers with peptide reactivity data had low or moderate peptide reactivity. The 12 traditional LLNA sensitizers with peptide reactivity data, across both relevant LLNA: BrdU-ELISA SI categories (i.e., whether 1.3 < SI < 1.9 or SI ≥ 1.9), were generally associated with moderate to high peptide reactivity (58% [7/12]); however, 25% (3/12) of the sensitizers were associated with low peptide reactivity, and 17% (2/12) of the sensitizers were associated with minimal peptide reactivity.

Table C-VIII-15 Peptide Reactivity for Sensitizers vs. Nonsensitizers[1]

Peptide Reactivity Category[2]	Sensitizer[3]/ LLNA: BrdU-ELISA SI ≥ 1.9	Nonsensitizer[3]/ LLNA: BrdU-ELISA SI ≤ 1.3	Sensitizer[3]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9	Nonsensitizer[3]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9
High	50% (5/10)	0% (0/3)	50% (1/2)	0% (0/5)
Moderate	10% (1/10)	0% (0/3)	0% (0/2)	0% (0/5)
Low	20% (2/10)	33% (1/3)	50% (1/2)	0% (0/5)
Minimal	20% (2/10)	67% (2/3)	0% (0/2)	100% (5/5)
NA	8	1	2	0
Total Substances	18	4	4	5

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; NA = peptide reactivity information was not available; SI = stimulation index.

[1] Number of substances shown. Proportion in parentheses based on number of substances with peptide reactivity data.

[2] Determined using data in Gerberick et al. (2007).

[3] Based on traditional LLNA.

There are insufficient data to definitively choose a single "breakpoint" for using peptide reactivity to predict sensitizers. However, a range of reactivity (i.e., low to high vs. minimal) could be useful since Fisher's exact test shows that peptide reactivity is highly associated ($p < 0.001$) with the traditional LLNA result using the low to high vs. minimal breakpoint (**Table C-VIII-16**).

Table C-VIII-16 Fisher's Exact Test for Association of Peptide Reactivity with Sensitizers and Nonsensitizers[1]

Peptide Reactivity Category	Sensitizer	Nonsensitizer	Peptide Reactivity Category	Sensitizer	Nonsensitizer
Low to High	10	1	Moderate to High	7	0
Minimal	2	7	Minimal to Low	5	8
p = 0.0045 (Fisher's Exact Test)			p = 0.0147 (Fisher's Exact Test)		

[1] Number of substances with peptide reactivity in each category shown.

Low to high vs. minimal would correctly classify 100% (7/7) of the substances in the range of uncertainty that have peptide reactivity data (**Table C-VIII-5**). Moderate to high vs. minimal to low would correctly classify 86% (6/7) substances in the range of uncertainty. The association is highly

significant, and peptide reactivity could be used as a "tiebreaker" for those substances for which the LLNA: BrdU-ELISA assay produces SI values in the range of uncertainty.

5.2.2 Numerical Analysis

Peptide reactivity data as percent cysteine depletion were available for 27/31 substances tested in the LLNA: BrdU-ELISA. Most of the cysteine depletion data were obtained from Natsch et al. (2009). Natsch et al. (2009) measured peptide depletion with methods similar to Gerberick et al. (2007) using a cysteine-containing peptide at a 1:10 molar ratio with the test substance. Thus, cysteine depletion data was obtained from Gerberick et al. (2007) for substances that were not included in Natsch et al. (2009). Natsch et al. (2009) demonstrated that using >15% cysteine-containing peptide depletion to classify sensitizers yielded an overall accuracy of 80% (93/116). The cysteine depletion data were used to analyze sensitizer/nonsensitizer classification using various peptide depletion cutoff values. Cysteine depletion data were available for 8/9 substances in the range of uncertainty (see **Table C-VIII-14**).

