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Non-Radioactive Murine Local Lymph Node Assay: Modified by Daicel Chemical Industries, Ltd. Based on ATP Content Test Method Protocol (LLNA: DA)

Revised Draft Background Review Document

March 2009

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147   **List of Abbreviations and Acronyms**

148	ACD	Allergic contact dermatitis
149	ANOVA	Analysis of variance
150	AOO	Acetone: olive oil (4:1)
151	aq.	Aqueous
152	ATP	Adenosine triphosphate
153	BRD	Background review document
154	CASRN	Chemical Abstracts Service Registry Number
155	CPSC	U.S. Consumer Product Safety Commission
156	CI	Confidence interval
157	Conc.	Concentration
158	CV	Coefficient of variation
159	DMF	*N,N*-dimethylformamide
160	DMSO	Dimethyl sulfoxide
161	EC2	Estimated concentration needed to produce a stimulation index
162		of two
163	EC2.5	Estimated concentration needed to produce a stimulation index
164		of 2.5
165	EC3	Estimated concentration needed to produce a stimulation index
166		of three
167	ECt	Estimated concentration needed to produce a stimulation index
168		of a specified threshold
169	ECETOC	European Centre for Ecotoxicology and Toxicology of
170		Chemicals
171	EPA	U.S. Environmental Protection Agency
172	FN	False negative
173	FP	False positive
174	GP	Guinea pig
175	HMT	Human maximization test
176	ICCVAM	Interagency Coordinating Committee on the Validation of
177		Alternative Methods
178	ILS	Integrated Laboratory Systems
179	ISO	International Organization for Standardization
180	IWG	Immunotoxicity Working Group
181	JaCVAM	Japanese Center for the Validation of Alternative Methods
182	K_{ow}	Octanol-water partition coefficient
183	LLNA	Murine local lymph node assay
184	LLNA: DA	Murine LLNA modified by Daicel Chemical Industries, Ltd.
185		based on ATP content
186	MEK	Methyl ethyl ketone
187	Min	Minimal
188	Mod	Moderate
189	Mol.	Molecular
190	NA	Not applicable
191	NICEATM	National Toxicology Program Interagency Center for the
192		Evaluation of Alternative Toxicological Methods

193	NT	Not tested
194	NTP	National Toxicology Program
195	OECD	Organisation for Economic Co-operation and Development
196	PBS	Phosphate buffered saline
197	Ref.	Reference
198	RLU	Relative luminescence units
199	SD	Standard deviation
200	SI	Stimulation index
201	SLS	Sodium lauryl sulfate
202	Stats.	Statistics
203	TG	Test guideline
204	Trad.	Traditional
205	U.S.	United States
206	Unk	Unknown
207	vs.	Versus

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179   Public Health (Kyoto, Japan) for submitting data to NICEATM used for the evaluation of the
180   LLNA: DA test method.

Preface

181

182 In 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative

183 Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (LLNA) as a

184 valid test method to assess the skin sensitization potential of most types of substances

185 (ICCVAM 1999). ICCVAM concluded that the LLNA (referred to herein as the "traditional

186 LLNA") provided several advantages compared to the guinea pig method, including

187 elimination of potential pain and distress, use of fewer animals, less time required to perform,

188 and availability of dose-response information. United States and international regulatory

189 authorities subsequently accepted the traditional LLNA as an alternative test method for

190 allergic contact dermatitis testing. It is now commonly used around the world.

191 One disadvantage of the traditional LLNA is that it requires injection of a radioactive marker

192 to measure cell proliferation in lymph nodes. To avoid the use of radioactive markers,

193 scientists have recently developed several non-radioactive versions of the LLNA. In 2007,

194 the U.S. Consumer Product Safety Commission (CPSC) asked ICCVAM and the National

195 Toxicology Program Interagency Center for the Evaluation of Alternative Methods

196 (NICEATM) to evaluate the scientific validity of these non-radioactive versions. ICCVAM

197 assigned the nomination a high priority, and established the ICCVAM Immunotoxicity

198 Working Group (IWG) to work with NICEATM to review the current literature and evaluate

199 available data to assess the validity of three such test methods. A comprehensive draft

200 background review document (BRD) provided the information, data, and analyses supporting

201 the validation status of each of the non-radioactive test methods. ICCVAM also developed

202 draft test method recommendations for each test method regarding its usefulness and

203 limitations, test method protocol, performance standards, and future studies.

204 NICEATM and ICCVAM provided the draft BRDs and draft test method recommendations

205 to an international independent scientific peer review panel (referred to hereafter as "Panel")

206 for their consideration at a public meeting on March 4-6, 2008. A report of the Panel meeting

207 was subsequently published on the NICEATM-ICCVAM website.[1] Both the Panel and

208 ICCVAM concluded that more information was needed before a recommendation on the

209 usefulness and limitations of each of the three test methods could be made. The Panel

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[1] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm.

210   recommended that NICEATM obtain additional existing data that was not available to the

211   Panel and reanalyze the performance of each non-radioactive LLNA test method. NICEATM

212   subsequently obtained additional data and prepared revised draft BRDs. ICCVAM also

213   prepared revised draft test method recommendations based on the revised draft BRDs. This

214   revised draft BRD addresses the validation database for the LLNA developed by Daicel

215   Chemical Industries, Ltd., based on adenosine triphosphate content (LLNA: DA).

216   The Panel will meet to consider the revised draft BRDs and to evaluate the extent to which

217   the available information supports the revised ICCVAM draft test method recommendations.

218   ICCVAM will consider the conclusions and recommendations of the Panel, along with

219   comments received from the public and the Scientific Advisory Committee on Alternative

220   Toxicological Methods (i.e., the ICCVAM-NICEATM advisory committee), and then

221   finalize the BRDs and test method recommendations. These will then be forwarded to

222   Federal agencies for their consideration and acceptance decisions, where appropriate.

223   We gratefully acknowledge the organizations and scientists who provided data and

224   information for this document. We also acknowledge the efforts of those individuals

225   contributing to the preparation of this revised draft BRD, including the following staff from

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231   and provided comments throughout the process leading to this final revised draft version.

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Executive Summary

244   *Background*

245   In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods

246   (ICCVAM) recommended to U.S. Federal agencies that the murine local lymph node assay

247   (LLNA) is a valid substitute for currently accepted guinea pig (GP) test methods to assess the

248   allergic contact dermatitis (ACD) potential of many, but not all, types of substances. ACD is

249   an allergic skin reaction characterized by redness, swelling, and itching that can result from

250   contact with a sensitizing chemical or product. The recommendation was based on a

251   comprehensive evaluation that included an independent scientific peer review panel (Panel)

252   assessment of the validation status of the LLNA. The Panel report and the ICCVAM

253   recommendations (ICCVAM 1999) are available at the National Toxicology Program

254   Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-

255   ICCVAM website.[2] The LLNA was subsequently incorporated into national and international

256   test guidelines for the assessment of skin sensitization (Organisation for Economic Co-

257   operation and Development [OECD] Test Guideline 429 [OECD 2002]; International

258   Organization for Standardization [ISO] 10993-10: Tests for Irritation and Sensitization [ISO

259   2002]; U.S. Environmental Protection Agency [EPA] Health Effect Testing Guidelines on

260   Skin Sensitization [EPA 2003]).

261   In 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several

262   activities related to the LLNA for evaluation by ICCVAM and NICEATM.[3] One of the

263   nominated activities was assessment of the validation status of non-radioactive modifications

264   to the current version of the LLNA ([ICCVAM 1999; Dean et al. 2001] referred to hereafter

265   as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information

266   described in the original (i.e., January 2008) and this background review document (BRD)

267   was compiled by ICCVAM and NICEATM in response to this nomination. The BRD

268   provides a comprehensive review of available data and information regarding the usefulness

269   and limitations of one of these test methods, the LLNA based on adenosine triphosphate

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[2] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf.
[3] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf.

270   (ATP) content in the draining auricular lymph nodes (referred to hereafter as the "LLNA:

271   DA").

Revisions to the LLNA: DA Evaluation

273   NICEATM and ICCVAM convened an independent scientific peer review panel meeting on

274   March 4-6, 2008. The Panel peer reviewed the draft BRD and commented on the extent that

275   it supported the draft ICCVAM test method recommendations on the usefulness and

276   limitations of the LLNA: DA. Both ICCVAM and the Panel concluded that more information

277   was needed before a recommendation on the usefulness and limitations of the LLNA: DA

278   could be made.[4] The Panel indicated that the following information was needed: a detailed

279   protocol, individual animal data, and an evaluation of interlaboratory reproducibility. The

280   Panel recommended that NICEATM obtain additional data in order to reanalyze the

281   performance of the LLNA: DA. In response to this recommendation, NICEATM obtained

282   additional LLNA: DA data from the test sponsor, which were used to update the evaluation.

283   These data include:

284   •   Individual animal data for the LLNA: DA intralaboratory validation study of
285   31 substances (Idehara et al. 2008). These data were used in the updated
286   accuracy analyses represented in **Section 6.0**

287   •   Individual animal data for 14 additional LLNA: DA substances tested in the
288   intralaboratory validation study (Idehara unpublished). These data were used
289   in the updated accuracy analyses represented in **Section 6.0**

290   •   Individual animal data for the LLNA: DA two-phased interlaboratory
291   validation study of 14 substances (Omori et al. 2008). These data were used in
292   the updated accuracy analyses represented in **Section 6.0** and the additional
293   quantitative analyses of test method reproducibility, which are detailed in
294   **Section 7.0** of this BRD.

Test Method Protocol

296   The test method protocol in this revised draft BRD is the same as the test method protocol

297   discussed in the January 2008 draft BRD. Daicel Chemical Industries, Ltd. developed the

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[4] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm.

298   LLNA: DA test method based on modifications to the traditional LLNA (Yamashita et al.

299   2005). While the traditional LLNA assesses cellular proliferation by measuring the

300   incorporation of radioactivity into the DNA of dividing lymph node cells, the LLNA: DA

301   assesses cellular proliferation by measuring increases in ATP content in the lymph node as an

302   indicator of the cell number. In addition, the LLNA: DA also differs from the traditional

303   LLNA in the timing and administration of the test substance. In the traditional LLNA, the

304   test substance is applied on days 1, 2, and 3 and the auricular lymph nodes are excised on day

305   6. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and 7 and the auricular

306   lymph nodes are excised on day 8. Furthermore, one hour prior to each application of the test

307   substance, 1% sodium lauryl sulfate is applied to increase absorption of the test substance

308   through the skin. A stimulation index (SI) is used to identify a substance as a sensitizer (i.e.,

309   the ratio of the mean ATP content of the substance treatment group to the mean ATP content

310   of the vehicle treatment group).

311   ***Validation Database***

312   The validation database in this revised draft BRD has been updated from the January 2008

313   draft BRD to include 15 additional substances. The accuracy and reliability of the LLNA:

314   DA was assessed using data submitted to NICEATM for 45 substances tested in one

315   laboratory (Idehara et al. 2008; Idehara unpublished) and 14 substances, one not previously

316   examined, tested in a two-phased interlaboratory validation study (17 laboratories). The

317   reference test data for these substances were obtained from the traditional LLNA, GP skin

318   sensitization tests, and/or human skin sensitization tests. One substance, benzocaine, yielded

319   both positive and negative results in the traditional LLNA and therefore was not considered

320   in the performance evaluation of the LLNA: DA. LLNA studies for another substance,

321   toluene 2,4-diisocyanate, were not conducted according to the traditional LLNA test method

322   protocol described (ICCVAM 1999; Dean et al. 2001). Of the remaining 44 substances with

323   sufficient traditional LLNA data, 32 were classified by the traditional LLNA as skin

324   sensitizers and 12 were classified as nonsensitizers.

