Annex VIII

Reproducibility Analyses for the LLNA: DA
Using a Single Decision Criterion of SI ≥ 3.0 or SI ≥ 2.0

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1.0 LLNA: DA Test Method Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period (ICCVAM 1997, 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. With regard to the murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") test method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional murine local lymph node assay (LLNA).

The LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of a single decision criterion in Section 6.5 of this background review document (BRD) showed that stimulation index (SI) $\geq$ 1.8 produced the most optimum results (i.e., 93% accuracy and 0% false negative rate) among the alternate decision criteria evaluated. Thus Section 7.0 of this BRD provides an assessment of reproducibility for the decision criterion of SI $\geq$ 1.8 to identify potential sensitizers. Further, since SI $\geq$ 3.0 was used by the validation management team in the intralaboratory and interlaboratory validation studies, and SI $\geq$ 2.0 was previously evaluated as an optimum decision criterion in the March 2009 draft BRD reviewed by the independent scientific peer review Panel, this annex details additional reproducibility analyses for SI $\geq$ 3.0 and SI $\geq$ 2.0.

1.1 Intralaboratory Reproducibility (SI $\geq$ 3.0 or SI $\geq$ 2.0)

Idehara et al. (2008) evaluated the intralaboratory reproducibility of EC3 values (estimated concentration needed to produce an SI of three) for the LLNA: DA using two substances (isoeugenol and eugenol) that were each tested in three different experiments (**Table C-VIII-1**). The data indicate coefficients of variation (CVs) of 21% and 11% for isoeugenol and eugenol, respectively. The authors state that for both compounds the EC3 values appeared to be close and that for each test substance the SI values for the same concentration were fairly reproducible (Idehara et al. 2008). The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) also determined the intralaboratory reproducibility of EC2 values (estimated concentration needed to produce an SI of two) for the same set of data. The results for EC2 values with CV values of 35% and 20% for isoeugenol and eugenol, respectively, indicate slightly larger intralaboratory variability compared to EC3 value results.

1.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (Annex IV of this BRD) from a two-phased interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the first phase of the interlaboratory validation study, a blinded test of 12 substances was conducted in 10 laboratories. Three substances (i.e. 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances were randomly assigned to subsets of three of the 10 laboratories **(Table C-VIII-2)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were predetermined (i.e., the participating laboratories did not determine their own dose levels for testing). Nine substances are sensitizers and three substances are nonsensitizers according to traditional LLNA results. Six substances are ICCVAM-recommended LLNA performance standards

reference substances: cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate (ICCVAM 2009).

Table C-VIII-1Intralaboratory Reproducibility of EC3 and EC2 Values Using the LLNA: DA[1]

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54	-------	1.22 ± 0.13
1	2.28 ± 0.60	-------	2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98
5	3.39 ± 0.69	4.39 ± 1.25	-------
10	5.68 ± 1.19	6.77 ± 0.23	-------
EC3	3.40%	2.35%	2.46%
EC2	0.82%	1.37%	0.75%
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC2: 0.98% ± 0.34% and 35% CV*			
Eugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	5.09%	5.59%	4.50%
EC2	4.33%	3.59%	2.87%
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC2: 3.60% ± 0.73% and 20% CV*			

Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] Based on results discussed in Idehara et al. 2008; the number per group was not specified.

[2] Mean stimulation index value ± standard deviation.

Table C-VIII-2 Substances and Allocation for the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitrochloro-benzene (+)	AOO	0.03	0.10	0.30	X	X	X	X	X	X	X	X	X	X
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X	X	X	X
Isopropanol (-)	AOO	10	25	50	X	X	X	X	X	X	X	X	X	X
Abietic acid (+)	AOO	5	10	25		X				X	X			
3-Aminophenol (+)	AOO	1	3	10	X		X					X		
Dimethyl isophthalate (-)	AOO	5	10	25	X		X				X			
Isoeugenol (+)	AOO	1	3	10				X	X				X	
Methyl salicylate (-)	AOO	5	10	25			X				X			X
Formaldehyde (+)	ACE	0.5	1.5	5.0	X	X			X					
Glutaraldehyde (+)	ACE	0.05	0.15	0.50	X	X			X					
Cobalt chloride[2] (+)	DMSO	0.3	1.0	3.0				X		X		X		
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10				X		X		X		