The analysis evaluated the performance of several different % cysteine depletion values by determining the accuracy, false negative rate, and false positive rate for classifying substances as sensitizers and nonsensitizers. The results indicated that the highest accuracy (81% [22/27]) occurred for three different cysteine depletion cutoffs, >0.55%, >1.40, or >4.75%, that were used to classify substances as sensitizers. The associated false positive rates were 38% (3/8), 25% (2/8), and 13% (1/8), respectively. False negative rates were 11% (2/19), 16% (3/19), and 22% (4/19), respectively. Thus, the cutoff with the lowest false negative rate was >0.55%. See **Annex VIIIb** for the performance of other cysteine depletion cutoffs.

Table C-VIII-17 shows that the percentages of sensitizers with LLNA: BrdU-ELISA SI ≥ 1.9 with cysteine depletion values of >0.55%, >1.40, or >4.75% were 88% (14/16), 81% (13/16), and 81% (13/16), respectively. The percentages of nonsensitizers with LLNA: BrdU-ELISA SI ≤ 1.3 for cysteine depletion values ≤0.55%, ≤1.40 or ≤4.75% were 0% (0/3), 67% (2/3), and 67% (2/3), respectively. For the substances with 1.3 < SI < 1.9, 100% (3/3) of the sensitizers had cysteine depletion values >0.55% or >1.40%, and 100% (5/5) of the nonsensitizers had cysteine depletion ≤4.75.

Table C-VIII-17 Correct Classification Rate of Sensitizers vs. Nonsensitizers by Cysteine Depletion[1]

Cysteine Depletion Cutoff	Sensitizer[2]/ LLNA: BrdU-ELISA SI ≥ 1.9	Nonsensitizer[2]/ LLNA: BrdU-ELISA SI ≤ 1.3	Sensitizer[2]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9	Nonsensitizer[2]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9
≤0.55%	12% (2/16)	0% (0/3)	0% (0/3)	80% (4/5)
>0.55%	88% (14/16)	100% (3/3)	100% (3/3)	20% (1/5)
≤1.40%	19% (3/16)	67% (2/3)	0% (0/3)	80% (4/5)
>1.40%	81% (13/16)	33% (1/3)	100% (3/3)	20% (1/5)
≤4.75%	19% (3/16)	67% (2/3)	33% (1/3)	100% (5/5)
>4.75%	81% (13/16)	33% (1/3)	67% (2/3)	0% (0/5)
≤ 15%	25% (4/16)	67% (2/3)	33% (1/3)	100% (5/5)
> 15%	75% (12/16)	33% (1/3)	67% (2/3)	0% (0/5)
NA	3	1	1	0
Total Substances	18	4	4	5

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; NA = peptide reactivity information was not available; SI = stimulation index.

[1] Number of substances shown. Proportion in parentheses based on the total number of substances with peptide reactivity data.

[2] Based on traditional LLNA.

Natsch et al. (2009) indicated that at least 15% peptide depletion is needed for significant results. The percentage of sensitizers and nonsensitizers associated with peptide depletion ≤15% and >15% are also shown in **Table C-VIII-17**. The results were similar to the cutoff value of 4.75% cysteine depletion. Of the sensitizers with SI ≥ 1.9, 75% (12/16) had cysteine depletion values >15%, and 67% (2/3) of the nonsensitizers with SI ≤ 1.3 had cysteine depletion values ≤15%. For the substances with 1.3 < SI < 1.9, 67% (2/3) of the sensitizers had cysteine depletion >15%, and 100% (5/5) of the nonsensitizers had cysteine depletion ≤15%.

The cysteine depletion cutoffs of 4.75% and 15% (evaluated in **Table C-VIII-17)** would have accurately classified 88% (7/8) of the substances in the range of uncertainty that had cysteine depletion data. This is similar to the result yielded by the categorical analysis when using low to high peptide reactivity to classify sensitizers and minimal peptide reactivity to classify nonsensitizers, which classified 100% (7/7) of the substances (with categorical peptide reactivity data) in the range of uncertainty.

5.3 Molecular Weight

The molecular weights of the 22 sensitizers and nine nonsensitizers were not different, as shown by the means and standard deviations in **Table C-VIII-18**. The standard deviations for sensitizers and nonsensitizers have a large range of overlap.