325   ***Test Method Accuracy***

326   The accuracy evaluation in this revised draft BRD has been updated from the January 2008

327   draft BRD to include the results for 15 additional substances. Other revisions include the

328   evaluation of multiple decision criteria compared to traditional LLNA results (SI ≥ 2.0 was

329   further compared with GP and human outcomes) and the additional evaluation of two

330   different criteria used simultaneously to classify sensitizers and nonsensitizers compared to

331   traditional LLNA results. Based on the evaluation of multiple decision criteria, the optimal

332   performance was achieved using SI ≥ 2.5 to classify sensitizers and SI ≤ 1.7 to classify

333   nonsensitizers. When these two criteria are used, false positive results (0/12) and false

334   negative results (0/32) are eliminated compared with the traditional LLNA. However, using

335   these criteria, 10 substances have an SI > 1.7 and an SI < 2.5, which includes five substances

336   that were sensitizers and five substances that were nonsensitizers in the traditional LLNA.

337   Other available information could be used to interpret LLNA: DA results when the SI falls

338   between 1.7 and 2.5, such as peptide reactivity. Forty percent (2/5) of the traditional LLNA

339   sensitizers in this range had peptide reactivity data (i.e., one substance had minimal peptide

340   reactivity and one substance had high peptide reactivity). Eighty percent (4/5) of the

341   traditional LLNA nonsensitizers in this range had peptide reactivity data (i.e., all four

342   substances had minimal peptide reactivity).

343   When using the decision criterion of SI ≥ 2.5 to classify sensitizers versus nonsensitizers,

344   compared to the traditional LLNA, accuracy was 91% (40/44), with a false positive rate of

345   0% (0/12), and a false negative rate of 13% (4/32). Among the discordant substances, no

346   unique characteristics were identified that could be used as rationale for excluding any

347   particular types of substances from testing in the LLNA: DA.

348   ***Test Method Reliability – Intralaboratory Reproducibility***

349   The intralaboratory evaluation in this revised draft BRD has been updated from the January

350   2008 draft BRD to include, in addition to SI ≥ 3.0, an evaluation of SI ≥ 2.5 for the same

351   substances. Intralaboratory reproducibility for the LLNA: DA was assessed using data for

352   two substances (isoeugenol and eugenol) that were tested at varying concentrations in three

353   different experiments. The EC3 (estimated concentration needed to produce an SI of three)

354   coefficient of variation (CV) for the reproducibility of isoeugenol and eugenol was 21% and

355   11%, respectively. The EC2.5 (estimated concentration needed to produce an SI of 2.5) CV

356   for the reproducibility of isoeugenol and eugenol was 33% and 13%, respectively.

357   ***Test Method Reliability – Interlaboratory Reproducibility***

358   The interlaboratory reproducibility evaluation in this revised draft BRD is a new addition

359   because interlaboratory data were not available for evaluation in the January 2008 draft BRD.

360   This revised draft BRD also includes a reproducibility analysis using separate SI criteria to

361   identify sensitizers and nonsensitizers. The two-phased multilaboratory validation study

362   included 17 different laboratories in which 14 different substances were examined. In the

363   first phase of the study, 10 laboratories each tested up to 12 substances, while in the second

364   phase of the study seven laboratories (different from the 10 laboratories in the first phase of

365   the interlaboratory validation study) each tested up to five substances. In both studies, each

366   substance was tested once at three different doses, which were provided to the participating

367   laboratories by the validation study management team.

368   When using SI ≥ 2.5 as the decision criterion, the qualitative (positive/negative)

369   interlaboratory concordance analysis for the 12 substances that were tested in up to 10

370   laboratories during the first phase of the LLNA: DA interlaboratory validation study resulted

371   in 100% (3/3 or 10/10) concordance for 10 substances (i.e., seven sensitizers and three

372   nonsensitizers in the traditional LLNA) and 67% (2/3) concordance for two substances (i.e.,

373   two sensitizers in the traditional LLNA). The CV values for the EC2.5 ranged from 26% (i.e.,

374   hexyl cinnamic aldehyde) to 133% (i.e., cobalt chloride) and the mean CV was 79%. The

375   qualitative interlaboratory concordance analysis for the five substances tested in up to seven

376   laboratories during the second phase of the validation study resulted in 100% (4/4 or 7/7)

377   concordance for four substances (i.e., three sensitizers and one nonsensitizer in the traditional

378   LLNA) and 75% (3/4) concordance for one substance (i.e., a sensitizer in the traditional

379   LLNA). The CV values for the EC2.5 ranged from 20% (i.e., hexyl cinnamic aldehyde) to

380   92% (i.e., cobalt chloride) and the mean CV was 62%.

381   When using SI ≥ 2.5 to classify sensitizers and SI ≤ 1.7 to classify nonsensitizers, the

382   concordance analysis for the 14 substances with multiple tests indicated that the SI results for

383   87% (27/31) of the tests that yielded SI ≤ 1.7 were for substances that were classified as

384   nonsensitizers by the traditional LLNA; 13% (4/31) of the tests that yielded SI ≤ 1.7 were for

385   substances that were classified as sensitizers by the traditional LLNA. Fifty-eight percent

386   (7/12) of the tests that yielded 1.7 < SI < 2.5 were for substances that were classified as

387   sensitizers by the traditional LLNA.

388   ***Animal Welfare Considerations***

389   The animal welfare considerations in this revised draft BRD have not changed from the

390   January 2008 draft BRD. The LLNA: DA will use the same number of animals when

391   compared to the updated ICCVAM-recommended LLNA protocol (ICCVAM 2009).

392   However, since use of the traditional LLNA is restricted in some institutions because it

393   involves radioactivity, availability and use of the non-radioactive LLNA: DA may lead to

394   further reduction in use of the GP tests, which would provide for reduced animal use and

395   increased refinement due to the avoidance of pain and distress in the LLNA procedure.

396   ***Test Method Transferability***

397   The test method transferability considerations in this revised draft BRD have not changed

398   from the January 2008 draft BRD. The transferability of the LLNA: DA is expected to be

399   similar to the traditional LLNA. Notably, the test method developer indicates that when the

400   LLNA: DA test method is conducted, all the procedural steps from lymph node excision to

401   the determination of ATP content should be performed without delay since ATP content

402   decreases over time (Idehara et al. 2008; Omori et al. 2008). Compared to the traditional

403   LLNA, the LLNA: DA will not require laboratories, equipment, and licensing permits for

404   handling radioactive materials. The level of training and expertise needed to conduct the

405   LLNA: DA should be similar to the traditional LLNA except that the understanding and

406   practice of luciferase methodology is required.

407   ***ICCVAM Revised Draft Test Method Recommendations***

408   ICCVAM developed revised draft test method recommendations for the LLNA: DA based on

409   the new data and analyses. Test method recommendations are provided for test method

410   usefulness and limitations, test method protocol, and future studies, in order to further

411   characterize its usefulness and limitations. These are provided in a separate document, *Draft*

412   *ICCVAM Test Method Recommendations, Non-Radioactive Murine Local Lymph Node*

413   *Assay: Modified by Daicel Chemical Industries, Ltd. Based on ATP Content Test Method*

414   *Protocol.*

415   **1.0   Introduction**

416   **1.1   Public Health Perspective**

417   Allergic contact dermatitis (ACD) is a frequent occupational health problem. According to

418   the U.S. Department of Labor Bureau of Labor Statistics, in 2005, 980 cases of ACD

419   involved days away from work.[5] ACD develops in two phases, induction and elicitation. The

420   induction phase occurs when a susceptible individual is exposed topically to a skin-

421   sensitizing substance. Induction depends on the substance passing through the epidermis,

422   where it forms a hapten complex with dermal proteins. The Langerhans cells, the resident

423   antigen-presenting cells in the skin, process the hapten complex. The processed hapten

424   complex then migrates to the draining lymph nodes. Antigen presentation to T-lymphocytes

425   follows, which leads to the clonal expansion of these cells. At this point, the individual is

426   sensitized to the substance (Basketter et al. 2003; Jowsey et al. 2006). Studies have shown

427   that the magnitude of lymphocyte proliferation correlates with the extent to which

428   sensitization develops (Kimber and Dearman 1991, 1996).

429   The elicitation phase occurs when the individual is again topically exposed to the same

430   substance. As in the induction phase, the substance penetrates the epidermis, is processed by

431   the Langerhans cells, and presented to circulating T-lymphocytes. The antigen-specific T-

432   lymphocytes are then activated, which causes release of cytokines and other inflammatory

433   mediators. This release produces a rapid dermal immune response that can lead to ACD

434   (ICCVAM 1999; Basketter et al. 2003; Jowsey et al. 2006).

435   **1.2   Historical Background for the Murine Local Lymph Node Assay**

436   In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods

437   (ICCVAM) recommended that the murine local lymph node assay (LLNA) is a valid

438   substitute for currently accepted guinea pig (GP) test methods to assess the ACD potential of

439   many, but not all, types of substances. The recommendation was based on a comprehensive

440   evaluation that included an independent scientific peer review panel (Panel) assessment of

441   the validation status of the LLNA. The Panel report and the ICCVAM recommendations

442   (ICCVAM 1999) are available at the National Toxicology Program (NTP) Interagency

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[5] http://www.bls.gov/.

443   Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM

444   website.[6] ICCVAM forwarded recommendations to U.S. Federal agencies that the LLNA

445   should be considered for regulatory acceptance or other non-regulatory applications for

446   assessing the ACD potential of substances, while recognizing that some testing situations

447   would still require the use of traditional GP test methods (ICCVAM 1999; Sailstad et al.

448   2001). The LLNA was subsequently incorporated into national and international test

449   guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation

450   and Development [OECD] Test Guideline [TG] 429 [OECD 2002]; International Standards

451   Organization [ISO] 10993-10: Tests for Irritation and Sensitization [ISO 2002]; U.S.

452   Environmental Protection Agency [EPA] Health Effect Testing Guidelines on Skin

453   Sensitization [EPA 2003]).

454   On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally

455   nominated several activities related to the LLNA for evaluation by ICCVAM and

456   NICEATM.[7] One of the nominated activities was an assessment of the validation status of

457   non-radioactive modifications to the current version of the LLNA ([ICCVAM 1999; Dean et

458   al. 2001] referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect

459   sensitizers. The information described in this draft background review document (BRD) was

460   compiled by ICCVAM and NICEATM in response to this nomination. The draft BRD

461   provides a comprehensive review of available data and information regarding the usefulness

462   and limitations of one of these test methods, the LLNA based on adenosine triphosphate

463   (ATP) content in the draining auricular lymph nodes (referred to hereafter as the "LLNA:

464   DA"). Further, ICCVAM and its IWG developed draft test method recommendations based

465   on this evaluation.

466   A Panel reviewed the original draft BRD in March 2008 to evaluate the extent to which the

467   information contained in the draft BRD supported the draft test method recommendations.

468   The Panel concluded that additional information was needed to evaluate the test method,

469   including a detailed test method protocol, quantitative data for the test method, and an

470   evaluation of interlaboratory reproducibility. In response to this recommendation, NICEATM

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[6] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf.
[7] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf.