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The second phase of the interlaboratory validation study was designed to evaluate the reliability of the LLNA: DA for testing metallic salts using dimethyl sulfoxide (DMSO) as a vehicle since two metal salts dissolved in DMSO (cobalt chloride and nickel [II] sulfate hexahydrate) from the first phase of the interlaboratory validation study yielded inconsistent results. Five coded substances (two of the five substances were unique to the second phase of the interlaboratory validation study) were tested in seven laboratories (different from the 10 laboratories that performed the first interlaboratory validation study) **(Table C-VIII-3)**. One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (cobalt chloride, nickel [II] sulfate hexahydrate, lactic acid, and potassium dichromate) were randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the substance one time at three different dose levels. Again, the dose levels for each substance were predetermined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (lactic acid and potassium dichromate), one is a nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively. In addition, lactic acid is a reference substance included in performance standards recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM; ICCVAM 2009).

The LLNA: DA test results from the two-phased interlaboratory validation study are amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive) or nonsensitizer

(negative) classification (based on SI ≥ 3.0 and SI ≥ 2.0), and EC3 and EC2 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer based on SI ≥ 3.0 and SI ≥ 2.0) (**Sections 1.2.1** and **1.2.3,** respectively) and a CV analysis for the quantitative results (EC3 and EC2 values) (**Sections 1.2.2** and **1.2.4,** respectively).

Table C-VIII-3 Substances and Allocation for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory						
					11	12	13	14	15	16	17
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X
Cobalt chloride[2] (+)	DMSO	1	3	5	X		X	X			X
Lactic acid (-)	DMSO	5	10	25	X		X		X	X	
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10	X	X		X		X	
Potassium dichromate (+)	DMSO	0.1	0.3	1.0	X	X			X		X

Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional Murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

1.2.1 Interlaboratory Reproducibility – Qualitative Results (SI ≥ 3.0)

The qualitative (i.e., positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-4** using SI ≥ 3.0 as the decision criterion to distinguish sensitizers from nonsensitizers. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), eight substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were four discordant substances (formaldehyde, glutaraldehyde, cobalt chloride, and nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). One of the three laboratories that tested formaldehyde reported a maximum SI = 2.69 while the other two laboratories produced at least one SI ≥ 3.0. Similarly, one of the three laboratories that tested glutaraldehyde reported a maximum SI = 2.57 while the other two laboratories had at least one SI ≥ 3.0. Two of the three laboratories that tested cobalt chloride yielded an SI ≥ 3.0 at all three doses tested (0.3%, 1.0%, and 3.0%) and therefore classified the substance as a sensitizer similar to the traditional LLNA test method. Notably, the laboratory that did not generate an SI ≥ 3.0 did not test cobalt chloride at the highest dose and the middle dose yielded an SI = 2.66. One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories had at least two doses that yielded an SI ≥ 3.0. Since the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the first phase of the interlaboratory validation study.

Table C-VIII-4Qualitative Results for the First Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 3.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]										Concordance
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	
2,4-Dinitrochlorobenzene (+)	+ (11.97)	+ (9.23)	+ (9.96)	+ (8.53)	+ (7.86)	+ (15.14)	+ (13.18)	+ (12.60)	+ (10.89)	+ (4.71)	10/10
Hexyl cinnamic aldehyde (+)	+ (5.78)	+ (4.82)	+ (4.44)	+ (5.11)	+ (3.97)	+ (5.50)	+ (7.09)	+ (10.22)	+ (3.88)	+ (3.51)	10/10
Isopropanol (-)	- (1.54)	- (0.91)	- (1.01)	- (1.57)	- (0.76)	- (1.97)	- (1.45)	- (1.21)	- (0.70)	- (1.25)	10/10
Abietic acid (+)		+ (4.64)				+ (7.96)	+ (3.98)				3/3
3-Aminophenol (+)	- (2.83)		- (1.76)					- (2.38)			3/3
Dimethyl isophthalate (-)	- (1.34)		- (1.29)				- (1.26)				3/3
Isoeugenol (+)				+ (6.11)	+ (5.54)				+ (7.09)		3/3
Methyl salicylate (-)			- (1.55)				- (1.77)			- (0.83)	3/3
Formaldehyde (+)	**+ (4.84)**	**+ (3.18)**			**- (2.69)**						**2/3**
Glutaraldehyde (+)	**+ (5.00)**	**+ (3.39)**			**- (2.57)**						**2/3**
Cobalt chloride[3] (+)				**-[4] (2.66)**		**+ (20.55)**		**+ (8.07)**			**2/3**
Nickel (II) sulfate hexahydrate (+)				**-[5] (1.52)**		**+ (11.78)**		**+[5] (3.49)**			**2/3**