Table C-VIII-18 Molecular Weight (g/mol) for Sensitizers vs. Nonsensitizers

	Sensitizer[1]/ LLNA: BrdU-ELISA SI ≥ 1.9	**Nonsensitizer[1]/ LLNA: BrdU-ELISA SI ≤ 1.3**	**Sensitizer[1]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9**	**Nonsensitizer[1]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9**
Mean	153.4	163.2	146.7	92.9
Standard Deviation	55.1	57.3	36.5	35.1
Total	18	4	4	5

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; SI = stimulation index.

[1] Based on traditional LLNA.

5.4 Octanol-Water Partition Coefficient (log K_{ow})

The octanol-water partition coefficients (log K_{ow}) of the sensitizers and nonsensitizers were not different, as shown by the means and overlapping standard deviations in **Table C-VIII-19**. The log K_{ow} value was unavailable for one substance.

Table C-VIII-19 Log K_{ow} for Sensitizers vs. Nonsensitizers

	Sensitizer[1]/ LLNA: BrdU-ELISA SI ≥ 1.9	Nonsensitizer[1]/ LLNA: BrdU-ELISA SI ≤ 1.3	Sensitizer[1]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9	Nonsensitizer[1]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9
Mean	1.98	1.15	2.01	0.90
Standard Deviation	1.13	0.82	0.43	0.74
Total	17[2]	4	4	5

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; SI = stimulation index.

[1] Based on traditional LLNA.

[2] No log K_{ow} available for nickel sulfate.

5.5 Physical Form

Table C-VIII-20 shows the association of physical form with traditional LLNA sensitizer/ nonsensitizer outcome. The sensitizers with SI ≥ 1.9 and the nonsensitizers with SI ≤ 1.3 were divided approximately equally into solids and liquids. The majority of the substances (89% [8/9]) with 1.3 < SI < 1.9 were liquids regardless of whether they were sensitizers or nonsensitizers.

Table C-VIII-20 Physical Form for Sensitizers vs. Nonsensitizers[1]

Physical Form	Sensitizer[2]/ LLNA: BrdU-ELISA SI ≥1.9	Nonsensitizer[2]/ LLNA: BrdU-ELISA SI ≤ 1.3	Sensitizer[2]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9	Nonsensitizer/[2] 1.3 < LLNA: BrdU-ELISA SI < 1.9
Solid	7 (39%)	2 (50%)	1 (25%)	0 (0%)
Liquid	11 (61%)	2 (50%)	3 (75%)	5 (100%)
Total	18	4	4	5

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; SI = stimulation index.

[1] Number of substances shown. Proportion in parentheses is based on the total number of substances.

[2] Based on traditional LLNA.

5.6 Vehicle Control Substances

Table C-VIII-21 shows the proportions of sensitizers and nonsensitizers for each vehicle control substance used for traditional LLNA and LLNA: BrdU-ELISA testing. Because there were too many vehicles with few substances to make an adequate comparison, the substances tested in AOO were compared with all other vehicles combined. The proportions of sensitizers and nonsensitizers tested in AOO vs. all other vehicles were similar.

Table C-VIII-21 Vehicle Control for Sensitizers vs. Nonsensitizers[1]

Vehicle	Sensitizer[2]/ LLNA: BrdU-ELISA SI ≥ 1.9	Nonsensitizer[2]/ LLNA: BrdU-ELISA SI ≤ 1.3	Sensitizer[2]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9	Nonsensitizer[2]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9
Acetone: olive oil (4:1)	15 (83%)	3 (75%)	3 (75%)	3 (60%)
Dimethylformamide	0 (0%)	1 (25%)	1 (25%)	0 (0%)
Acetone	2 (11%)	0 (0%)	0 (0%)	0 (0%)
Dimethyl sulfoxide	1 (6%)	0 (0%)	0 (0%)	1 (20%)
Water	0 (0%)	0 (0%)	0 (0%)	1 (20%)
Total	18	4	4	5
Acetone: Olive Oil vs. Other Vehicles				
Acetone: olive oil (4:1)	15 (83%)	3 (75%)	3 (75%)	3 (60%)
Other	3 (17%)	1 (25%)	1 (25%)	2 (40%)
Total	18	4	4	5

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; SI = stimulation index.

