Appendix F

Reproducibility Analyses for the LLNA: DA Using a 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 inter-laboratory 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 reproducibility evaluation in this revised draft background review document (BRD) has been updated from the January 2008 draft BRD to include an interlaboratory reproducibility evaluation and a reproducibility analysis using separate stimulation index (SI) criteria to identify sensitizers and nonsensitizers (see **Section 7.0**). The available LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of a single decision criterion in **Section 6.6** showed that SI $\geq$ 2.0 was the SI value that produced the lowest false negative rate among the alternative decision criteria evaluated (i.e., 3% [1/32]) when the traditional LLNA was the reference test (**Table 6-6)**. Thus, this appendix describes the evaluation of reproducibility for the decision criterion of SI $\geq$ 2.0 to identify sensitizers, which was evaluated in **Section 6.6**. In addition the reproducibility for SI $\geq$ 3.0, the SI cut-off used in the LLNA: DA validation studies, is also evaluated in this appendix.

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

Idehara et al. (2008) evaluated the intralaboratory reproducibility of EC3 (i.e., estimated concentration needed to produce a stimulation index of three) values for the LLNA: DA

using two substances (i.e., isoeugenol and eugenol) that were each tested in three different experiments (**Table F-1**). The data indicate coefficient of variations (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 (i.e., estimated concentration needed to produce a stimulation index of two) values for the same set of data. The EC2 results indicate slightly larger intralaboratory variability compared to EC3 results with CVs of 35% and 20% for isoeugenol and eugenol, respectively.

Table F-1 Intralaboratory 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.

1.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (**Appendix D**) 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 F-2)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were pre-determined (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 the traditional LLNA. Six substances are recommended LLNA performance standards reference substances: cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate (ICCVAM 2009).

The second phase of the interlaboratory validation study was designed to determine the reason for inconsistencies obtained from the two metals dissolved in dimethyl sulfoxide (DMSO) (i.e., cobalt chloride and nickel [II] sulfate hexahydrate) and thus to further evaluate the reliability of the LLNA: DA for testing metallic salts using DMSO as a vehicle. A blinded test of five substances (two of the five substances were unique to the second phase of the interlaboratory validation study) was conducted in seven laboratories (different from the 10 laboratories that performed the first interlaboratory validation study) **(Table F-3)**. One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (i.e., 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 pre-determined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (i.e., 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 recommended LLNA performance standards reference substance (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 EC2values) (**Sections 1.2.2** and **1.2.4, respectively**).

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

Substance[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitrochlorobenzene (+)	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 LLNA tests.

[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.

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

Substance[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 LLNA tests.
[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 F-4** using SI ≥ 3.0 as the decision criterion to distinguish sensitizers from nonsensitizers. In a qualitative comparison of LLNA: DA calls (i.e., positive/negative), 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 (i.e., 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 F-4 Qualitative Results for the First Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 3.0)

Substance[1]	Laboratory[2]										Concordance
	1	2	3	4	5	6	7	8	9	10	
2,4-Dinitrochlorobenzene (+)	+	+	+	+	+	+	+	+	+	+	10/10
Hexyl cinnamic aldehyde (+)	+	+	+	+	+	+	+	+	+	+	10/10
Isopropanol (-)	-	-	-	-	-	-	-	-	-	-	10/10
Abietic acid (+)		+				+	+				3/3
3-Aminophenol (+)	-		-					-			3/3
Dimethyl isophthalate (-)	-		-				-				3/3
Isoeugenol (+)				+	+				+		3/3
Methyl salicylate (-)			-				-			-	3/3
Formaldehyde (+)	+	+			-						**2/3**
Glutaraldehyde (+)	+	+			-						**2/3**
Cobalt chloride[3] (+)				-[4]		+		+			**2/3**
Nickel (II) sulfate hexahydrate (+)				-[5]		+		+[5]			**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 LLNA tests.
[2](+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests.
[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 (i.e., 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 F-5** using SI ≥ 3.0 as the decision criterion to distinguish sensitizers from nonsensitizers. In a qualitative comparison of LLNA: DA calls (i.e., positive/negative), four substances (i.e., 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 (i.e., 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 F-5 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 3.0)

