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11 **Appendix E**

12 **Accuracy Analyses Using Additional Approaches for Combining**

13 **Multiple Test Results**

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30 **1.0   LLNA: BrdU-ELISA Accuracy Analysis Using Alternative**

31 **   Decision Criteria and Alternate Methods for Combining Data for**

32 **   Substances Tested Multiple Times**

33 This appendix shows performance analyses for the LLNA: BrdU-ELISA using single

34 alternative decision criteria and two different approaches for combining test results for the 14

35 substances with multiple LLNA: BrdU-ELISA tests:

36   1. The positive/negative outcome for each substance for each criterion was

37   determined by the outcome of the test with the highest maximum SI of the

38   multiple tests.

39   2. The positive/negative outcome for each substance for each criterion was

40   determined by the outcome of the test with the lowest maximum SI of the

41   multiple tests.

42 **Section 6.4** provides the results for the analysis when the most prevalent outcome was used

43 as the result for each substance tested multiple times (for each criterion).

44 **1.1   Results of LLNA: BrdU-ELISA Accuracy Analysis Using Single Alternative**

45 **   Decision Criteria and the Highest Maximum SI for the Outcome of Multiple**

46 **   Tests**

47 When combining multiple test results for a single substance using the outcome of the test

48 with the highest maximum SI, the decision criterion of SI $\geq$ 2.0 to identify sensitizers yielded

49 an accuracy of 77% (24/31), a sensitivity of 77% (17/22), a specificity of 78% (7/9), a false

50 positive rate of 22% (2/9), and a false negative rate of 23% (5/22) (**Table E-1)**. SI $\geq$ 2.0 was

51 the decision criterion used by the JSAAE interlaboratory validation study of the LLNA:

52 BrdU-ELISA. The performance for the additional decision criteria described in **Section 6.4**,

53 are also shown in **Table E-1**. Over the range of SI cutoffs evaluated, increasing the SI cutoff

54 decreased accuracy (84% at SI $\geq$ 1.3 to 68% at SI $\geq$ 5.0), decreased sensitivity (100% at

55 SI $\geq$ 1.3 to 54% at SI $\geq$ 5.0), increased specificity (44% at SI $\geq$ 1.3 to 100% at SI $\geq$ 5.0),

56 decreased the false positive rate (56% at SI $\geq$ 1.3 to 0% at SI $\geq$ 5.0), and increased the false

57 negative rate (0% at SI $\geq$ 1.3 to 46% at SI $\geq$ 5.0) (**Figure E-1** and **Table E-1)**. Use of

58 ANOVA and summary statistics (i.e., mean absorbance values of treated groups $\geq$95%

59 confidence interval of the control group, or ≥2 or ≥3 SD from the control group mean),

60 yielded accuracy values of 74 to 81%, with sensitivity values of 86 to 100%, and false

61 negative rates of 0 to 14%. The specificity for these criteria ranged from 33 to 56% and the

62 false positive rates were 44 to 67%.

63 The highest accuracy and lowest false negative rate, for the approach using the highest

64 maximum SI for the substances with more than one test, was achieved using an SI ≥ 1.3. The

65 accuracy for SI ≥ 1.3 was 84% (26/31), with sensitivity of 100% (22/22), specificity of 44%

66 (5/9), a false positive rate of 56% (5/9), and false negative rate of 0% (0/22). However, using

67 an SI ≥ 1.3 incorrectly classified lactic acid, isopropanol, and methyl salicylate, three of the

68 ICCVAM performance standards reference substances, as sensitizers. Use of mean

69 absorbance values of treated groups ≥95% confidence interval of the control group to

70 identify sensitizers also produced the lowest false negative rate 0% (0/9), with a slightly

71 lower accuracy of 81% (25/31), and a higher false positive rate of 67% (6/9). This criterion

72 also incorrectly classified lactic acid and isopropanol as sensitizers. The lowest false positive

73 rates (0% [0/9]) were produced by SI cutoffs of 3.0 to 5.0, however the false negative rates at

74 those cutoffs were 27% (6/22) to 46% (10/22).