Bolded substances did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[4] Data not reported for the highest dose (3%), only for 0.3% and 1%.

[5] Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-5** using SI ≥ 3.0 as the decision criterion to distinguish sensitizers from nonsensitizers. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (hexyl cinnamic aldehyde, lactic acid, nickel [II] sulfate hexahydrate, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (cobalt chloride) for which interlaboratory concordance was 50% (2/4). Two of the four laboratories that tested cobalt chloride reported a maximum SI = 2.01 and 2.54, respectively, while the other two laboratories had at least two doses that yielded an SI ≥ 3.0. As was discussed previously, cobalt chloride was also discordant among the laboratories that tested the substance in the first phase of the interlaboratory validation study and interlaboratory concordance was 67% (2/3). Notably, different doses of cobalt chloride were tested in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study. Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table C-VIII-5 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 3.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]							Concordance
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	
Hexyl cinnamic aldehyde (+)	+ (4.47)	+ (5.71)	+ (5.41)	+ (7.60)	+ (3.92)	+ (8.42)	+ (6.45)	7/7
Cobalt chloride[3] (+)	**- (2.01)**		**- (2.54)**	**+ (4.25)**			**+ (5.06)**	**2/4**
Lactic acid (-)	- (0.93)		- (0.99)		- (0.97)	- (0.91)		4/4
Nickel (II) sulfate hexahydrate (+)	- (0.79)	- (1.24)		- (2.13)		- (1.56)		4/4
Potassium dichromate (+)	+ (4.78)	+ (4.08)			+ (6.01)		+ (6.37)	4/4

Boldface type indicates substances that did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

1.2.2 Interlaboratory Reproducibility – EC3 Values

The available quantitative (i.e., EC3 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA tests that yielded positive results (SI ≥ 3.0) during the first and second phase of the LLNA: DA interlaboratory validation study. The method for calculating EC3 values for the positive results was based on the method of linear interpolation reported by Gerberick et al. (2004) according to the equation:

$$EC3 = c + \left[\frac{(3-d)}{(b-d)}\right] \times (a-c)$$

where the data points lying immediately above and below the SI = 3.0 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI ≥ 3.0, an EC3 value was extrapolated according to the equation:

$$EC3_{ex} = 2^{\left\{\log_2(c) + \frac{(3-d)}{(b-d)} \times [\log_2(a) - \log_2(c)]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC3 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phase of the interlaboratory validation study are shown in **Tables C-VIII-6** and **C-VIII-7**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 4% (abietic acid) to 84% (glutaraldehyde) and the mean CV was 48% (**Table C-VIII-6**). Notably, although nickel (II) sulfate hexahydrate was a sensitizer in two of three laboratories, a CV could not be determined because one of the two laboratories that yielded a positive test demonstrated an insufficient dose response (i.e., an inverse dose response curve) from which to calculate an EC3 value. In the second phase of the interlaboratory validation study, CV values ranged from 32% (cobalt chloride) to 71% (potassium dichromate) and the mean CV was 45% (**Table C-VIII-7**).

The ICCVAM-recommended LLNA performance standards (ICCVAM 2009) indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. Acceptable reproducibility is attained when each laboratory obtains ECt values (estimated concentration needed to produce an SI of a specified threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, four laboratories reported EC3 values outside the range indicated for 2,4-dinitrochlorobenzene; one laboratory obtained an EC3 value that was lower than the specified acceptance range (0.025%) and three laboratories obtained EC3 values that were higher than the specified acceptance range (0.1%) (**Table C-VIII-6**). For hexyl cinnamic aldehyde, all the laboratories obtained an EC3 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and all seven laboratories obtained EC3 values that were within the acceptance range indicated (**Table C-VIII-7**).