Substance[1]	Laboratory[2]							Concordance
	11	12	13	14	15	16	17	
Hexyl cinnamic aldehyde (+)	+	+	+	+	+	+	+	7/7
Cobalt chloride[3] (+)	**-**		**-**	**+**			**+**	**2/4**
Lactic acid (-)	-		-		-	-		4/4
Nickel (II) sulfate hexahydrate (+)	-	-		-		-		4/4
Potassium dichromate (+)	+	+			+		+	4/4

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 LLNA tests.
[2](+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests.
[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 results for the nine sensitizers that were tested 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 F-6** and **F-7**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 4% (i.e., abietic acid) to 84% (i.e., glutaraldehyde) and the mean CV was 48% (**Table F-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 from which to calculate an EC3 (i.e., an inverse dose response curve). In the second phase of the interlaboratory validation study, CV values ranged from 32% (i.e., cobalt chloride) to 71% (i.e., potassium dichromate) and the mean CV was 45% (**Table F-7**).

Recommended Performance Standards: Murine Local Lymph Node Assay (ICCVAM 2009) indicates 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 (i.e., estimated concentration needed to produce a stimulation index 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 (i.e., 0.025%) and three laboratories obtained EC3 values that were higher than the specified acceptance range (i.e., 0.1%) (**Table F-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 F-7**).

231 **Table F-6 EC3 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA**

Substance[1]	Laboratory										Mean EC3 (%)	CV (%)
	1	2	3	4	5	6	7	8	9	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**	**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**	**30**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		8.196				7.544	7.676				7.805	4
3-Aminophenol (+)	NA		NA					NA			NA	NA
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				1.112	5.983				2.300		3.131	81
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	1.747	1.480			NA						1.614	12
Glutaraldehyde (+)	0.110	0.435			NA						0.272	84
Cobalt chloride[2] (+)				NA[3]		0.063		0.137			0.100	53
Nickel (II) sulfate hexahydrate (+)				NA[4]		0.469		IDR			0.469	NA

232 Note: Bolded text indicates recommended LLNA performance standards reference substances (ICCVAM 2009). Values in parentheses are highest SI values achieved. For both 2,4-
233 dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved are from the highest dose tested (i.e., 0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl
234 cinnamic aldehyde). Shading shows EC3 values that are outside of the acceptable range indicated by the recommended LLNA performance standards: 5 - 20% for hexyl cinnamic
235 aldehyde and 0.025 - 0.1% for 2,4-dinitrochlorobenzene.
236 Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay
237 modified by Daicel Chemical Industries, Ltd., based on ATP content; IDR = insufficient dose response; NA = not available.
238 [1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
239 [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.
240 [3]Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.
241 [4]Insufficient dose response.

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

Substance[1]	Laboratory							Mean EC3 (%)	CV (%)
	11	12	13	14	15	16	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**	**33**
Cobalt chloride[2] (+)	NA		NA	1.761			1.109	1.435	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	71

Bolded text indicates a recommended LLNA performance standards reference substance (ICCVAM 2009). Values in parentheses are highest SI values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved are from the highest dose tested (i.e., 25%). None of the EC3 values are outside of the acceptable range indicated by the recommended LLNA performance standards (i.e., 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 available.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[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 F-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (i.e., 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 F-8 Interlaboratory Reproducibility of the EC3 for Substances Tested in the Traditional LLNA[1]

Substance	Laboratory					CV (%)
	1	2	3	4	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 F-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 (i.e., 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 (i.e., SI = 2.83 and 2.38, respectively) but one lab did not achieve SI ≥ 2.0 at any dose tested (**Appendix D**). 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 (**Appendix D**). 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 F-9 Qualitative Results for the First Phase of the Interlaboratory Validation Studies for the LLNA: DA (SI ≥ 2.0)