471 obtained additional LLNA: DA data and information, which were used in this revised draft

472 BRD for review by the Panel. These data and information include:

473 • A detailed description of the standard operating procedure of the LLNA: DA

474 test method used for the two-phased interlaboratory validation study (see

475 **Appendix A**)

476 • Individual animal data for the LLNA: DA intralaboratory validation study of

477 31 substances (Idehara et al. 2008). These data were used in the updated

478 accuracy analyses represented in **Section 6.0**

479 • Data for 14 additional LLNA: DA intralaboratory substances (Idehara

480 unpublished). These data were used in the updated accuracy analyses

481 represented in **Section 6.0**

482 • Individual animal data for the LLNA: DA two-phased interlaboratory

483 validation study of 14 substances (Omori et al. 2008). These data were used in

484 the updated accuracy analyses represented in **Section 6.0** and the additional

485 quantitative analyses of test method reproducibility, which are detailed in

486 **Section 7.0** of this BRD.

487 ICCVAM will consider the conclusions and recommendations of the Panel, along with

488 comments received from the public and its advisory committee (i.e., the Scientific Advisory

489 Committee on Alternative Toxicological Methods), when developing the final BRD and final

490 test method recommendations on the usefulness and limitations of each non-radioactive

491 alternative LLNA test method that is being considered.

492 **1.3   The LLNA: DA**

493 The LLNA: DA was developed by Daicel Chemical Industries, Ltd. as a non-radioactive

494 modification (Yamashita et al. 2005) to the current version of the LLNA. The traditional

495 LLNA assesses cellular proliferation by measuring the incorporation of radioactive

496 thymidine or iodine into the DNA of dividing lymph node cells. In contrast, the LLNA: DA

497 assesses ATP content in the lymph node by employing a luciferin-luciferase assay to measure

498 bioluminescence. Since ATP content is linearly related to living cell number, this

499 measurement serves as a surrogate for cell number at the time of sampling.

500   This document provides:

501   •   A comprehensive summary of the LLNA: DA test method protocol
502   •   The substances used in the validation of the test method and the test results
503   •   The performance characteristics (accuracy and reliability) of the test method
504   •   Animal welfare considerations
505   •   Other considerations relevant to the usefulness and limitations of this test
506   method (e.g., transferability, cost of the test method).

507   **2.0   LLNA: DA Test Method Protocol**

508   The test method protocol in this revised draft BRD is the same as the test method protocol
509   discussed in the January 2008 draft BRD. Notably, this revised draft BRD now includes a
510   detailed standard operating procedure for the LLNA: DA test method and supplemental data
511   evaluating the effect of 1% sodium lauryl sulfate (SLS) pre-treatment on lymph node
512   proliferation that was not available for inclusion in the January 2008 draft BRD (**Appendix**
513   **A**). The LLNA: DA test method protocol (**Appendix A**) differs from the ICCVAM-
514   recommended test method protocol for the traditional LLNA (ICCVAM 2009) in the method
515   used to assess lymphocyte proliferation in the auricular lymph nodes (**Table 2-1**). In
516   addition, there are substantive differences between the two test method protocols regarding
517   test substance application and timing for the collection of the lymph nodes. In the traditional
518   LLNA, the test substance is administered on three consecutive days (days 1, 2, and 3). On
519   day 6, radiolabeled thymidine or iodine is administered via the tail vein and the lymph nodes
520   are excised five hours later. A lymph node cell suspension is then prepared and radioactive
521   thymidine or iodine incorporation is determined by β-scintillation or γ-scintillation counting,
522   respectively. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and additionally
523   on day 7. During the initial development of the LLNA: DA, the study group (Yamashita et al.
524   2005) determined the optimal dosing schedule by evaluating whether the addition of a fourth
525   application (day 7) was useful for increasing lymph node proliferation. Based on a
526   statistically significant increase in lymph node weight-based stimulation indexes (SIs) for
527   mice that received a fourth application (day 7) of the test substance, this test method protocol
528   was chosen. Furthermore, one hour prior to each application of the test substance, a solution
529   of 1% SLS is applied to the dorsum of the treated ears to increase absorption of the test

530   substance across the skin (van Och et al. 2000). Various researchers have shown that a

531   solution of 1% SLS does not elicit a positive response in the traditional LLNA but when

532   applied prior to test substance administration there is generally an increased response

533   compared to the test substance alone (van Och et al. 2000; De Jong et al. 2002). Similar

534   results were observed by Idehara et al. (2008) (see also **Appendix A**). Lastly, twenty-four to

535   30 hours after the last test substance application (day 7), the auricular lymph nodes are

536   excised and a lymph node cell suspension is prepared, and the ATP content is measured by

537   luciferin-luciferase assay.

538

538 **Table 2-1   Comparison of the LLNA: DA and Traditional LLNA Experimental**
539 **Procedure**

	Days 1, 2, & 3	Days 4 & 5	Day 6	Day 7	Day 8
LLNA: DA	• Pretreat with 1% SLS solution • After one hour, apply 25 μL of test substance or vehicle to dorsum of each ear	No Treatment	No Treatment	• Pretreat with 1% SLS solution • After one hour, apply 25 μL of test substance or vehicle to dorsum of each ear	• Excision of auricular lymph nodes • Measurement of ATP content in lymph node cells
Traditional LLNA	• Apply 25 μL of test substance or vehicle to dorsum of each ear	No Treatment	• Administer ^{3}H-thymidine or ^{125}I via tail vein • Excision of auricular lymph nodes • Measurement of radioactivity incorporated into lymph node cells	No Treatment	No Treatment

540 Abbreviations: ATP = adenosine triphosphate; ^{3}H = tritiated; ^{125}I = iodine-125; LLNA = murine local lymph node assay;
541 LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SLS =
542 sodium lauryl sulfate.
543

544 **2.1.   Decision Criteria**

545 Similar to the traditional LLNA, an SI is used in the LLNA: DA to distinguish skin

546 sensitizers from nonsensitizers. The formula for calculating the SI in the LLNA: DA is the

547 ratio of the mean ATP content of the auricular lymph nodes collected from the test substance

548 treatment group to the mean ATP content of the auricular lymph nodes collected from the

549 vehicle treatment group (measured in relative luminescence units; RLU)

550
$$SI = \frac{mean\ ATP\ content\ of\ auricular\ lymph\ nodes\ in\ test\ treatment\ group\ (RLU)}{mean\ ATP\ content\ of\ auricular\ lymph\ nodes\ in\ vehicle\ treatment\ group\ (RLU)}$$

551 In the intra- and interlaboratory validation studies for the LLNA: DA, an SI ≥ 3.0 was used

552 as the threshold for labeling a substance as a sensitizer, which is the same threshold used in

553 the traditional LLNA. As noted in **Section 6.0**, alternative decision criteria are evaluated in

554 this revised draft BRD to determine the threshold that provides optimum performance.

555

555   **3.0   LLNA: DA Validation Database**

556   The validation database in this revised draft BRD has been updated from the January 2008

557   draft BRD to include 15 additional substances. To evaluate the usefulness and limitations of

558   the LLNA: DA, Daicel Chemical Industries, Ltd., tested a total of 45 substances in one

559   laboratory (Idehara et al. 2008; Idehara unpublished data). They further evaluated two of the

560   45 substances (i.e., isoeugenol and eugenol) in the LLNA: DA at varying concentrations in

561   three different experiments in order to assess intralaboratory reproducibility. In addition, a

562   two-phased interlaboratory validation study evaluated the reproducibility of the LLNA: DA

563   (**Section 7.0**). In the first phase, 10 laboratories tested 12 coded substances (**Table 7-2**) and

564   in the second phase, seven different laboratories tested five coded substances (**Table 7-3**).

565   Between the 17 laboratories, 14 different substances were examined and one of those

566   substances, 3-aminophenol, was not previously tested among the 45 substances in the

567   intralaboratory validation study.

568   Taken together, all 46 substances tested in the LLNA: DA were previously tested in the

569   traditional LLNA and data for 39 of the substances were considered in the original ICCVAM

570   evaluation (ICCVAM 1999). Cinnamic alcohol, diethyl maleate, diethyl phthalate, ethyl

571   acrylate, glutaraldehyde, methyl methacrylate, and toluene 2,4-diisocyanate were the seven

572   substances tested in the LLNA: DA not evaluated in the ICCVAM 1999 report. Of the 46

573   substances tested in the LLNA: DA, 33 were classified by the traditional LLNA as skin

574   sensitizers,[8] 12 were classified as nonsensitizers, and one (i.e., benzocaine) was classified as

575   equivocal due to highly variable results and therefore was not included in the performance

576   analyses (ICCVAM 1999)[9] (**Table 3-1**). For the sensitizers in the traditional LLNA, the

577   range of traditional LLNA EC3 values (estimated concentrations needed to produce a

578   stimulation index of three) was from 0.009% to 90% (**Table 3-1**). Similar to benzocaine,

579   traditional LLNA data for toluene 2,4-diisocyanate, not evaluated in the original ICCVAM

580   1999 report, were not suitable for comparison. The LLNA test method protocol followed for

581   the study that tested toluene 2,4-diisocyanate (van Och et al. 2000) was a modified version of

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[8] Resorcinol was classified as a nonsensitizer based on original LLNA data (ICCVAM 1999) but recent LLNA data have instead suggested that it is actually a sensitizer (Basketter et al. 2007) and is therefore classified as a sensitizer for this evaluation.

[9] A series of 12 tests conducted in two laboratories resulted in some positive results that were not reproducible (Basketter et al. 1995).

582   the traditional LLNA which was not performed in accordance with OECD TG 429 (OECD

583   2002) or ICCVAM 1999 and Dean et al. 2001. One variation was that the BALB/c strain of

584   mouse was used for the experiments, and not the CBA/Ca or CBA/J strains as specified by

585   ICCVAM (1999), Dean et al. (2001) or OECD TG 429 (2002). In addition, the ears of the

586   mice were pretreated with a solution of 1% SLS before treatment with the test substance. The

587   authors also stated that the auricular lymph nodes were excised and pooled for each animal.

588   Thus, of the 46 substances with LLNA: DA data and traditional LLNA data, 44 were

589   included in the accuracy analyses described in **Section 6.0**.

590   **Appendix B** provides information on the physico-chemical properties (e.g., physical form),

591   Chemical Abstracts Service Registry Number (CASRN), and chemical class for each

592   substance tested. When available, chemical classes for each substance were retrieved from

593   the National Library of Medicine's ChemID Plus database. If chemical classes were not

594   located, they were assigned for each test substance using a standard classification scheme,

595   based on the National Library of Medicine Medical Subject Headings classification system.[10]

596   A substance could be assigned to more than one chemical class; however, no substance was

597   assigned to more than three classes. Classification of substances into chemical classes is not

598   intended to indicate the impact of structure on biological activity with respect to sensitization

599   potential. Instead, chemical class information is being presented to provide an indication of

600   the variety of structural elements that are present in the substances that were evaluated in this

601   analysis.

602

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[10] http://www.nlm.nih.gov/mesh/meshhome.html.