75 **Table E-1   Performance of the LLNA: BrdU-ELISA Compared with the Traditional LLNA Using Alternative Decision**
76 **Criteria to Identify Sensitizers and the Highest Maximum SI for Substances with Multiple Tests**

Alternate Criterion	N[1]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
Statistics[3]	31	74	23/31	86	19/22	44	4/9	56	5/9	14	3/22	79	19/24	57	4/7
≥ 95% CI[4]	31	81	25/31	100	22/22	33	3/9	67	6/9	0	0/22	79	22/28	100	3/3
≥ 2 SD[5]	31	77	24/31	96	21/22	33	3/9	67	6/9	5	1/22	78	21/27	75	3/4
≥ 3 SD[6]	31	81	25/31	91	20/22	56	5/9	44	4/9	9	2/22	83	20/24	71	5/7
SI ≥ 5.0	31	68	21/31	54	12/22	100	9/9	0	0/9	46	10/22	100	12/12	47	9/19
SI ≥ 4.5	31	71	22/31	59	13/22	100	9/9	0	0/9	41	9/22	100	13/13	50	9/18
SI ≥ 4.0	31	77	24/31	68	15/22	100	9/9	0	0/9	32	7/22	100	15/15	56	9/16
SI ≥ 3.5	31	77	24/31	68	15/22	100	9/9	0	0/9	32	7/22	100	15/15	56	9/16
SI ≥ 3.0	31	81	25/31	73	16/22	100	9/9	0	0/9	27	6/22	100	16/16	60	9/15
SI ≥ 2.5	31	81	25/31	77	17/22	89	8/9	11	1/9	23	5/22	94	17/18	62	8/13
SI ≥ 2.0	31	77	24/31	77	17/22	78	7/9	22	2/9	23	5/22	90	17/19	58	7/12
SI ≥ 1.5	31	81	25/31	91	20/22	56	5/9	44	4/9	9	2/22	83	20/24	71	5/7
SI ≥ 1.3	31	84	26/31	100	22/22	44	4/9	56	5/9	0	0/22	82	22/27	100	4/4

77 Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA)
78 detection of bromodeoxyuridine (BrdU); CI = confidence interval; No. = number; SD = standard deviation; SI = stimulation index
79 [1] N = Number of substances included in this analysis.
80 [2] The proportion on which the percentage calculation is based.
81 [3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data
82 were log-transformed prior to analysis of variance. Significance at p < 0.05 was further tested by Dunnett's test.
83 [4] The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.
84 [5] The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.
85 [6] The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

86   **Figure E-1   Performance of the LLNA: BrdU-ELISA with SI Compared to the**
87   **Traditional LLNA Using the Highest Maximum SI for Substances with**
88   **Multiple Tests**
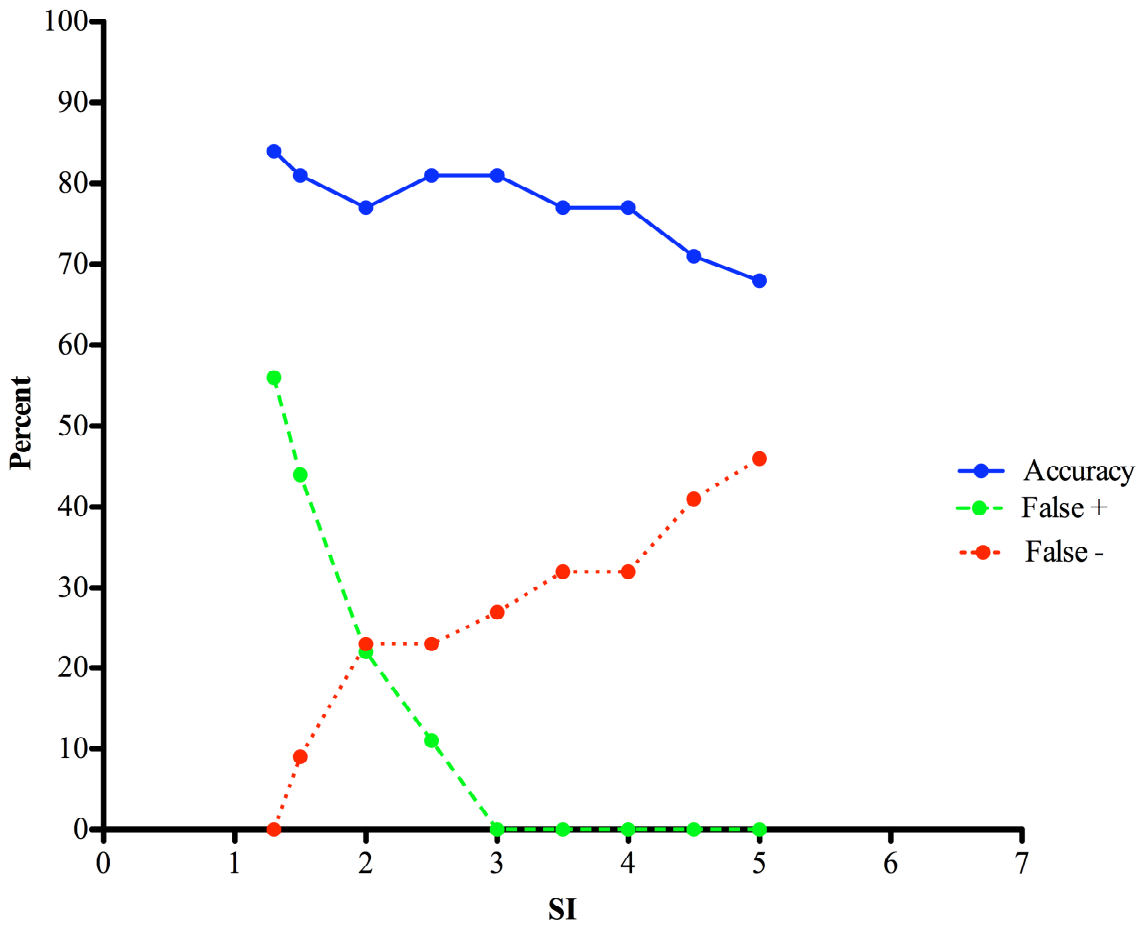
89