Table C-VIII-6EC3 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC3 (%)										Mean EC3 (%) ± SD	CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10		
2,4-Dinitrochlorobenzene (+)	**0.034 (11.97)**	**0.109 (9.23)**	**0.056 (9.96)**	**0.031 (8.53)**	**0.129 (7.86)**	**0.042 (15.14)**	**0.016 (13.18)**	**0.095 (12.60)**	**0.040 (10.89)**	**0.169 (4.71)**	**0.072 ± 0.051**	**70**
Hexyl cinnamic aldehyde (+)	**9.983 (5.78)**	**12.412 (4.82)**	**14.90 (4.44)**	**9.340 (5.11)**	**18.131 (3.97)**	**13.130 (5.50)**	**7.706 (7.09)**	**7.924 (10.22)**	**17.070 (3.88)**	**15.235 (3.51)**	**12.583 ± 3.748**	**30**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		8.196				7.544	7.676				7.805 ± 0.345	4
3-Aminophenol (+)	NA		NA					NA			NA	NA
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				1.112	5.983				2.300		3.131 ± 2.540	81
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	1.747	1.480			NA						1.614 ± 0.189	12
Glutaraldehyde (+)	0.110	0.435			NA						0.272 ± 0.230	84
Cobalt chloride[2] (+)				NA[3]		0.063		0.137			0.100 ± 0.053	53
Nickel (II) sulfate hexahydrate (+)				NA		0.469		IDR			0.469 ± NA	NA

Note: Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved are from the highest dose tested (0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC3 values (estimated concentration needed to produce an SI of three) that are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards: 5 - 20% for hexyl cinnamic aldehyde and 0.025 - 0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; IDR = insufficient dose response; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[3] Data not reported for the highest dose (3%), only for 0.3% and 1%.

Table C-VIII-7EC3 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC3 (%)							Mean EC3 (%) ± SD	CV (%)
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17		
Hexyl cinnamic aldehyde (+)	**9.127 (4.47)**	**8.764 (5.71)**	**7.590 (5.41)**	**7.938 (7.60)**	**15.184 (3.92)**	**6.230 (8.42)**	**7.542 (6.45)**	**8.911 ± 2.920**	**33**
Cobalt chloride[2] (+)	NA		NA	1.761			1.109	1.435 ± 0.461	32
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		NA		NA	NA
Potassium dichromate (+)	0.509	0.485			0.156		0.086	0.309 ± 0.219	71

Bolded text indicates a substance that is an ICCVAM-recommended murine local lymph node assay performance standards reference substance for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved are from the highest dose tested (25%). None of the EC3 values are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards (5 - 20% for hexyl cinnamic aldehyde).

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC3 values were higher than that for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 6.8 to 83.7% for five substances tested in five laboratories (**Table C-VIII-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for the LLNA: DA were greater than that for the traditional LLNA. The CV of 70% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2%, calculated from five values each, reported by ICCVAM (1999). The CV values of 30% and 33% for hexyl cinnamic aldehyde tested in the first and second phase of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 81% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

Table C-VIII-8 Interlaboratory Reproducibility of the EC3 Values for Substances Tested in the Traditional LLNA[1]

Substance Name	EC3 (%)					CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	
2,4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37.4
	0.5	0.6	0.4	0.6	0.3	27.2
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	6.8
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41.2
Eugenol	5.8	14.5	8.9	13.8	6.0	42.5
Sodium lauryl sulfate	13.4	4.4	1.5	17.1	4.0	83.7

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

[1] From ICCVAM 1999 report.

1.2.3 Interlaboratory Reproducibility – Qualitative Results (SI ≥ 2.0)

The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-9** for SI ≥ 2.0. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), ten substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were two discordant substances (3-aminophenol and nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). Two of the three laboratories that tested 3-aminophenol reported SI ≥ 2.0, at least at the highest dose tested (SI = 2.83 and 2.38, respectively) but one lab did not achieve SI ≥ 2.0 at any dose tested (Annex IV of this BRD). One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories produced SI ≥ 2.0 at all three doses tested (Annex IV of this BRD). Since the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the first phase of the interlaboratory validation study.