602 **Table 3-1   Traditional LLNA EC3 Values and Chemical Classifications of**
603   **Substances Tested in the LLNA: DA**

Substance Name	Chemical Class[1]	Traditional LLNA EC3 (%)[2]	No.[3]
5-Chloro-2-methyl-4-isothiazolin-3-one[b]	Sulfur Compounds; Heterocyclic Compounds	0.009	1
p-Benzoquinone[b]	Quinones	0.010	1
2,4-Dinitrochlorobenzene[a, c]	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	0.049	15
Benzalkonium chloride[a]	Amines; Onium Compounds	0.070[4]	1
Glutaraldehyde[a, c]	Aldehydes	0.080	3
p-Phenylenediamine[a]	Amines	0.110	6
Toluene 2,4-diisocyanate[5, a]	Hydrocarbons, Cyclic; Isocyanates	0.110	1
Potassium dichromate[a, d]	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	0.170	12
Propyl gallate[b]	Carboxylic Acids	0.320	1
Phthalic anhydride[a]	Anhydrides; Carboxylic Acids	0.360	1
Formaldehyde[a, c]	Aldehydes	0.500	4
Cobalt chloride[a, c, d]	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.600	2
Isoeugenol[a, c]	Carboxylic Acids	1.540	47
2-Mercaptobenzothiazole[a]	Heterocyclic Compounds	1.700	1
Cinnamic aldehyde[a]	Aldehydes	1.910	6
3-Aminophenol[c]	Amines; Phenols	3.200	1
Benzocaine[a]	Carboxylic Acids	3.400[6]	1
Diethyl maleate[b]	Carboxylic Acids	3.600	4
Trimellitic anhydride[a]	Anhydride; Carboxylic Acids	4.710	2
Nickel (II) sulfate hexahydrate[a, c, d]	Inorganic Chemical, Elements; Inorganic Chemical, Metals	4.800	1
Resorcinol[a]	Phenols	6.330	1
Sodium lauryl sulfate[a]	Alcohols; Sulfur Compounds; Lipids	8.080	5
Citral[a]	Hydrocarbons, Other	9.170	6
Hexyl cinnamic aldehyde[a, c, d]	Aldehydes	9.740	21
Eugenol[a]	Carboxylic Acids	10.090	11
Abietic acid[a, c]	Hydrocarbons, Cyclic; Polycyclic Compounds	11.920	5
Phenyl benzoate[b]	Carboxylic Acids	13.600	3
Cinnamic alcohol[b]	Alcohols	21.000	1
Hydroxycitronellal[a]	Hydrocarbons, Other	23.750	6
Imidazolidinyl urea[a]	Urea	24.000	1
Ethylene glycol dimethacrylate[b]	Carboxylic Acids	28.000	1
Butyl glycidyl ether[b]	Ethers	30.900	1
Ethyl acrylate[b]	Carboxylic Acids	32.800	2
Methyl methacrylate[b]	Carboxylic Acids	90.000	1
1-Bromobutane[a]	Hydrocarbons, Halogenated	NA	1
Chlorobenzene[a]	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA	1
Diethyl phthalate[a]	Carboxylic Acids	NA	1
Dimethyl isophthalate[b, c]	Carboxylic Acids	NA	1
Hexane[a]	Hydrocarbons, Acyclic	NA	1
Isopropanol[a, c]	Alcohols	NA	1
Lactic acid[a, d]	Carboxylic Acids	NA	1

Substance Name	Chemical Class[1]	Traditional LLNA EC3 (%)[2]	No.[3]
Methyl salicylate[a, c]	Carboxylic Acids; Phenols	NA	9
Propylparaben[a]	Carboxylic Acids; Phenols	NA	1
Nickel (II) chloride[b]	Inorganic Chemical, Elements; Inorganic Chemical, Metals	NA	2
Salicylic acid[b]	Phenols; Carboxylic Acids	NA	1
Sulfanilamide[b]	Hydrocarbons, Cyclic; Sulfur Compounds	NA	1

604   Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; LLNA = murine local lymph
605   node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP
606   content; NA = not applicable; No. = number.
607   [1]Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the
608   National Library of Medicine: http://www.nlm.nih.gov/mesh/meshhome.html.
609   [2]The traditional LLNA EC3 (stimulation index needed to produce a threshold of three) listed for each substance is from
610   traditional LLNA studies that used the same vehicle as the LLNA: DA (**Appendix D)**, except where noted.
611   [3]Number of traditional LLNA studies from which the data were obtained.
612   [4]Benzalkonium chloride was tested in the LLNA: DA using acetone: olive oil (4:1) as the vehicle (**Appendix D**) but is
613   classified as a sensitizer in the traditional LLNA based on results using acetone as the vehicle.
614   [5]Not included in accuracy analyses. Comparable LLNA reference data from modified LLNA test (van Och et al. 2000).
615   [6]Not included in accuracy analyses. EC3 value reported in **Table 3-1** for benzocaine is based on data from the NICEATM
616   database but variable and equivocal responses were reported by Basketter et al. (1995) and in the 1999 ICCVAM report.
617   [a]Substance tested in intralaboratory validation study (Idehara et al. 2008).
618   [b]Substance tested in intralaboratory validation study (Idehara unpublished data).
619   [c]Substance tested in phase one of two-phased interlaboratory validation study (Omori et al. 2008).
620   [d]Substance tested in phase two of two-phased interlaboratory validation study (Omori et al. 2008).
621
622

622 **4.0   Reference Data**

623   As mentioned in **Section 3.0**, 44 of the 46 substances tested in the LLNA: DA are included in

624   the accuracy analyses described in **Section 6.0.** The traditional LLNA reference data used for

625   the accuracy analyses comparisons are from ICCVAM (1999) (**Appendix C**) for 11 of those

626   44 substances. The traditional LLNA reference data for the remaining substances (i.e.,

627   benzalkonium chloride, cinnamic alcohol, diethyl maleate, diethyl phthalate, ethyl acrylate,

628   formaldehyde, glutaraldehyde, imidazolidinyl urea, methyl methacrylate, and nickel [II]

629   sulfate hexahydrate) were obtained from other sources (**Appendix C**) (Gerberick et al. 1992;

630   Hilton et al. 1998; Ryan et al. 2002; Basketter et al. 2005; Gerberick et al. 2005; Betts et al.

631   2006). In addition, Basketter et al. (2007) reassessed the skin sensitization potential of

632   resorcinol in the LLNA, in accordance with OECD TG 429 (2002), which updates

633   information in the ICCVAM 1999 report and from Gerberick et al. (2005) that had

634   previously stated that this substance tested negative in the LLNA.

635   The reference data for the GP tests (guinea pig maximization test or Buehler test) and human

636   tests (human maximization test, human patch test allergen, or other human data) were

637   obtained from Vandenberg and Epstein (1963), Kligman (1966), Marzulli and Maibach

638   (1974), Jordan and King (1977), Klecak et al. (1977), Marzulli and Maibach (1980), Van der

639   Walle et al. (1982), Gad et al. (1986), Robinson et al. (1990), Gerberick et al. (1992),

640   ICCVAM (1999), Basketter et al. (1999, 2001, 2005, 2007), Kwon et al. (2003), Schneider

641   and Akkan (2004), or Betts et al. (2006).

642   An independent quality assurance contractor for the NTP audited the traditional LLNA data

643   provided in the ICCVAM 1999 report. Audit procedures and findings are presented in the

644   quality assurance report on file at the National Institute of Environmental Health Sciences.

645   The audit supports the conclusion that the transcribed test data in the submission were

646   accurate, consistent, and complete as compared to the original study records.

647

647   **5.0   LLNA: DA Test Method Data and Results**

648   The test method data in this revised draft BRD has been updated from the January 2008 draft

649   BRD to include the individual animal data for all the LLNA: DA results evaluated in this

650   BRD that are from published studies (Idehara et al. 2008; Omori et al. 2008). **Appendix C**

651   represents a summary of substances for which there are LLNA: DA data. Forty-five of the

652   substances are from an intralaboratory validation study (Idehara et al. 2008; Idehara

653   unpublished data). In addition, 14 substances evaluated in an independent two-phased

654   interlaboratory validation study are included (Omori et al. 2008). One of the 14 substances

655   (3-aminophenol) was not assessed among the 45 substances evaluated in the intralaboratory

656   validation study. Taking these studies together, **Appendix C** contains information for 46

657   different substances, all with available LLNA: DA and traditional LLNA data, although

658   sufficient comparative LLNA data is only available for 44 of the 46 substances (**Section 3.0**).

659   In addition, 42 of the 46 substances examined in the LLNA: DA have GP data and 43 of the

660   46 substances tested have human skin sensitization data. Based on Idehara et al. (2008,

661   unpublished data), the 45 substances tested in the intralaboratory study were not coded prior

662   to testing. However, the two-phased interlaboratory validation study used coded substances

663   (Omori et al. 2008). Original data for these studies have been received.

664

664   **6.0   LLNA: DA Test Method Accuracy**

665   The accuracy evaluation in this revised draft BRD has been updated from the January 2008

666   draft BRD to include the results for 15 additional substances. Other revisions include the

667   evaluation of multiple decision criteria of which SI ≥ 2.0 was chosen, based on performance

668   in the LLNA: DA, to be further analyzed and the additional evaluation of two different

669   criteria used simultaneously to classify sensitizers and nonsensitizers.

670   A critical component of a formal evaluation of the validation status of a test method is an

671   assessment of the accuracy of the proposed test method when compared to the current

672   reference test method (ICCVAM 2003). Additional comparisons should also be made against

673   any available human data or experience from testing or accidental exposures. This aspect of

674   assay performance is typically evaluated by calculating:

675   • Accuracy (concordance): the proportion of correct outcomes (positive and
676   negative) of a test method

677   • Sensitivity: the proportion of all positive substances that are classified as
678   positive

679   • Specificity: the proportion of all negative substances that are classified as
680   negative

681   • False positive rate: the proportion of all negative substances that are
682   incorrectly identified as positive

683   • False negative rate: the proportion of all positive substances that are
684   incorrectly identified as negative.

685   **6.1   LLNA: DA Database Used for the Accuracy Analysis**

686   An accuracy analysis for the LLNA: DA test method was conducted using data from the

687   intralaboratory validation study and the two-phased interlaboratory validation study. Taken

688   together, LLNA: DA test data were available for 46 different substances, 44 of which had

689   sufficient comparative LLNA: DA and traditional LLNA data to conduct an accuracy

690   analysis (**Section 3.0**). Thus, of the 44 substances included in the accuracy analysis, 40 had

691   available LLNA: DA, traditional LLNA, and GP data and 41 had available LLNA: DA,

692   traditional LLNA, and human data. Classification of substances and data available for each

693   substance are provided in **Appendix C**.

694   Multiple LLNA: DA tests were available for 14 substances tested in the intralaboratory

695   (Idehara et al. 2008; Idehara unpublished data) and the two-phased interlaboratory LLNA:

696   DA studies (Omori et al. 2008). For the accuracy analysis, the test results were combined so

697   that each substance was represented by one overall result for the SI analyzed and represented

698   the outcome that was most prevalent. For example, when using SI ≥ 3.0 as the decision

699   criterion, cobalt chloride was positive because five of the eight LLNA: DA results were

700   positive (**Appendix D**).

701   **6.2   Accuracy Analysis Using the SI ≥ 3.0 Decision Criterion**

702   The performance characteristics of the LLNA: DA test method were first evaluated using the

703   decision criterion of SI ≥ 3.0 to identify sensitizers, which was the threshold for a positive

704   response used in both the intralaboratory and two-phased interlaboratory validation studies

705   (**Appendix A**).

706   *6.2.1   Accuracy vs. the Traditional LLNA*

707   Based on the available data (i.e., 44 substances), when compared to the traditional LLNA, the

708   LLNA: DA had an accuracy of 91% (40/44), a sensitivity of 88% (28/32), a specificity of

709   100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32)

710   (**Table 6-1**).