90   As compared to traditional LLNA results, the lines show the change in performance characteristics
91   for the LLNA: BrdU-ELISA with the SI cutoff used to identify sensitizers. This analysis used LLNA:
92   BrdU-ELISA and traditional LLNA results for 31 substances (22 sensitizers and nine nonsensitizers
93   based on traditional LLNA results). For the 14 substances with multiple test results, the results for
94   each substance were combined by using the outcome for the test with the highest maximum SI value.
95   The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows
96   the false negative rate.

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99   **1.2   Results of LLNA: BrdU-ELISA Accuracy Analysis Using Alternative**

100   **Decision Criteria and Lowest Maximum SI for the Outcome of Multiple Tests**

101   When combining multiple test results for a single substance using the outcome of the test

102   with the lowest maximum SI, the decision criterion of SI ≥ 2.0 to identify sensitizers for

103   these 31 substances yielded an accuracy of 81% (25/31), a sensitivity of 73% (16/22), a

104   specificity of 100% (9/9), a false positive rate of 0% (0/9), and a false negative rate of 27%

105   (6/22) (**Table E-2)**. SI ≥ 2.0 was the decision criterion used by the JSAAE interlaboratory

106   validation study of the LLNA: BrdU-ELISA. The performance for the additional decision

107   criteria described in **Section 6.4**, are shown in **Table E-2**.

108   Over the range of SI cutoffs evaluated, increasing the SI cutoff decreased accuracy (90% at

109   SI ≥ 1.3 to 45% at SI ≥ 5.0), decreased sensitivity (100% at SI ≥ 1.3 to 23% at SI ≥ 5.0),

110   increased specificity (67% at SI ≥ 1.3 to 100% at SI ≥ 5.0), decreased the false positive rate

111   (33% at SI ≥ 1.3 to 0% at SI ≥ 5.0), and increased the false negative rate (0% at SI ≥ 1.3 to

112   77% at SI ≥ 5.0) (**Figure E-2** and **Table E-2**). Use of ANOVA and summary statistics (i.e.,

113   mean absorbance values of treated groups ≥95% confidence interval of the control group, or

114   ≥2 or 3 SD from the control group mean), yielded accuracy of 84 to 90%, with sensitivity

115   values of 86 to 100%, and false negative rates of 0 to 14%. The specificity for these criteria

116   ranged from 67 to 89% and the false positive rates were 11 to 33%.

117   The highest accuracy and lowest false negative rate, for the approach using the lowest

118   maximum SI for the substances with more than one test, was achieved using an SI ≥ 1.3 and

119   mean absorbance values of treated groups ≥95% confidence interval of the control group.