[1] Number of substances shown. Proportion of total is shown in parentheses.

[2] Based on traditional LLNA.

5.7 Skin Irritation Data

The maximum concentrations tested in the traditional LLNA were compared with concentrations known to produce skin irritation to determine whether there was a relationship between skin irritation and sensitizer or nonsensitizer results in the traditional LLNA. For the sensitizers, 73% (16/22) were tested at potentially irritating concentrations while 56% (5/9) of the nonsensitizers were tested at irritating concentrations. For the entire group of substances tested, 68% (21/31) were tested at irritating concentrations.

5.8 Conclusion

Based on the available data, peptide reactivity is the only promising characteristic for a positive association with LLNA sensitizer/nonsensitizer results that could be used to assist in classifying substances that produce LLNA: BrdU-ELISA SI values in the range of uncertainty. While there are insufficient data to definitively choose a single "breakpoint" for using peptide reactivity to predict sensitizers, ranges of peptide reactivity were highly associated ($p < 0.001$) with the traditional LLNA results using the low to high vs. minimal breakpoints. Thus, peptide reactivity could be used as a "tiebreaker" for those substances for which the LLNA: BrdU-ELISA produces SI values in the range of uncertainty. The numerical analysis using different cysteine depletion cutoffs also supports the conclusion that peptide reactivity is associated with sensitization outcomes.

5.9 References

Gerberick GF, Vassallo JD, Foertsch LM, Price BB, Chaney JG, Lepoittevin JP. 2007. Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. Toxicol Sci 97:417-427.

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Annex VIIIa Data for 31 Substances Tested Using the LLNA: BrdU-ELISA Method

Substance	Vehicle	Trad. LLNA SI	LLNA: BrdU-ELISA SI and Highest Conc. Tested (%)[1]	MW (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Cys Depletion (%)[3]	Physical Form	Chemical Class[4]	Skin Irritant[5]	Highest Conc. Tested (%)[6]	Maximum Non-Irritating Conc. (%) (unless noted)[7]
1,4-Phenylene-diamine	AOO	26.4	11.70, 14.70; (2, 10)	108.141	1.17	NA	95.2	Solid	Amines	YES	1	0.5
2,4-Dinitrochloro-benzene	AOO	43.9	4.30, 8.37, 6.26, 5.50, 18.80, 4.83, 12.98, 17.90, 6.84; (1, 1, 0.3, 1, 0.3, 1, 1, 2, 2)	202.55	-0.057	High	100	Solid	Hydrocarbon, halogenated; Nitro compounds; Hydrocarbons, cyclic	YES	0.25	0.1
3-Aminophenol	AOO	5.7	3.06; (25)	109.126	1.17	NA	7	Solid	Amines; Phenols	YES	10	5
4-Chloroaniline	AOO	+NA	2.53; (25)	127.57	1.8	NA	NA	Liquid	Amines	NA	NA	2.5
Benzoquinone	AOO	52.3	6.90; (1)	108.095	1.17	High	91.8	Solid	Quinones	YES	2.5	2.5
Cinnamic aldehyde	AOO	18.4	3.97; (50)	132.16	2.29	High	90.5	Liquid	Aldehydes	NO	25	100
Citral	AOO	20.5	16.35, 1.84; (50, 10)	152.233	2.54	NA	34.7	Liquid	Hydrocarbons, other	YES	20	0.5
Cyclamen aldehyde	AOO	5.2	1.97; (100)	190.28	3.28	Low	59.9	Liquid	Carboxylic acids	NO	50	100
Diphenylcyclo-propenone	AOO	+NA	19.10, 9.34; (2, 10)	206.24	3.25	High	98.8	Solid	Hydrocarbons, cyclic	NA	NA	NA
Eugenol	AOO	17	3.18, 3.30, 3.83, 12.30, 3.10, 7.09, 3.17, 3.18, 17.70; (10, 30, 30, 50, 50, 50, 50, 50, 50)	164.201	2.15	NA	54	Liquid	Carboxylic acids	YES	50	25
Formaldehyde	ACE	11.9	16.59, 4.40, 1.97; (10)	30.03	0.33	Moderate	56.5	Liquid	Aldehydes	YES	25	2
Glutaraldehyde	ACE	18	28.64, 3.72, 2.25, 14.60, 15.50; (1, 1, 1, 2, 10)	100.12	0.92	High	30	Liquid	Aldehydes	NA	2.5	NA