Table C-VIII-9 Qualitative Results for the First Phase of the Interlaboratory Validation Studies for the LLNA: DA (SI ≥ 2.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]										Concordance
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	
2,4-Dinitrochlorobenzene (+)	+ (11.97)	+ (9.23)	+ (9.96)	+ (8.53)	+ (7.86)	+ (15.14)	+ (13.18)	+ (12.60)	+ (10.89)	+ (4.71)	10/10
Hexyl cinnamic aldehyde (+)	+ (5.78)	+ (4.82)	+ (4.44)	+ (5.11)	+ (3.97)	+ (5.50)	+ (7.09)	+ (10.22)	+ (3.88)	+ (3.51)	10/10
Isopropanol (-)	- (1.54)	- (0.91)	- (1.01)	- (1.57)	- (0.76)	- (1.97)	- (1.45)	- (1.21)	- (0.70)	- (1.25)	10/10
Abietic acid (+)		+ (4.64)				+ (7.96)	+ (3.98)				3/3
3-Aminophenol (+)	**+ (2.83)**		**- (1.76)**					**+ (2.38)**			**2/3**
Dimethyl isophthalate (-)	- (1.34)		- (1.29)				- (1.26)				3/3
Isoeugenol (+)				+ (6.11)	+ (5.54)				+ (7.09)		3/3
Methyl salicylate (-)			- (1.55)				- (1.77)			- (0.83)	3/3
Formaldehyde (+)	+ (4.84)	+ (3.18)			+ (2.69)						3/3
Glutaraldehyde (+)	+ (5.00)	+ (3.39)			+ (2.57)						3/3
Cobalt chloride[3] (+)				+[4] (2.66)		+ (20.55)		+ (8.07)			3/3
Nickel (II) sulfate hexahydrate (+)				**-[5] (1.52)**		**+ (11.78)**		**+[5] (3.49)**			**2/3**

Boldface text indicates substances did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizer result and (-) indicates nonsensitizer result in the LLNA: DA test. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[4] Data not reported for the highest dose (3%), only for 0.3% and 1%.

[5] Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-10**. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (hexyl cinnamic aldehyde, cobalt chloride, lactic acid, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 75% (3/4). Three of the four laboratories that tested nickel (II) sulfate hexahydrate did not report a maximum SI $\geq$ 2.0, while the other laboratory produced an SI $\geq$ 2.0 at the highest dose tested. As was discussed previously, nickel (II) sulfate hexahydrate was also discordant among the laboratories that tested the substance in the first phase of the interlaboratory validation study and interlaboratory concordance was 67% (2/3). Notably, when analyzing the dose response curves for the seven tests performed for nickel (II) sulfate hexahydrate in the two-phased interlaboratory validation study, only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative to increase in concentration). Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table C-VIII-10 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 2.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]							Concordance
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	
Hexyl cinnamic aldehyde (+)	+ (4.47)	+ (5.71)	+ (5.41)	+ (7.60)	+ (3.92)	+ (8.42)	+ (6.45)	7/7
Cobalt chloride[3] (+)	+ (2.01)		+ (2.54)	+ (4.25)			+ (5.06)	4/4
Lactic acid (-)	- (0.93)		- (0.99)		- (0.97)	- (0.91)		4/4
Nickel (II) sulfate hexahydrate (+)	**- (0.79)**	**- (1.24)**		**+ (2.13)**		**- (1.56)**		**3/4**
Potassium dichromate (+)	+ (4.78)	+ (4.08)			+ (6.01)		+ (6.37)	4/4

Boldface text indicates substance that did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizer result and (-) indicates nonsensitizer result in the LLNA: DA test. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) interlaboratory validation studies.

1.2.4 Interlaboratory Reproducibility – EC2 Values

The available quantitative (i.e., EC2 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA tests that yielded positive results (i.e., SI ≥ 2.0) during the first and second phase of the LLNA: DA interlaboratory validation study. The equation used for calculating EC2 values for the positive results was modified based on the method of linear interpolation reported by Gerberick et al. (2004) for the EC3 value:

$$EC2 = c + \left[\frac{(2-d)}{(b-d)}\right] \times (a-c)$$

where the data points lying immediately above and below the SI = 2.0 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI ≥ 2.0, an EC2 value was extrapolated according to the equation:

$$EC2_{ex} = 2^{\left\{\log_2(c) + \frac{(2-d)}{(b-d)} \times [\log_2(a) - \log_2(c)]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC2 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phase of the interlaboratory validation study are shown in **Tables C-VIII-11** and **C-VIII-12**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 14% (abietic acid) to 134% (isoeugenol) and the mean CV was 70% (**Table C-VIII-11**). In the second phase of the interlaboratory validation study, CV values ranged from 16% (hexyl cinnamic aldehyde) to 100% (cobalt chloride) and the mean CV was 57% (**Table C-VIII-12**).