120   Both criteria yielded an accuracy of 90% (26/31) and a false negative rate of 0% (0/22). Both

121   criteria also yielded sensitivity =100% (22/22), specificity = 67% (6/9), and false positive

122   rate = 33% (3/9). However, these criteria incorrectly classified lactic acid, isopropanol, and

123   methyl salicylate, three of the ICCVAM performance standards reference substances, as

124   sensitizers. The lowest false positive rate (0% [0/9]) was produced by SI cutoffs of 2.0 to 5.0,

125   however the false negative rates at those cutoffs were 27% to 77%. Of those cutoffs, SI ≥ 2.0

126   produced the highest accuracy, 81% (25/31), and the lowest false negative rate, 27% (6/22).

127 **Table E-2 Performance of the LLNA: BrdU-ELISA Compared with the Traditional LLNA Using Alternative Decision**
128 **Criteria to Identify Sensitizers and the Lowest Maximum SI for Substances with Multiple Tests**

Alternate Criterion	N[1]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
Statistics[3]	31	84	26/31	86	19/22	78	7/9	22	2/9	14	3/22	91	19/21	70	7/10
≥ 95% CI[4]	31	90	28/31	100	22/22	67	6/9	33	3/9	0	0/22	88	22/25	100	6/6
≥ 2 SD[5]	31	87	27/31	96	21/22	67	6/9	33	3/9	5	1/22	88	21/24	86	6/7
≥ 3 SD[6]	31	87	27/31	86	19/22	89	8/9	11	1/9	14	3/22	95	19/20	73	8/11
SI ≥ 5.0	31	45	14/31	23	5/22	100	9/9	0	0/9	77	17/22	100	5/5	35	9/26
SI ≥ 4.5	31	45	14/31	23	5/22	100	9/9	0	0/9	77	17/22	100	5/5	35	9/26
SI ≥ 4.0	31	55	17/31	36	8/22	100	9/9	0	0/9	64	14/22	100	8/8	39	9/23
SI ≥ 3.5	31	55	17/31	36	8/22	100	9/9	0	0/9	64	14/22	100	8/8	39	9/23
SI ≥ 3.0	31	64	20/31	50	11/22	100	9/9	0	0/9	50	11/22	100	11/11	45	9/20
SI ≥ 2.5	31	71	22/31	59	13/22	100	9/9	0	0/9	41	9/22	100	13/13	50	9/18
SI ≥ 2.0	31	81	25/31	73	16/22	100	9/9	0	0/9	27	6/22	100	16/16	60	9/15
SI ≥ 1.5	31	87	27/31	91	20/22	78	7/9	22	2/9	9	2/22	91	20/22	78	7/9
SI ≥ 1.3	31	90	28/31	100	22/22	67	6/9	33	3/9	0	0/22	88	22/25	100	6/6

129 Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA)
130 detection of bromodeoxyuridine (BrdU); CI = confidence interval; No. = number; SD = standard deviation; SI = stimulation index
131 [1]N = Number of substances included in this analysis.
132 [2]The proportion on which the percentage calculation is based.
133 [3]Analysis of variance for difference of group means when substances were tested at multiple doses or t-test when substances were tested at one dose. The absorbance data
134 were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.
135 [4] The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.
136 [5] The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.
137 [6] The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