Substance	Vehicle	Trad. LLNA SI	LLNA: BrdU-ELISA SI and Highest Conc. Tested (%)[1]	MW (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Cys Depletion (%)[3]	Physical Form	Chemical Class[4]	Skin Irritant[5]	Highest Conc. Tested (%)[6]	Maximum Non-Irritating Conc. (%) (unless noted)[7]
Hexyl cinnamic aldehyde	AOO	20	3.60, 5.90, 3.64, 2.72, 3.02, 3.40, 2.07, 6.11, 3.43, 5.15, 2.52, 2.87, 3.34, 3.54, 2.18, 3.34, 2.69, 3.17, 6.58, 13.50, 12.46, 4.24, 6.07, 3.27, 5.30, 2.41, 2.52, 3.84, 6.86, 4.39, 4.78; (50)	216.319	3.77	Minimal	-0.3	Liquid	Aldehydes	YES	50	10
Isoeugenol	AOO	31	8.40, 2.40, 6.73; (10, 10, 30)	164.201	2.15	NA	98.4	Liquid	Carboxylic acids	YES	5	5
Isopropyl myristate	AOO	3.4	4.20, 1.10 (50, 100)	270.46	3.88	Minimal	0.8	Liquid	Lipids	YES	100	100
Nickel Sulfate	DMSO	3.1	2.58, 4.53, 2.66; (10)	154.76	NA	NA	35.5	Solid	Inorganic chemicals, metals; Inorganic chemicals, elements	YES	5	0.15
trans-Cinnamaldehyde	AOO	13.1	4.11, 3.50, 3.37; (10)	132.6	1.82	NA	NA	Liquid	Aldehydes	NA	25	NA
Trimellitic anhydride	AOO	4.6	7.85; (10)	192.13	1.95	Low	-1.1	Solid	Anhydrides, Carboxylic acids	YES	25	10
2-Mercaptobenzo-thiazole	DMF	8.6	1.62; (50)	167.253	1.8	High	100	Solid	Heterocyclic compounds	YES	10	10
Aniline	AOO	3.6	1.50; (50)	93.1265	1.56	NA	NA	Liquid	Amines	YES	100	100
Hexane	AOO	2.2	1.76, 0.73, 1.89; (100, 10, 50)	86.1754	1.94	Minimal	-0.4[8]	Liquid	Hydrocarbons, acyclic	YES	100	100
Hydroxycitronellal	AOO	8.5	1.30; (100)	172.26	2.15	Low	46.7	Liquid	Hydrocarbons, other	YES	100	50
Isopropanol	AOO	1.7	2.22, 0.98, 1.57, 0.94, 2.04, 1.01, 0.92; (50, 50, 50, 50, 50, 50, 100)	60.095	0.82	Minimal	0.3	Liquid	Alcohols	NO	50	100
Lactic acid	DMSO	2.2	2.53, 1.89, 1.80; (50)	90.08	0.05	Minimal	2.5	Liquid	Carboxylic acids	YES	25	10
Linalool	AOO	8.3	1.45; (100)	154.25	2.54	NA	2	Liquid	Hydrocarbons	YES	100	100
Methyl salicylate	AOO	2.9	1.44, 1.44, 1.40; (50)	152.15	1.28	Minimal	0.3	Liquid	Phenols; Carboxylic acids	YES	20	10
Propylene glycol	H2O	1.6	1.2, 1.57, 0.87; (10, 50, 50)	76.0944	0.43	Minimal	-0.9	Liquid	Alcohols	NA	100	NA