711   *6.2.2   Accuracy vs. Guinea Pig Data*

712   When the accuracy statistics for the LLNA: DA and the traditional LLNA were compared for

713   substances with available LLNA: DA, traditional LLNA, and GP data, and GP results served

714   as the reference data, the LLNA: DA had a lower accuracy (78% [31/40] vs. 85% [34/40]),

715   sensitivity (85% [22/26] vs. 96% [25/26]), the same specificity (64% [9/14]) and false

716   positive rate (36% [5/14]), and higher false negative rate (15% [4/26] vs. 4% [1/26]) relative

717   to the traditional LLNA (**Table 6-1**).

718   *6.2.3   Accuracy vs. Human Data*

719   When substances with only comparative LLNA: DA, traditional LLNA, and human data

720   were evaluated, and human outcomes served as the reference point, the LLNA: DA had

721   lower accuracy (78% [32/41] vs. 88% [36/41]) and sensitivity (76% [26/34] vs. 88%

722   [30/34]), the same specificity (86% [6/7]) and false positive rate (14% [1/7]), and higher false

723   negative rate (24% [8/34] vs. 12% [4/34]) relative to the traditional LLNA (**Table 6-1**).

724   **Table 6-1   Performance of the LLNA: DA in Predicting Skin Sensitization Potential Using Decision Criterion of SI ≥ 3.0 to**
725   **Identify Sensitizers**

Comparison	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]
LLNA: DA vs. Traditional LLNA	44	91	40/44	88	28/32	100	12/12	0	0/12	13	4/32	100	28/28	75	12/16
Substances *with LLNA: DA, Traditional LLNA, and GP Data*															
LLNA: DA vs. Traditional LLNA	40	93	37/40	90	27/30	100	10/10	0	0/10	10	3/30	100	27/27	77	10/13
LLNA: DA vs. GP[3]	40	78	31/40	85	22/26	64	9/14	36	5/14	15	4/26	81	22/27	69	9/13
Traditional LLNA vs. GP[3]	40	85	34/40	96	25/26	64	9/14	36	5/14	4	1/26	83	25/30	90	9/10
Substances *with LLNA: DA, Traditional LLNA, and Human Data*															
LLNA: DA vs. Traditional LLNA	41	90	37/41	87	27/31	100	10/10	0	0/10	13	4/31	100	27/27	71	10/14
LLNA: DA vs. Human[4]	41	78	32/41	76	26/34	86	6/7	14	1/7	24	8/34	96	26/27	43	6/14
Traditional LLNA vs. Human[4]	41	88	36/41	88	30/34	86	6/7	14	1/7	12	4/34	97	30/31	60	6/10

726   Abbreviations: GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on
727   ATP content; No. = number; vs. = versus.
728   [1]n = Number of substances included in this analysis.
729   [2]The proportion on which the percentage calculation is based.
730   [3]GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.
731   [4]Human refers to outcomes obtained by studies conducted using the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published
732   clinical case studies/reports.

733 **6.3** **Accuracy Analysis (SI ≥ 3.0) Based on ICCVAM-recommended LLNA**
734 **Performance Standards Reference Substances**

735 ICCVAM has developed recommended test method performance standards for the traditional
736 LLNA (ICCVAM 2009),[11] which are proposed to evaluate the performance of modified
737 LLNA test methods that are mechanistically and functionally similar to the traditional
738 LLNA. Since the validation studies for the LLNA: DA test method were completed prior to
739 the development of LLNA performance standards, the LLNA: DA is not being evaluated
740 using the ICCVAM-recommended LLNA performance standards. Thus, evaluations of the
741 LLNA: DA test substances to the ICCVAM-recommended LLNA performance standards test
742 substances are shown to provide a general comparison to a set list of reference substances (18
743 required reference substances and four optional reference substances) that represent a diverse
744 substance group. In addition, the ICCVAM-recommended LLNA performance standards are
745 not applicable to the LLNA: DA test method due to two main differences between the
746 LLNA: DA and traditional LLNA test method protocols (i.e., 1% SLS pre-treatment prior to
747 test substance application and an additional test substance application on day 7) (**Section**
748 **2.0**).

749 As shown in **Table 6-2**, all of the 18 required reference substances and three of the four
750 optional reference substances included in the ICCVAM-recommended LLNA performance
751 standards have been tested in the LLNA: DA. When compared to the traditional LLNA, the
752 LLNA: DA at SI ≥ 3.0 predicted the same sensitization classification for 16 of the 18
753 required ICCVAM-recommended reference substances tested. One discordant substance, 2-
754 mercaptobenzothiazole, was classified as a sensitizer based on traditional LLNA results (i.e.,
755 EC3 of 1.7%) but as a nonsensitizer based on LLNA: DA data. As indicated in **Table 6-2**,
756 *N,N*-dimethylformamide (DMF) was the vehicle used in both the traditional LLNA and the
757 LLNA: DA tests for 2-mercaptobenzothiazole. The positive result for 2-
758 mercaptobenzothiazole reported in the ICCVAM LLNA performance standards was based on
759 one LLNA experiment that tested the substance at 1%, 3%, and 10% (Gerberick et al. 2005).
760 By comparison, the negative result for 2-mercaptobenzothiazole obtained with the LLNA:
761 DA test method was based on one LLNA: DA experiment that tested the substance at 10%,

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[11] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm.

762   25%, and 50% (Idehara et al. 2008). The highest dose tested for 2-mercaptobenzothiazole in

763   the traditional LLNA was the lowest dose tested in the LLNA: DA (i.e., 10%) and resulted in

764   an SI of 8.6 versus 2.0, respectively.

765   Notably, a review of the original LLNA: DA laboratory records for 2-mercaptobenzothiazole

766   indicated that the concurrent positive control (i.e., 10% eugenol in DMF) failed to yield an

767   SI $\geq$ 3.0. Consequently the test method developers should have repeated the test for 2-

768   mercaptobenzothiazole to ensure that the result obtained was correctly classified as negative

769   and not the result of a failed experiment. This could explain the discordant result obtained

770   between the traditional LLNA and the LLNA: DA test method for this test substance.

771   The second discordant substance, methyl methacrylate, was classified as a sensitizer based on

772   traditional LLNA results (i.e., EC3 of 90%) but as a nonsensitizer based on LLNA: DA data.

773   As indicated in **Table 6-2**, acetone: olive oil (4:1; AOO) was the vehicle used in both the

774   traditional LLNA and the LLNA: DA tests for methyl methacrylate. The positive result for

775   methyl methacrylate reported in the ICCVAM LLNA performance standards was based on

776   one LLNA experiment that tested the substance at 10%, 30%, 50%, and 100% (Betts et al.

777   2006). By comparison, the negative result for 2-mercaptobenzothiazole obtained with the

778   LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at

779   25%, 50%, 75%, and 100% (Idehara, unpublished data). The highest dose tested for 2-

780   mercaptobenzothiazole in the traditional LLNA was the same in the LLNA: DA (i.e., 100%)

781   and resulted in an SI of 3.6 versus 1.8, respectively.

782   As shown in **Table 6-2**, when compared to the traditional LLNA, the LLNA: DA at SI $\geq$ 3.0

783   predicted the same sensitization for all three of the optional reference substances tested. The

784   optional reference substances, SLS and ethylene glycol dimethacrylate, were categorized as

785   nonsensitizers based on GP and human data but as sensitizers by the LLNA: DA. Thus,

786   similar to the traditional LLNA, these substances were false positive in the LLNA: DA. SLS

787   was tested in the same vehicle (i.e., DMF) in both the traditional LLNA and the LLNA: DA.

788   In addition, the positive results for SLS reported in the ICCVAM LLNA performance

789   standards were based on five LLNA studies that tested SLS at 1%, 2.5%, 5%, 10%, and 20%

790   (Loveless et al. 1996). In comparison, the positive result for SLS obtained with the LLNA:

791   DA test method was based on one LLNA: DA experiment that tested the substance at 1%,

792   2.5%, 5%, and 10% (Idehara et al. 2008). The EC3 values for SLS in the traditional LLNA

793   (i.e., 8.1%) and the LLNA: DA (6.9%) were comparable. In addition, ethylene glycol

794   dimethacrylate was tested in the same vehicle (i.e., methyl ethyl ketone) in both the

795   traditional LLNA and the LLNA: DA. The positive result for ethylene glycol dimethacrylate

796   reported in the ICCVAM LLNA performance standards was based on one LLNA study that

797   tested ethylene glycol dimethacrylate at 10%, 25%, and 50% (Gerberick et al. 2005). In

798   comparison, the positive result for ethylene glycol dimethacrylate obtained with the LLNA:

799   DA test method was based on one LLNA: DA experiment that also tested the substance at

800   10%, 25%, and 50% (Idehara, unpublished data). The EC3 values for ethylene glycol

801   dimethacrylate in the traditional LLNA (i.e., 28%) and the LLNA: DA (34%) were

802   comparable.

803   Lastly, the optional reference substance, nickel (II) chloride, was categorized as a sensitizer

804   based on GP and human data but as a nonsensitizer by the LLNA: DA. Thus, similar to the

805   traditional LLNA, this substance was false negative in the LLNA: DA. Nickel (II) chloride

806   was tested in the same vehicle (i.e., dimethyl sulfoxide [DMSO]) in both the traditional

807   LLNA and the LLNA: DA. In addition, the negative results for nickel (II) chloride reported

808   in the ICCVAM LLNA performance standards were based on two independent LLNA

809   studies that tested the substance at 0.5%, 1%, and 2.5% (Basketter et al. 1999) and at 1%,

810   2.5%, and 5% (Basketter and Scholes 1992). In comparison, the negative result for nickel (II)

811   chloride obtained with the LLNA: DA test method was based on one LLNA: DA experiment

812   that tested the substance at 2.5%, 5%, and 10% (Idehara, unpublished data). The highest dose

813   tested for nickel (II) chloride in the traditional LLNA was the same in the LLNA: DA (i.e.,

814   5%) and resulted in an SI of 2.4 versus 1.3, respectively.

815

815 **Table 6-2**   **Performance of the LLNA: DA (SI ≥ 3.0) Compared to the ICCVAM-**
816   **recommended LLNA Performance Standards Reference Substances[1]**
817   **(Sorted by Traditional LLNA EC3 Value)**

Substance	ICCVAM-Recommended LLNA Performance Standards				LLNA: DA[2]			
	Vehicle	Result	EC3 (%)[3]	N[4]	Vehicle	Result	EC3 (%)[3]	N[4]
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	+	0.009	1	DMF	+	0.03	1
2,4-Dinitrochlorobenzene	AOO	+	0.049	15	AOO	+	0.08	11
4-Phenylenediamine	AOO	+	0.11	6	AOO	+	0.07	1
Cobalt chloride	DMSO	+	0.60	2	DMSO	+	1.27	5
Isoeugenol	AOO	+	1.5	47	AOO	+	2.94	4
2-Mercaptobenzothiazole	*DMF*	*+*	*1.7*	*1*	*DMF*	*-*	*NA*	*1*
Citral	AOO	+	9.2	6	AOO	+	15.63	1
Hexyl cinnamic aldehyde	AOO	+	9.7	21	AOO	+	11.10	18
Eugenol	AOO	+	10.1	11	AOO	+	4.50	1
Phenyl benzoate	AOO	+	13.6	3	AOO	+	2.26	1
Cinnamic alcohol	AOO	+	21.0	1	AOO	+	21.34	1
Imidazolidinyl urea	DMF	+	24.0	1	DMF	+	18.77	1
Methyl methacrylate	*AOO*	*+*	*90.0*	*1*	*AOO*	*-*	*NA*	*1*
Chlorobenzene	AOO	-	NA	1	AOO	-	NA	1
Isopropanol	AOO	-	NA	1	AOO	-	NA	11
Lactic acid	DMSO	-	NA	1	DMSO	-	NA	5
Methyl salicylate	AOO	-	NA	9	AOO	-	NA	4
Salicylic acid	AOO	-	NA	1	AOO	-	NA	1
Sodium lauryl sulfate	DMF	FP	8.1	5	DMF	+	6.88	1
Ethylene glycol dimethylacrylate	MEK	FP	28	1	MEK	+	34.03	1
Xylene	AOO	FP	95.8	1	NT	NT	NT	NT
Nickel chloride	DMSO	FN	NA	2	DMSO	-	NA	1