138   **Figure E-2   Performance of the LLNA: BrdU-ELISA with SI Compared to the**
139   **Traditional LLNA Using the Lowest Maximum SI for Substances with**
140   **Multiple Tests**
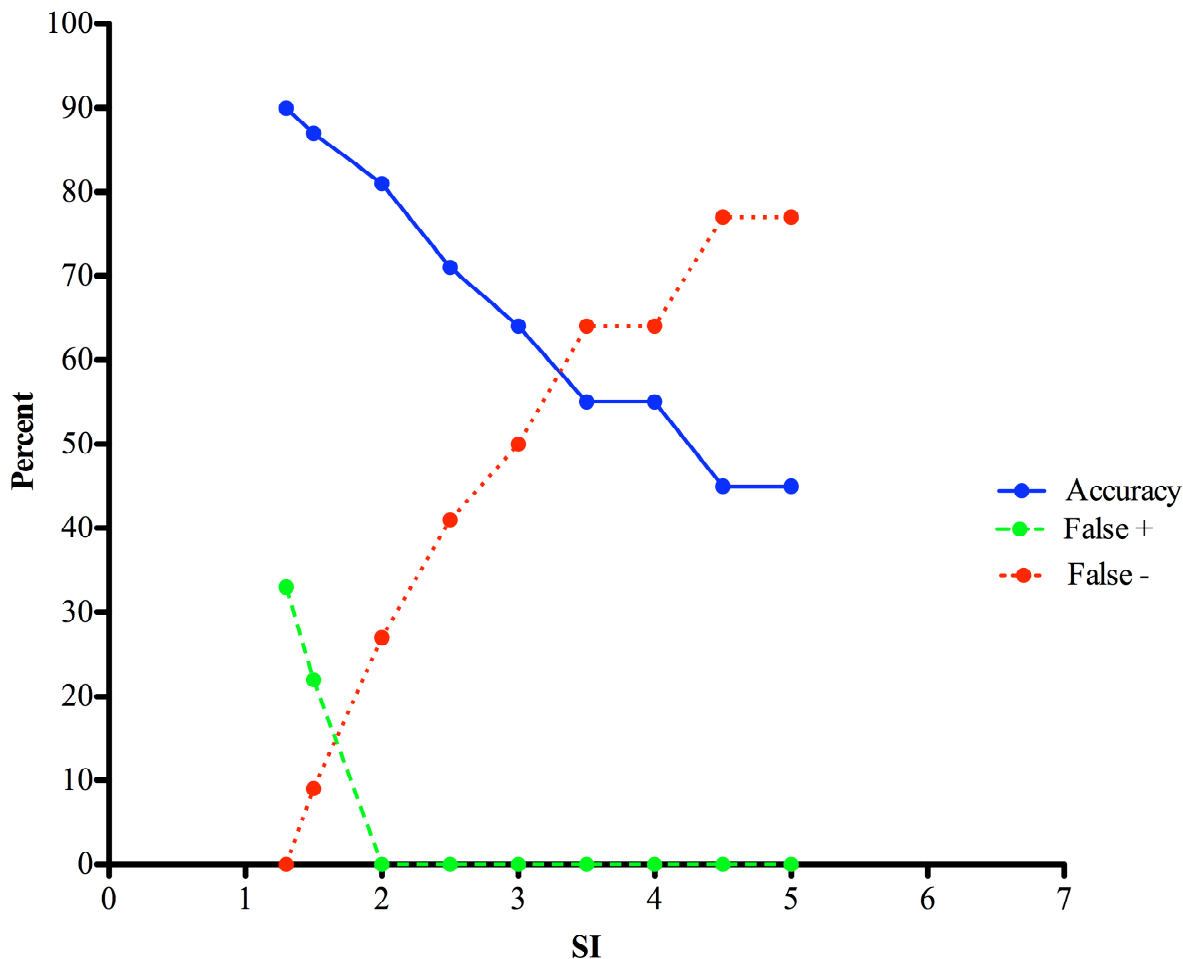
141
142   As compared to traditional LLNA results, the lines show the change in performance characteristics for the
143   LLNA: BrdU-ELISA with the SI cutoff used to identify sensitizers. This analysis used LLNA: BrdU-
144   ELISA and traditional LLNA results for 31 substances (22 sensitizers and nine nonsensitizers based on
145   traditional LLNA results). For the 14 substances with multiple test results, the results for each substance
146   were combined by using the outcome for the test with the lowest maximum SI value. The solid line shows
147   accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.
148

148   ## 2.0   Discordant Results For Accuracy Analysis of Alternative Decision
149   Criteria

150   Using the decision criteria of SI ≥ 2.0 to identify sensitizers and the most prevalent outcome for
151   the substances with multiple tests for the analysis of alternative decision criteria, the five
152   discordant substances (when compared to the traditional LLNA) were aniline, cyclamen
153   aldehyde, hydroxycitronellal, 2-mercaptobenzothiazole, and linalool (**Table 6-4**). As indicated in
154   **Section 6.4.1**, all five substances were misclassified as nonsensitizers when compared to the
155   traditional LLNA, which classified them as sensitizers.