The ICCVAM-recommended 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 (estimated concentration needed to produce an SI of a specific threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, seven laboratories reported EC2 values outside the range indicated for 2,4-dinitrochlorobenzene; all seven laboratories obtained EC2 values that were lower than the specified acceptance range (0.025%) (**Table C-VIII-11**). For hexyl cinnamic aldehyde, all the laboratories obtained an EC2 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and two of the seven laboratories obtained EC2 values that were below the acceptance range indicated (**Table C-VIII-12**).

Table C-VIII-11 EC2 Values from the First Phase Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC2 (%)										Mean EC2 (%) ± SD	CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10		
2,4-Dinitrochlorobenzene (+)	**0.020 (11.97)**	**0.023 (9.23)**	**0.026 (9.96)**	**0.016 (8.53)**	**0.091 (7.86)**	**0.016 (15.14)**	**0.007 (13.18)**	**0.013 (12.60)**	**0.019 (10.89)**	**0.093 (4.71)**	**0.032 ± 0.032**	**98**
Hexyl cinnamic aldehyde (+)	**6.962 (5.78)**	**7.461 (4.82)**	**8.404 (4.44)**	**6.460 (5.11)**	**11.057 (3.97)**	**7.463 (5.50)**	**5.850 (7.09)**	**6.140 (10.22)**	**9.191 (3.88)**	**7.256 (3.51)**	**7.624 ± 1.570**	**21**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		4.760				5.393	6.333				5.495 ± 0.791	14
3-Aminophenol (+)	1.877		NA					3.179			2.528 ± 0.921	36
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				0.407	4.399				0.375		1.727 ± 2.314	134
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	0.262	0.729			2.019						1.003 ± 0.910	91
Glutaraldehyde (+)	0.072	0.268			0.118						0.153 ± 0.103	67
Cobalt chloride[2] (+)				0.283[3]		0.032		0.079			0.131 ± 0.134	102
Nickel (II) sulfate hexahydrate (+)				IDR		0.235		IDR			0.235 ± NA	NA

Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC2 values that are outside of the acceptable range indicated by the ICCVAM-recommended LLNA performance standards: 5 - 20% for hexyl cinnamic aldehyde and 0.025 - 0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; IDR = insufficient dose response; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) interlaboratory validation studies.

[3] Data not reported for the highest dose (3%), only for 0.3% and 1%.

Table C-VIII-12 EC2 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC2 (%)							Mean EC2 (%) ± SD	CV (%)
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17		
Hexyl cinnamic aldehyde (+)	**6.348 (4.47)**	**5.983 (5.71)**	**5.954 (5.41)**	**4.849 (7.60)**	**7.451 (3.92)**	**4.662 (8.42)**	**6.024 (6.45)**	**5.896 ± 0.937**	**16**
Cobalt chloride[2] (+)	4.929		1.875	0.821			0.461	2.021 ± 2.029	100
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		8.404		8.404 ± NA	NA
Potassium dichromate (+)	0.159	0.128			0.055		0.047	0.097 ± 0.055	56

Bolded text indicates a substance that is an ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substance for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (25%). Two of the EC2 values are outside of the acceptable range indicated by the ICCVAM-recommended LLNA performance standards (5 - 20% for hexyl cinnamic aldehyde), indicated by shading.

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC2 values were higher than that for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 6.8 to 83.7% for five substances tested in five laboratories (**Table C-VIII-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for LLNA: DA EC2 values were greater than that for the traditional LLNA. The CV of 98% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2% (which were calculated from five values each), reported by ICCVAM (1999). The CV of 21% and 16% for hexyl cinnamic aldehyde tested in the first and second phase of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 134% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

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