818 Bolded and italicized text highlights discordant LLNA: DA vs. traditional LLNA test results.
819 Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 =
820 estimated concentration needed to produce a stimulation index of three; FN = false negative in traditional LLNA when
821 compared to guinea pig and/or human results; FP = false positive in traditional LLNA when compared to guinea pig and/or
822 human results; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine
823 local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based
824 on ATP content; MEK = methyl ethyl ketone; NA = not applicable (stimulation index < 3.0); NT = not tested; SI =
825 stimulation index.
826 "+" = Sensitizer.
827 "-" = Nonsensitizer.
828 [1]From *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available at:
829 http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm. The table lists the 18 required reference substances
830 first (sorted from lowest to highest EC3), followed by the four optional reference substances (sorted from lowest to highest
831 EC3).
832 [2]Substances tested in LLNA: DA intralaboratory validation study (Idehara et al. 2008; Idehara unpublished data) and/or two-
833 phased interlaboratory validation study (Omori et al. 2008).
834 [3]Based on mean EC3 when more than one value was available.
835 [4]Number of LLNA studies from which data were obtained.
836

836 **Table 6-3** provides the range and characteristics for 44 substances tested in the LLNA: DA

837 based on traditional LLNA data. These substances are compared to the range of 18 required

838 reference substances included on the ICCVAM-recommended LLNA performance standards

839 reference substances list (ICCVAM 2009). The table indicates that the range of the

840 substances tested in the LLNA: DA is similar to that included in the performance standards

841 list. In general, there are a proportionally increased number of substances tested in the

842 LLNA: DA in each of the categories included in the table.

843 **Table 6-3** **Characteristics of the Substances Tested in the LLNA: DA Compared to**
844 **the ICCVAM-recommended LLNA Performance Standards Reference**
845 **Substances[1]**

EC3 (%) Range in the Traditional LLNA	No. Substances	Solid/ Liquid	Actual EC3 Range (%)[2]	Human Data	Peptide Reactivity (High/Mod/Min/Low/Unk)[3]
<0.1	**5**	**4/2[4]**	**0.009-0.080**	**5**	**4/0/0/0/1**
	2	1/1	0.009-0.049	2	2/0/0/0/0
≥0.1 to <1	**7**	**5/2**	**0.11-0.60**	**7**	**1/2/0/0/4**
	2	2/0	0.11-0.60	2	0/0/0/0/2
≥1 to <10	**12**	**7/5**	**1.54-9.74**	**11**	**4/0/3/1/4**
	4	1/3	1.54-9.74	4	2/0/1/0/1
≥10 to <100	**10**	**4/6**	**10.09-90.00**	**10**	**2/1/0/1/6**
	5	3/2	10.09-90.00	5	0/1/0/0/4
Negative	**12**	**6/6**	**NA**	**10**	**0/0/8/1/3**
	5	1/4	NA	3	0/0/2/0/3
Overall	**46**	**26/21[4]**	**0.009-90.00**	**28**	**11/3/11/3/18**
	18	10/8	0.009-90.00	16	4/1/3/0/10

846 Bolded text represents characteristics of the LLNA: DA database, which includes the 44 substances tested in the
847 intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and/or the two-phased interlaboratory
848 validation study (Omori et al. 2008).
849 Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; ICCVAM =
850 Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine local lymph
851 node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based
852 on ATP Content; NA = not applicable because maximum SI < 3.0; No. = number; Min = minimal; Mod =
853 moderate; SI = stimulation index; Unk = unknown.
854 [1]From the ICCVAM-recommended performance standards for the LLNA (ICCVAM 2009), based on the 18
855 required reference substances.
856 [2]Based on traditional LLNA studies for substances tested in the LLNA: DA (bold values) and for the 18
857 required reference substances in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009).
858 [3]Data obtained from Gerberick et al. 2007.
859 [4]One substance tested in the LLNA: DA, benzalkonium chloride, is categorized as both a solid and a liquid.
860

860   **6.4   Discordant Results for Accuracy Analysis Using the SI ≥ 3.0 Decision Criterion**

861   *6.4.1   Discordance between the LLNA: DA and the Traditional LLNA*

862   When the outcomes for the 44 substances tested in the LLNA: DA (using SI ≥ 3.0) and the

863   traditional LLNA were compared, the classifications for four substances were different. The

864   LLNA: DA classified 3-aminophenol, 2-mercaptobenzothiazole, methyl methacrylate, and

865   nickel (II) sulfate hexahydrate as nonsensitizers while the traditional LLNA classified them

866   as sensitizers (**Tables 6-4** and **6-5**). These substances were tested in the same vehicle in both

867   the LLNA: DA and the traditional LLNA tests. One commonality noted between three of the

868   four discordant substances is that they are solids. Furthermore, the molecular weights for 3-

869   aminophenol and methyl methacrylate are both about 100 g/mol and those for 2-

870   mercaptobenzothiazole and nickel (II) sulfate hexahydrate are comparable at 160 g/mol

871   (**Appendix B**). In addition, all four discordant substances are considered nonirritants based

872   on GP data.

873   *6.4.2   Discordance among the LLNA: DA, the Traditional LLNA, and/or the Guinea Pig*

874   *   Test*

875   When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional

876   LLNA, and GP data, the LLNA: DA at SI ≥ 3.0 classified three substances differently

877   compared with the traditional LLNA (**Table 6-4**). 2-Mercaptobenzothiazole, methyl

878   methacrylate, and nickel (II) sulfate hexahydrate were identified as nonsensitizers by the

879   LLNA: DA while the traditional LLNA and GP tests classified these substances as

880   sensitizers. The discordant substances were tested at the same or higher concentrations in the

881   LLNA: DA and in the traditional LLNA yet the substances were still classified as

882   nonsensitizers (**Table 6-4**). There are few commonalities among these substances with regard

883   to chemical class, physical form, molecular weight, peptide reactivity (see **Appendix B** for

884   physico-chemical information), EC3 range (based on traditional LLNA, see **Table 3-1**) and

885   potential for skin irritation (**Appendix C**) as follows:

886   •   2-Mercaptobenzothiazole is a heterocyclic compound, methyl methacrylate is

887   carboxylic acid, and nickel (II) sulfate hexahydrate is a metal

888   •   2-Mercaptobenzothiazole and nickel (II) sulfate hexahydrate exist as solids and

889   methyl methacrylate exists as a liquid

890 • Nickel (II) sulfate hexahydrate and methyl methacrylate are soluble in water whereas
891   2-mercaptobenzothizole is not

892 • All three discordant substances have similar molecular weights (approximately 100 to
893   160 g/mol)

894 • 2-Mercaptobenzothaizole has a high peptide reactivity, whereas the peptide reactivity
895   for methyl methacrylate and nickel (II) sulfate hexahydrate is not known

896 • All three discordant substances are classified as sensitizers by the traditional LLNA
897   (EC3 values were 90.00 for methyl methacrylate, 1.70 for 2-mercaptobenzothiazole,
898   and 4.80 for nickel [II] sulfate hexahydrate)

899 • All three discordant substances are nonirritants based on data from guinea pig studies
900   (**Table 6-4**).

901 In addition, benzalkonium chloride, ethyl acrylate, ethylene glycol dimethacrylate,
902 resorcinol, and SLS were positive in both the LLNA: DA and the traditional LLNA, but were
903 negative in the GP test (**Table 6-4**). In contrast, nickel (II) chloride was negative in both the
904 LLNA: DA and the traditional LLNA but was positive in the GP test. There are few
905 commonalities among these substances with regard to chemical class, physical form,
906 molecular weight, peptide reactivity (see **Appendix B** for physico-chemical information),
907 and potential for skin irritation (**Appendix C**) as follows:

908 • Benzalkonium chloride is an amine, ethyl acrylate and ethylene glycol dimethacrylate
909   are carboxylic acids, resorcinol is a phenol, and SLS is an alcohol, sulfur, and lipid
910   compound; nickel (II) chloride is a metal.

911 • Resorcinol and SLS exist as solids in their physical state and ethyl acrylate and
912   ethylene glycol dimethacrylate exist as liquids in their physical state, whereas
913   benzalkonium chloride can exist in both a solid and liquid physical state; nickel (II)
914   chloride exists as a solid in its physical state.

915 • These five substances have varying molecular weights (100 g/mol for ethyl acrylate,
916   110 g/mol for resorcinol, 171 g/mol for benzalkonium chloride, 198 g/mol for
917   ethylene glycol dimethacrylate, and 288 g/mol for SLS); the molecular weight for
918   nickel (II) chloride is about 130 g/mol.

919   • These five discordant substances are soluble in water; nickel (II) chloride is slightly
920   soluble in water.

921   • Peptide reactivity is identified as minimal for resorcinol, and high for ethyl acrylate
922   and ethylene glycol dimethacrylate, but is not identified for benzalkonium chloride
923   and SLS; peptide reactivity for nickel (II) chloride is also not identified.

924   • Benzalkonium chloride and SLS have been found to be skin irritants based on results
925   in mice, rabbits, or humans, while resorcinol is considered a nonirritant based on
926   studies in humans, and ethyl acrylate and ethylene glycol dimethacrylate are
927   considered nonirritants based on studies in guinea pigs; nickel (II) chloride is
928   identified as negative at ≤0.15% based on GP studies (**Table 6-4**).

929

929   **Table 6-4**   **Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers)**
930   **Compared to Traditional LLNA and Guinea Pig Reference Data[1]**

Substance Name	Vehicle[2]	LLNA: DA[3]	Traditional LLNA[3]	Guinea Pig Studies[4]	Skin Irritant?
Benzalkonium chloride	AOO ACE[5]	+ (6.7, 2.5%)	+ (11.1, 2%)[6]	-	Irritant at 2% and 1% ACE (mice)
Ethyl acrylate	AOO	+ (4.2, 50%)[7]	+ (4.0, 50%)	-	Nonirritant at 0.3 Molar (GP)
Ethylene glycol dimethacrylate	MEK	+ (4.5, 50%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)
Resorcinol	AOO	+ (4.3, 25%)[8]	+ (10.4, 50%)	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate	DMF	+ (3.4, 10%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)
2-Mercaptobenzothiazole	DMF	- (2.0, 50%)[8]	+ (8.6, 10%)	+	Nonirritant at 10% (GP); Nonirritant at 25% (humans)
Methyl methacrylate	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 Molar (GP)
Nickel (II) sulfate hexahydrate	DMSO	- (11.8, 10%)	+ (3.1, 5%)	+	Irritant at 10% (humans); Nonirritant at 0.15% (GP)

931   Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-
932   dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay;
933   LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP
934   content; MEK = methyl ethyl ketone; SI = stimulation index.
935   "+" = Sensitizer.
936   "-" = Nonsensitizer.
937   [1]Data source indicated in **Appendix C.**
938   [2]Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.
939   [3]Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum
940   concentration test, unless otherwise noted.
941   [4]Based on studies using either the guinea pig maximization test or the Buehler test.
942   [5]Tested in AOO in LLNA: DA and ACE in traditional LLNA.
943   [6]Highest SI occurred at concentration 1%.
944   [7]Highest SI occurred at concentration 25%.
945   [8]Highest SI occurred at concentration 10%.
946

947   *6.4.3   Discordance among the LLNA: DA, Traditional LLNA, and/or the Human Outcome*

948   When analyses were restricted to the 41 substances with unequivocal LLNA: DA, traditional

949   LLNA, and human outcomes, the LLNA: DA classified four substances differently compared

950   with the classification of the traditional LLNA (**Table 6-5**). 3-Aminophenol, 2-

951   mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate were

952   identified as nonsensitizers by the LLNA: DA while the traditional LLNA and human

953   outcomes classified these substances as sensitizers. All four discordant substances were

954   tested at similar or higher concentrations in the LLNA: DA and in the traditional LLNA yet

955   the substances were still classified as nonsensitizers (**Table 6-5**). There are few

956   commonalities among these substances with regard to chemical class, physical form,

957   molecular weight, peptide reactivity (see **Appendix B** for physico-chemical information),

958   EC3 range (based on traditional LLNA, see **Table 3-1**) and potential for skin irritation

959   (**Appendix C**):

960   • 3-Aminophenol is an amine and phenol compound, 2-mercaptobenzothiazole is a

961   heterocyclic compound, methyl methacrylate is a carboxylic acid, and nickel (II)

962   sulfate hexahydrate is a metal.