Substance[1]	Laboratory[2]										Concordance
	1	2	3	4	5	6	7	8	9	10	
2,4-Dinitrochlorobenzene (+)	+	+	+	+	+	+	+	+	+	+	10/10
Hexyl cinnamic aldehyde (+)	+	+	+	+	+	+	+	+	+	+	10/10
Isopropanol (-)	-	-	-	-	-	-	-	-	-	-	10/10
Abietic acid (+)		+				+	+				3/3
3-Aminophenol (+)	+		-					+			**2/3**
Dimethyl isophthalate (-)	-		-				-				3/3
Isoeugenol (+)				+	+				+		3/3
Methyl salicylate (-)			-				-			-	3/3
Formaldehyde (+)	+	+			+						3/3
Glutaraldehyde (+)	+	+			+						3/3
Cobalt chloride[3] (+)				+[4]		+		+			3/3
Nickel (II) sulfate hexahydrate (+)				-[5]		+		+[5]			**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 LLNA tests.
[2](+) indicates sensitizer result and (-) indicates nonsensitizer result in the LLNA: DA test.
[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 (i.e., 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 F-10**. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (i.e., 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 (i.e., 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 ≥ 2.0, while the other laboratory produced an SI ≥ 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 F-10 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 2.0)

Substance[1]	Laboratory[2]							Concordance
	11	12	13	14	15	16	17	
Hexyl cinnamic aldehyde (+)	+	+	+	+	+	+	+	7/7
Cobalt chloride[3] (+)	+		+	+			+	4/4
Lactic acid (-)	-		-		-	-		4/4
Nickel (II) sulfate hexahydrate (+)	**-**	**-**		**+**		**-**		**3/4**
Potassium dichromate (+)	+	+			+		+	4/4

Bolded substance 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 LLNA tests.
[2](+) indicates sensitizer result and (-) indicates nonsensitizer result in the LLNA: DA test.
[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 results for the ten sensitizers that were tested 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:

339 $$EC2 = c + \left[\frac{(2-d)}{(b-d)}\right] \times (a-c)$$

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

344 $$EC2_{ex} = 2^{\left\{\log_2(c) + \frac{(2-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}}$$

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

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

354 The recommended LLNA performance standards indicate that interlaboratory reproducibility
355 should be evaluated with at least two sensitizing chemicals with well-characterized activity in
356 the traditional LLNA (ICCVAM 2009). Acceptable reproducibility is attained when each
357 laboratory obtains ECt (i.e., estimated concentration needed to produce a stimulation index
358 threshold) values within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20%
359 for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory
360 validation study, seven laboratories reported EC2 values outside the range indicated for 2,4-
361 dinitrochlorobenzene; all seven laboratories obtained EC2 values that were lower than the
362 specified acceptance range (i.e., 0.025%) (**Table F-11**). For hexyl cinnamic aldehyde, all the
363 laboratories obtained an EC2 value within the acceptance range (5% to 20%). In the second
364 phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and
365 two of the seven laboratories obtained EC2 values that were below the acceptance range
366 indicated (**Table F-12**).

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

Substance[1]	Laboratory										Mean EC2 (%)	CV (%)
	1	2	3	4	5	6	7	8	9	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**	**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**	**21**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		4.760				5.393	6.333				5.495	14
3-Aminophenol (+)	1.877		NA					3.179			2.528	36
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				0.407	4.399				0.375		1.727	134
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	0.262	0.729			2.019						1.003	91
Glutaraldehyde (+)	0.072	0.268			0.118						0.153	67
Cobalt chloride[2] (+)				0.283[3]		0.032		0.079			0.131	102
Nickel (II) sulfate hexahydrate (+)				NA[4]		0.235		IDR			0.235	NA

Bolded text indicates substances that are recommended LLNA performance standards reference substances (ICCVAM 2009). Values in parentheses are highest SI values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (i.e., 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 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 available.

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

[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 (i.e., 3%), only for 0.3% and 1%.

[4]Insufficient dose response.

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

Substance[1]	Laboratory							Mean EC2 (%)	CV (%)
	11	12	13	14	15	16	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**	**16**
Cobalt chloride[2] (+)	4.929		1.875	0.821			0.461	2.021	100
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		8.404		8.404	
Potassium dichromate (+)	0.159	0.128			0.055		0.047	0.097	56

Bolded text indicates substances that are recommended LLNA performance standards reference substances. Values in parentheses are highest SI values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (i.e., 25%). Two of the EC2 values are outside of the acceptable range indicated by the recommended LLNA performance standards (i.e., 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 available.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[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 F-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (i.e., hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for LLNA: DA EC2 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).