156   ## 2.1   Discordant Results Using Alternative Decision Criteria and Highest Maximum SI
157   Outcome for Multiple Tests

158   Using the decision criteria of SI ≥ 2.0 to identify sensitizers and the test with the highest
159   maximum SI as the result for substances with multiple tests, yielded two additional discordant
160   substances: isopropanol and lactic acid, which were misclassified as sensitizers.

161   **Table E-3** shows how the number and identity of discordant substances changes with the
162   alternate decision criteria when using the test with the highest maximum SI as the result for
163   substances with multiple tests. Using an SI cutoff less than 2.0, SI ≥ 1.5, to identify sensitizers
164   yielded six discordant substances. Two substances, hydroxycitronellal and linalool, were
165   misclassified as nonsensitizers, and four substances, hexane, isopropanol, lactic acid and
166   propylene glycol, were misclassified as sensitizers. Using an even lower SI to identify
167   sensitizers, SI ≥ 1.3, yielded five discordant substances that were all misclassified as sensitizers
168   (hexane, isopropanol, lactic acid, methyl salicylate, and propylene glycol). Increasing the SI
169   cutoff to values greater than 2.0, increased the number of sensitizers that were misclassified as
170   nonsensitizers. At SI ≥ 2.0, five sensitizers were misclassified as nonsensitizers while at SI ≥ 5.0,
171   10 sensitizers were classified as nonsensitizers (**Table E-3)**. At SI ≥ 2.0, two nonsensitizers were
172   misclassified as sensitizers while, at SI ≥ 5.0, no nonsensitizers were classified as sensitizers.

173   Use of a statistical test (i.e., ANOVA or *t*-test) or summary statistics (i.e., ≥95% CI, ≥2 SD, or ≥3
174   SD) tended to misclassify more nonsensitizers than sensitizers. Using ANOVA or a *t*-test to
175   identify sensitizers misclassified three sensitizers (linalool, 2-mercaptobenzothiazole, and

176   aniline) as nonsensitizers and four nonsensitizers (glycerol, hexane, isopropanol, and lactic acid)

177   as sensitizers. Using treatment group absorbance ≥95% CI or ≥2 SD of vehicle control mean

178   misclassified six nonsensitizers as sensitizers (glycerol, hexane, isopropanol, lactic acid, methyl

179   salicylate, and propylene glycol). Treatment group absorbance ≥2 SD of vehicle control mean

180   also misclassified one weak sensitizer as a nonsensitizer (linalool). Using treatment group

181   absorbance ≥3 SD of vehicle control mean misclassified three nonsensitizers as sensitizers

182   (hexane, isopropanol, and lactic acid) and three weak sensitizers as nonsensitizers

183   (hydroxycitronellal, linalool, and aniline).

184   Four ICCVAM performance standards reference substances were discordant for the analysis of

185   alternate decision criteria using the test with the highest maximum SI as the result for substances

186   with multiple tests (**Table E-3**). One sensitizer, 2-mercaptobenzothiazole, was misclassified by

187   some criteria as a nonsensitizer, and three nonsensitizers, isopropanol, lactic acid, and methyl

188   salicylate, were misclassified as sensitizers by some criteria. The criteria that yielded the correct

189   results for 2-mercaptobenzothiazole included summary statistics (i.e., ≥95% CI, ≥2 SD, or

190   ≥3 SD), SI ≥ 1.5, and SI ≥ 1.3. The criteria that yielded the correct results for isopropanol

191   included SI ≥ 2.5 to 5.0. The criteria that yielded the correct results for lactic acid included SI ≥

192   3.0 to 5.0. All criteria yielded the correct results for methyl salicylate except for treatment group

193   absorbance ≥95% CI or ≥2 SD of vehicle control mean, and SI ≥ 1.3.