963   • All four discordant substances exist as solids in their physical state except methyl

964   methacrylate which is a liquid.

965   • All four discordant substances are soluble in water except 2-mercaptobenzothizole.

966   • Molecular weights range from 100 to 167 g/mol.

967   • 2-Mercaptobenzothaizole has high peptide reactivity and 3-aminophenol has minimal

968   peptide reactivity; peptide reactivity information for methyl methacrylate and nickel

969   (II) sulfate hexahydrate is not available.

970   • All four discordant substances are classified as sensitizers by the traditional LLNA

971   (EC3 values are 1.70 for 2-mercaptobenzothiazole, 3.20 for 3-aminophenol, 4.80 for

972   nickel [II] sulfate hexahydrate, and 90.0 for methyl methacrylate).

973   • All four discordant substances are classified as nonirritants based on data from guinea

974   pig studies, although human data indicates that nickel (II) sulfate hexahydrate is an

975   irritant at 10% (**Table 6-5**).

976   In addition, the LLNA: DA predicted the same outcome for SLS as the traditional LLNA

977   (i.e., sensitizer), but was discordant when compared to the negative human test result (**Table**

978   **6-5**). Isopropanol, nickel (II) chloride, propylparaben and sulfanilamide were also predicted

979   similarly by the LLNA: DA and the traditional LLNA (i.e., nonsensitizers), but were

980   discordant when compared to the positive human test result (**Table 6-5**). There are few

981   commonalities among these substances with regard to chemical class, physical form,

982   molecular weight, peptide reactivity (see **Appendix B** for physico-chemical information),

983   EC3 range (based on traditional LLNA, see **Table 3-1**) and potential for skin irritation

984   (**Appendix C**):

985   •   SLS is an alcohol, sulfur, and lipid compound; isopropanol is an alcohol, nickel (II)

986   chloride is a metal, propylparaben is a phenol compound, and sulfanilamide is a

987   cyclic hydrocarbon and sulfur compound.

988   •   SLS exists as a solid in its physical state; isopropanol is a liquid in its physical state,

989   whereas nickel (II) chloride, propylparaben, and sulfanilamide exist as solids in their

990   physical state.

991   •   These substances have varying molecular weights that range from 60 to 172 g/mol for

992   isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide to 288 g/mol for

993   SLS.

994   •   SLS, isopropanol, nickel (II) chloride, and sulfanilamide are soluble in water and

995   propylparaben is not.

996   •   Isopropanol, propylparaben, and sulfanilamide have minimal peptide reactivity;

997   peptide reactivity data for nickel (II) chloride and SLS is not available.

998   •   SLS has been found to be a skin irritant based on results in mice, rabbits, or humans;

999   isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide are considered

1000   negative or nonirritants based on studies in rabbits or GP (**Table 6-5**).

1001

1001 **Table 6-5** **Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers)**
1002 **Compared to Traditional LLNA and Human Reference Data[1]**

Substance	Vehicle[2]	LLNA: DA[3]	Traditional LLNA[3]	Human Outcomes[4]	Skin Irritant?
Sodium lauryl sulfate	DMF	+ (3.4, 10%)	+ (8.9, 20%)	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Isopropanol	AOO	- (1.97, 50%)	- (1.7, 50%)[5]	+ (case study at 0.001%)	Negative at 100% (rabbits)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+ (HMT, data expressed as nickel)	Negative at ≤0.15% (GP)
Propylparaben	AOO	- (1.3, 25%)	- (1.4, 25%)[6]	+ (HMT)	Nonirritant at 10% (GP)
Sulfanilamide	DMF	- (0.9, 50%)[5]	- (1.0, 50%)[7]	+ (20/25 at 25%)	Nonirritant at 25% (humans)
3-Aminophenol	AOO	- (2.8, 10%)	+ (5.7, 10%)	+	Nonirritant at 5% (GP)
2-Mercaptobenzothiazole	DMF	- (2.0, 50%)[8]	+ (8.6, 10%)	+ (24/63 at 25%)	Nonirritant at 10% (GP); Nonirritant at 25% (humans)
Methyl methacrylate	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 M (GP)
Nickel (II) sulfate hexahydrate	DMSO	- (11.8, 10%)	+ (3.1, 5%)	+ (23/88 at 1%)	Irritant at 10% (humans); Nonirritant at 0.15% (GP)

1003 Abbreviations: AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO =
1004 dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local
1005 lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.
1006 "+" = Sensitizer.
1007 "-" = Nonsensitizer.
1008 [1]Data source indicated in **Appendix C.**
1009 [2]Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.
1010 [3]Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum
1011 concentration tested, unless otherwise noted.
1012 [4]Based on studies using either the human maximization test, inclusion of the test substance in a human patch
1013 test allergen kit, and/or published clinical case studies/reports.
1014 [5]Highest SI occurred at concentration 25%.
1015 [6]Highest SI occurred at concentration 5%.
1016 [7]Highest SI occurred at concentration 10% and 25%.
1017 [8]Highest SI occurred at concentration 10%.
1018

1018   **6.5   Accuracy Analysis Using a Single Alternative Decision Criteria**

1019   In addition to the accuracy analysis using SI ≥ 3.0 to classify substances as sensitizers, other

1020   decision criteria were evaluated on the LLNA: DA test method performance, using the

1021   traditional LLNA (SI ≥ 3.0) as the comparative test (**Appendix C**). The performance

1022   characteristics presented in this section are for 13 decision criteria that were used to

1023   determine whether the skin sensitization potential for the substances were positive (i.e.,

1024   sensitizing) or negative (i.e., nonsensitizing). The substances evaluated were the 44

1025   substances discussed in **Section 6.1** with both LLNA: DA and sufficient comparative

1026   traditional LLNA data. The decision criteria analyzed included the following:

1027   1.   SI values ≥1.3, ≥1.5, ≥2.0, ≥2.5, ≥3.0, ≥3.5, ≥4.0, ≥4.5, or ≥5.0

1028   2.   ATP values of treated groups statistically different from control group based
1029   on analysis of variance (ANOVA) with a post-hoc Dunnett's test, when
1030   multiple treatment groups were tested, or Student's *t*-test when there was only
1031   one dosed group

1032   3.   Mean ATP values of treated groups ≥95% confidence interval (CI) of the
1033   control group mean

1034   4.   Mean ATP values of treated groups ≥2 standard deviations (SD) or ≥3 SD
1035   from the control group mean

1036   Multiple tests were available for 14 substances tested with the LLNA: DA. The results for

1037   each of these substances were combined so that each substance was represented by one

1038   positive or negative result for each criterion evaluated for the accuracy analysis. The results

1039   were combined in three ways and a separate accuracy analysis was performed for each

1040   approach.

1041   1.   The positive/negative outcome for each substance was the most prevalent
1042   outcome for each criterion. If the number of positive and negative outcomes
1043   were equal, the most conservative (i.e., positive) result was used for the
1044   accuracy analyses.

1045   2. The positive/negative outcome for each substance for each criterion was

1046   determined by the outcome of the test with the highest maximum SI of the

1047   multiple tests.

1048   3. The positive/negative outcome for each substance was determined by the

1049   outcome of the test with the lowest maximum SI of the multiple tests.

1050   The analysis using the most prevalent outcome for substances with multiple tests is presented

1051   in this section; the analyses using the highest maximum SI and the lowest maximum SI are

1052   included in **Appendix E**.

1053   When combining multiple test results for a single substance based on the most prevalent

1054   outcome, using the decision criterion of SI $\geq$ 3.0 to identify sensitizers, the 44 substances

1055   analyzed yielded an accuracy of 91% (40/44), a sensitivity of 88% (28/32), a specificity of

1056   100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32)

1057   (**Table 6-6**). The decision criterion of SI $\geq$ 2.5 was similar to SI $\geq$ 3.0 in its performance

1058   characteristics. In comparison, the decision criteria using higher SI values,

1059   SI $\geq$ 3.5 to SI $\geq$ 5.0, decreased performance except for specificity, which remained at 100%

1060   (12/12), and the false positive rate, which remained at 0% (0/12) (**Figure 6-1** and **Table 6-6**).

1061   Specifically, at SI $\geq$ 5.0, accuracy decreased to 57% (25/44) and the false negative rate

1062   increased to 59% (19/32).

1063   The decision criteria using lower SI values, SI $\geq$ 1.5 and SI $\geq$ 1.3, also decreased

1064   performance compared to SI $\geq$ 3.0 except for sensitivity, which increased to 100% (32/32),

1065   and the false negative rate, which decreased to 0% (0/32) (**Figure 6-1** and **Table 6-6**).

1066   Notably, the SI decision criterion that exhibited the best overall performance characteristics

1067   compared to SI $\geq$ 3.0 was the SI $\geq$ 2.0 (**Figure 6-1 and Table 6-6**). Compared to SI $\geq$ 3.0, the

1068   lower SI cutoff of 2.0 had the same accuracy (i.e., 91% [40/44]) but had an increased

1069   sensitivity of 97% (31/32), although specificity decreased to 75% (9/12) and the false

1070   positive rate increased to 25% (3/12) while the false negative rate decreased to 3% (1/32).

1071   Use of ANOVA and summary statistics (i.e., mean ATP values of treated groups $\geq$95%

1072   confidence interval of the control group mean, or $\geq$2 or 3 SD from the control group mean),

1073   yielded accuracy values of 75 to 84%, with sensitivity values of 88 to 100%, and false

1074   negative rates of 0 to 13%. The specificity for these criteria ranged from 8 to 58% and the

1075   false positive rates were 42 to 92%. None of the statistical criterion evaluated exhibited

1076   increased performance characteristics when compared to SI ≥ 3.0 (**Table 6-6**).