Substance	Vehicle	Trad. LLNA SI	LLNA: BrdU-ELISA SI and Highest Conc. Tested (%)[1]	MW (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Cys Depletion (%)[3]	Physical Form	Chemical Class[4]	Skin Irritant[5]	Highest Conc. Tested (%)[6]	Maximum Non-Irritating Conc. (%) (unless noted)[7]
2-Hydroxypropyl methacrylate	AOO	1.3	1.13; (50)	144.168	1.03	Low	58.4[8]	Solid	Carboxylic acids	YES	50	10
Diethyl phthalate	AOO	1.5	0.88; (50)	222.24	1.87	Minimal	0.8	Liquid	Carboxylic acids	YES	100	100
Dimethyl isophthalate	AOO	1	1.26; (50)	194.19	1.66	NA	NA	Solid	Carboxylic acids	NA	25	NA
Glycerol	DMF	1.1	1.29; (50)	92.09	0.05	Minimal	-3.8	Liquid	Alcohols; Carbohydrates	NA	100	NA

Note: Shaded cells contain substances in the range of certainty.

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); Cys = cysteine-containing peptide; DMF = *N*,*N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; K_{ow} = octanol/water partition coefficient; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; MEK = methyl ethyl ketone; MW = molecular weight; NA = not available; Trad. = traditional;+ = sensitizer; - = nonsensitizer.

[1] Highest SI value from LLNA: DA test(s); respective highest concentration tested for each SI value in parentheses.

2 Kow represents the estimated octanol-water partition coefficient (expressed on log scale) calculated by an interactive demo at the SRC website: http://www.srcinc.com/what-we-do/databaseforms.aspx?id=385.

[3] Peptide reactivity categories based on cysteine and lysine depletion as reported in Gerberick et al. (2007). Cysteine depletion values are primarily from Natsch et al. (2009) unless otherwise noted.

4 Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine: http://www.nlm.nih.gov/mesh/meshhome.html.

[5] Highest concentration tested compared to the maximum nonirritating concentration.

[6] Highest concentration tested in the traditional LLNA.

[7] Guinea pig data unless noted.

[8] Data from Gerberick et al. (2007).

Annex VIIIb Performance of Cysteine Depletion Cutoffs for Prediction of 19 Sensitizers and Eight Nonsensitizers Tested in the LLNA: BrdU-ELISA

Cys Depletion (%)	Accuracy (%)	Sensitivity (%)	Specificity (%)	False Positive (%)	False Negative (%)	Number of Substances Çorrect +	Number of Substances False +	Number of Substances False -	Number of Substances Çorrect -
> -2.450	74	100	13	88	0	19	7	0	1
> -1.000	70	95	13	88	5	18	7	1	1
> -0.6500	74	95	25	75	5	18	6	1	2
> -0.3500	78	95	38	63	5	18	5	1	3
> 0.0	74	89	38	63	11	17	5	2	3
> 0.5500	81	89	63	38	11	17	3	2	5
> 1.400	81	84	75	25	16	16	2	3	6
> 2.250	78	79	75	25	21	15	2	4	6
> 4.750	81	79	88	13	21	15	1	4	7
> 18.50	78	74	88	13	26	14	1	5	7
> 32.35	74	68	88	13	32	13	1	6	7
> 35.10	70	63	88	13	37	12	1	7	7
> 41.10	67	58	88	13	42	11	1	8	7
> 50.35	63	53	88	13	47	10	1	9	7
> 55.25	59	47	88	13	53	9	1	10	7
> 57.45	56	42	88	13	58	8	1	11	7
> 59.15	59	42	100	0	58	8	0	11	8
> 75.20	56	37	100	0	63	7	0	12	8
> 91.15	52	32	100	0	68	6	0	13	8
> 93.50	48	26	100	0	74	5	0	14	8
> 96.80	44	21	100	0	79	4	0	15	8
> 98.60	41	16	100	0	84	3	0	16	8
> 99.40	37	11	100	0	89	2	0	17	8

Abbreviations: Cys = cysteine-containing peptide; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; + = sensitizer; - = nonsensitizer.

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