194 **Table E-3   Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the**
195 **Traditional LLNA and the Highest Maximum SI for Substances with Multiple Tests**

Discordant Substance[1]	Alternate Decision Criterion[2]												
	Statistics[3]	≥ 95% CI[4]	≥ 2 SD[5]	≥ 3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-	-		
Cinnamic aldehyde (2.4%)					-	-							
3-Aminophenol (3.2%)					-	-	-	-					
Nickel sulfate (4.8%)					-								
4-Chloroaniline (6.5%)					-	-	-	-	-				
Cyclamen aldehyde (22.3%)					-	-	-	-	-	-	-		
Hydroxycitronellal (24%)				-	-	-	-	-	-	-	-	-	
Linalool (30%)	-		-	-	-	-	-	-	-	-	-	-	
Isopropyl myristate (44%)					-	-							
Aniline (63%)	-			-	-	-	-	-	-	-	-		
Glycerol (-)	+	+	+										
Hexane (-)	+	+	+	+								+	+
Isopropanol (-)	+	+	+	+							+	+	+
Lactic acid (-)	+	+	+	+						+	+	+	+
Methyl salicylate (-)		+	+										+
Propylene glycol (-)		+	+									+	+

196 Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA)
197 detection of bromodeoxyuridine (BrdU); CI = confidence interval; SD = standard deviation; SI = stimulation index.
198 [1]Compared to the traditional LLNA. Traditional LLNA result in parentheses: "-" for nonsensitizers and EC3 (%) for sensitizers.
199 [2]LLNA: BrdU result shown: "+" if the decision criterion was met and "-" if the decision criterion was not met.
200 [3]Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data
201 were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.
202 [4]The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.
203 [5]The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.
204 [6]The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

205 **2.2   Discordant Results Using Alternative Decision Criteria and Lowest Maximum SI
206   Outcome for Multiple Tests**

207 Using the decision criteria of SI ≥ 2.0 to identify sensitizers and the most prevalent outcome for

208 the substances with multiple tests for the analysis of alternative decision criteria yielded five

209 discordant substances (when compared to the traditional LLNA). Aniline, cyclamen aldehyde,

210 hydroxycitronellal, 2-mercaptobenzothiazole, and linalool were misclassified as nonsensitizers

211 (**Table 6-4**), while the traditional LLNA classified them as sensitizers. Using the test with the

212 lowest maximum SI as the result for substances with multiple tests yielded six discordant

213 substances at SI ≥ 2.0. One additional sensitizer, formaldehyde, was misclassified as a

214 nonsensitizer (**Table E-4**).

215 **Table E-4** shows how the number and identity of discordant substances changes with the

216 alternate decision criteria when using the test with the lowest maximum SI as the result for

217 substances with multiple tests. Using an SI cutoff less than 2.0, SI ≥ 1.5, to identify sensitizers

218 yielded four discordant substances. Two substances, hydroxycitronellal and linalool, were

219 misclassified as nonsensitizers, and two substances, hexane and lactic acid, were misclassified as

220 sensitizers. Using an even lower SI to identify sensitizers, SI ≥ 1.3, yielded three discordant

221 substances that were all misclassified as sensitizers (hexane, lactic acid, and methyl salicylate).

222 Increasing the SI cutoff to values greater than 2.0, increased the number of sensitizers that were

223 misclassified as nonsensitizers. At SI ≥ 2.0, six sensitizers were misclassified as nonsensitizers

224 while at SI ≥ 4.5 and SI ≥ 5.0, 17 sensitizers were classified as nonsensitizers (**Table E-4)**. From

225 SI ≥ 2.0 to SI ≥ 5.0 no nonsensitizers were misclassified as sensitizers.

226 Using the test with the lowest maximum SI as the result for substances with multiple tests caused

227 even potent sensitizers to be misclassified as nonsensitizers at the higher SI cutoffs. At SI ≥ 4.5

228 and SI ≥ 5.0, 2,4-dinitrochlorobenzene, glutaraldehyde, and formaldehyde were classified as

229 nonsensitizers. Glutaraldehyde was classified as a nonsensitizer at SI cutoffs as low as 2.5 and

230 formaldehyde was classified as a nonsensitizer at SI cutoffs as low as 2.0.

231 **Table E-4   Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the**
232 **Traditional LLNA and the Lowest Maximum SI for Substances with Multiple Tests**

Discordant Substance[1]	Alternate Decision Criterion[2]												
	Statistics[3]	≥ 95% CI[4]	≥ 2 SD[5]	≥ 3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3
2,4-Dinitrochlorobenzene (0.049%)					-	-							
Glutaraldehyde (0.14%)					-	-	-	-	-	-			
Formaldehyde (0.53%)					-	-	-	-	-	-	-		
trans-Cinnamic aldehyde (1.4%)					-	-	-	-					
Isoeugenol (1.5%)					-	-	-	-	-	-			
2-Mercaptobenzothiazole (1.7%)	-					-	-	-	-	-	-		
Cinnamic aldehyde (2.4%)					-	-							
3-Aminophenol (3.2%)					-	-	-	-					
Nickel sulfate (4.8%)					-	-	-	-	-				
4-Chloroaniline (6.5%)					-	-	-	-	-				
Hexyl cinnamic aldehyde (9.7%)					-	-	-	-	-	-			
Eugenol (10.1%)					-	-	-	-					
Cyclamen aldehyde (22.3%)					-	-	-	-	-	-	-		
Hydroxycitronellal (24%)			-		-	-	-	-	-	-	-	-	
Linalool (30%)	-		-	-	-	-	-	-	-	-	-	-	
Isopropyl myristate (44%)					-	-							
Aniline (63%)	-			-	-	-	-	-	-	-	-		
Glycerol (-)	+	+	+										
Hexane (-)	+	+										+	+
Lactic acid (-)		+	+									+	+
Methyl salicylate (-)													+

233 Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA)
234 detection of bromodeoxyuridine (BrdU); CI = confidence interval; SD = standard deviation; SI = stimulation index.
235 [1]Compared to the traditional LLNA. Traditional LLNA result in parentheses: "-" for nonsensitizers and EC3 (%) for sensitizers.

236   [2]LLNA: BrdU result shown: "+" if the decision criterion was met and "-" if the decision criterion was not met.
237   [3]Analysis of variance for difference of group means when substances were tested at multiple doses or t-test when substances were tested at one dose. The absorbance data
238   were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.
239   [4] The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.
240   [5] The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.
241   [6] The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.
242

243   Use of a statistical test (i.e., ANOVA or *t*-test) or summary statistics (i.e., ≥95% CI, ≥2

244   SD, or 3 ≥SD) more often misclassified nonsensitizers than sensitizers (**Table E-4**).

245   Using ANOVA or *t*-tests to identify sensitizers misclassified three sensitizers (2-

246   mercaptobenzothiazole, linalool, and aniline) as nonsensitizers and two nonsensitizers

247   (glycerol and hexane) as sensitizers. Using treatment group absorbance ≥95% CI or ≥2

248   SD of vehicle control mean misclassified glycerol and lactic acid as sensitizers. Using

249   treatment group absorbance ≥95% CI also misclassified hexane as a sensitizer.

250   Additionally, treatment group absorbance ≥2 SD of vehicle control mean misclassified

251   one weak sensitizer (linalool) as a nonsensitizer. Using treatment group absorbance ≥3

252   SD of vehicle control mean, however, misclassified three weak sensitizers as

253   nonsensitizers (hydroxycitronellal, linalool, and aniline).

254   Seven ICCVAM performance standards reference substances were discordant for the

255   analysis of alternate decision criteria using the test with the lowest maximum SI as the

256   result for substances with multiple tests (**Table E-4**). One strong sensitizer, 2,4-

257   dinitrochlorobenzene, was misclassified by some criteria as a nonsensitizer. Four

258   additional sensitizers, isoeugenol, 2-mercaptobenzothiazole, hexyl cinnamic aldehyde,

259   and eugenol, were also misclassified as nonsensitizers by some criteria. The criteria that

260   yielded the correct results for 2,4-dinitrochlorobenzene were all but the SI ≥ 4.5 to 5.0

261   criteria. The criteria that yielded the correct results for 2-mercaptobenzothiazole included

262   summary statistics (i.e., ≥95% CI, ≥2 SD, or ≥3 SD), SI ≥ 1.5 and SI ≥ 1.3. The criteria

263   that yielded the correct results for isoeugenol and hexyl cinnamic aldehyde were SI ≥ 1.5

264   to 2.0. The criteria that yielded the correct results for eugenol were SI ≥ 1.3 to 3.0.

265   Two nonsensitizers, lactic acid and methyl salicylate, from the list of ICCVAM

266   performance standards reference substances, were misclassified as sensitizers by some

267   criteria. The criteria that yielded the correct results for lactic acid were all except for

268   treatment group absorbance ≥95% CI or ≥2 SD of vehicle control mean, SI ≥ 1.5, and SI

269   ≥ 1.3. The criteria that yielded the correct results for methyl salicylate were all except for

270   SI ≥ 1.3.