1077   Since the decision criterion of SI ≥ 2.0 showed the best overall performance (i.e., similar

1078   accuracy, increased sensitivity, and decreased false negative rate compared to SI ≥ 3.0), it

1079   was further compared to SI ≥ 3.0 for accuracy against GP and human data (**Table 6-7)**. When

1080   the LLNA: DA was compared to GP outcomes for substances with available LLNA: DA,

1081   traditional LLNA, and GP data (i.e., 40 substances), SI ≥ 2.0 had the same accuracy (78%

1082   [31/40]), increased sensitivity (92% [24/26] vs. 85% [22/26]) and decreased specificity (50%

1083   [7/14] vs. 64% [9/14]) when compared with SI ≥ 3.0. Accordingly, the false positive rate was

1084   increased (50% [7/14] vs. 36% [5/14]) and the false negative rate was decreased (8% [2/26]

1085   vs. 15% [4/26]) for SI ≥ 2.0 compared to SI ≥ 3.0. The overall performance of the LLNA:

1086   DA (SI ≥ 2.0) compared to the traditional LLNA (SI ≥ 3.0) to predict GP outcomes was less

1087   (see **Table 6-7**).

1088   When the LLNA: DA was compared to human outcomes for substances with available

1089   LLNA: DA, traditional LLNA, and human data (i.e., 41 substances), SI ≥ 2.0 increased the

1090   accuracy (80% [31/41] vs. 78% [32/41]) and sensitivity (85% [29/34] vs. 76% [26/34]) and

1091   decreased the specificity (57% [4/7] vs. 86% [6/7]) when compared with SI ≥ 3.0.

1092   Accordingly, the false positive rate was increased (43% [3/7] vs. 14% [1/7]) and the false

1093   negative rate was decreased (15% [5/34] vs. 24% [8/34]). The overall performance of the

1094   LLNA: DA (SI ≥ 2.0) compared to the traditional LLNA (SI ≥ 3.0) to predict human

1095   outcomes was less (see **Table 6-7**).

1096

1096 **Figure 6-1   Performance of the LLNA: DA Compared to the Traditional LLNA in**
1097 **Predicting Skin Sensitization Potential Using Alternative SI Based on the**
1098 **Most Prevalent Outcome for Substances with Multiple Tests**
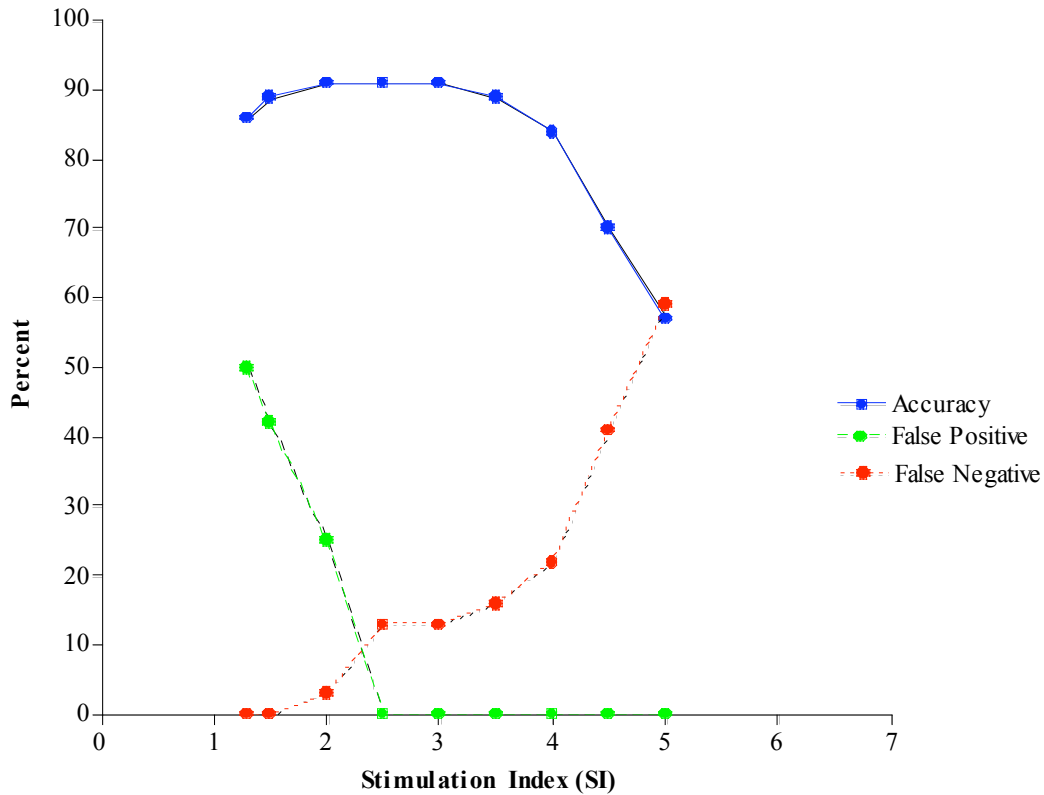
1099

1100 As compared to traditional LLNA results, the lines show the change in performance characteristics
1101 for the LLNA: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and
1102 traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA
1103 nonsensitizers). For the 14 substances with multiple test results, the results for each substance were
1104 combined by using the most prevalent outcome. The solid line shows accuracy, the dashed line shows
1105 the false positive rate, and the dotted line shows the false negative rate.

1106 **Table 6-6** **Performance of the LLNA: DA Compared to the Traditional LLNA in Predicting Skin Sensitization Potential**
1107 **Using Alternative Decision Criteria Based on the Most Prevalent Outcome 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]	44	84	37/44	94	30/32	58	7/12	42	5/12	6	2/32	86	30/35	78	7/9
≥95% CI[4]	44	75	33/44	100	32/32	8	1/12	92	11/12	0	0/32	74	32/43	100	1/1
≥2 SD[5]	44	77	34/44	91	29/32	42	5/12	58	7/12	9	3/32	81	29/36	63	5/8
≥3 SD[6]	44	80	35/44	88	28/32	58	7/12	42	5/12	13	4/32	85	28/33	64	7/11
SI ≥ 5.0	44	57	25/44	41	13/32	100	12/12	0	0/12	59	19/32	100	13/13	39	12/31
SI ≥ 4.5	44	70	31/44	59	19/32	100	12/12	0	0/12	41	13/32	100	19/19	48	12/25
SI ≥ 4.0	44	84	37/44	78	25/32	100	12/12	0	0/12	22	7/32	100	25/25	63	12/19
SI ≥ 3.5	44	89	39/44	84	27/32	100	12/12	0	0/12	16	5/32	100	27/27	71	12/17
SI ≥ 3.0	**44**	**91**	**40/44**	**88**	**28/32**	**100**	**12/12**	**0**	**0/12**	**13**	**4/32**	**100**	**28/28**	**75**	**12/16**
SI ≥ 2.5	45	91	40/44	88	28/32	100	12/12	0	0/12	13	4/32	100	28/28	75	12/16
SI ≥ 2.0	*44*	*91*	*40/44*	*97*	*31/32*	*75*	*9/12*	*25*	*3/12*	*3*	*1/32*	*91*	*31/34*	*90*	*9/10*
SI ≥ 1.5	44	89	39/44	100	32/32	58	7/12	42	5/12	0	0/32	86	32/37	100	7/7
SI ≥ 1.3	44	86	38/44	100	32/32	50	6/12	50	6/12	0	0/32	84	32/38	100	6/6

1108 Bolded text indicates the decision criterion chosen by the LLNA: DA validation study team; Italicized text indicates the single decision criterion that had an overall increased performance in predicting
1109 skin sensitization potential when compared to the traditional LLNA.
1110 Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP Content;
1111 No. = number; SD = standard deviation; SI = stimulation index.
1112 [1]N = Number of substances included in this analysis.
1113 [2]The proportion on which the percentage calculation is based.
1114 [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 ATP data were log-transformed prior to
1115 statistical analysis. For analysis of variance, significance at p < 0.05 was further tested by Dunnett's test.
1116 [4]The mean ATP of at least one treatment group was outside the 95% confidence interval for the mean ATP of the vehicle control group.

33

1117   [5]The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.
1118   [6]The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.
1119

1119 **Table 6-7   Performance of the LLNA: DA in Predicting Skin Sensitization Potential Comparing Decision Criteria of**
1120 **SI ≥ 3.0 versus SI ≥ 2.0 Based on the Most Prevalent Outcome for Substances with Multiple Tests**

Comparison	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]
LLNA: DA vs. Traditional LLNA	**44**	**91** / *91*	**40/44** / *40/44*	**88** / *97*	**28/32** / *31/32*	**100** / *75*	**12/12** / *9/12*	**0** / *25*	**0/12** / *3/12*	**13** / *3*	**4/32** / *1/32*	**100** / *91*	**28/28** / *31/34*	**75** / *90*	**12/16** / *9/10*
Substances with LLNA: DA, Traditional LLNA, and GP Data															
LLNA: DA vs. Traditional LLNA	**40**	**93** / *93*	**37/40** / *37/40*	**90** / *97*	**27/30** / *29/30*	**100** / *80*	**10/10** / *8/10*	**0** / *20*	**0/10** / *2/10*	**10** / *3*	**3/30** / *1/30*	**100** / *94*	**27/27** / *29/31*	**77** / *89*	**10/13** / *8/9*
LLNA: DA vs. GP[3]	**40**	**78** / *78*	**31/40** / *31/40*	**85** / *92*	**22/26** / *24/26*	**64** / *50*	**9/14** / *7/14*	**36** / *50*	**5/14** / *7/14*	**15** / *8*	**4/26** / *2/26*	**81** / *77*	**22/27** / *24/31*	**69** / *78*	**9/13** / *7/9*
Traditional LLNA vs. GP[3]	*40*	*85*	*34/40*	*96*	*25/26*	*64*	*9/14*	*36*	*5/14*	*4*	*1/26*	*83*	*25/30*	*90*	*9/10*
Substances with LLNA: DA, Traditional LLNA, and Human Data															
LLNA: DA vs. Traditional LLNA	**41**	**90** / *93*	**37/41** / *38/41*	**87** / *97*	**27/31** / *30/31*	**100** / *80*	**10/10** / *8/10*	**0** / *20*	**0/10** / *2/10*	**13** / *3*	**4/31** / *1/31*	**100** / *94*	**27/27** / *30/32*	**71** / *89*	**10/14** / *8/9*
LLNA: DA vs. Human[4]	**41**	**78** / *80*	**32/41** / *31/41*	**76** / *85*	**26/34** / *29/34*	**86** / *57*	**6/7** / *4/7*	**14** / *43*	**1/7** / *3/7*	**24** / *15*	**8/34** / *5/34*	**96** / *91*	**26/27** / *29/32*	**43** / *44*	**6/14** / *4/9*
Traditional LLNA vs. Human[4]	*41*	*88*	*36/41*	*88*	*30/34*	*86*	*6/7*	*14*	*1/7*	*12*	*4/34*	*97*	*30/31*	*60*	*6/10*

1121 Text is bolded for SI ≥ 3.0 and italicized for SI ≥ 2.0; performance for SI ≥ 3.0 is the same as SI ≥ 2.0 for traditional LLNA vs. GP and for traditional LLNA vs. human.
1122 Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical
1123 Industries, Ltd. based on ATP content; No. = number; SI = stimulation index; vs. = versus.
1124 [1]n = Number of substances included in this analysis.
1125 [2]The proportion on which the percentage calculation is based.
1126 [3]GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.
1127 [4]Human refers to outcomes obtained by studies conducted using the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published
1128 clinical case studies/reports.

1129   **6.6   Discordant Results for Accuracy Analysis Using a Single Alternative Decision**

1130   **Criteria**

1131   This section discusses the discordant results obtained for the analyses using the alternative

1132   decision criteria shown in **Tables 6-6** and **6-7,** in order to provide a comparison to the

1133   discordant substances identified when using the decision criterion of SI ≥ 3.0 to identify

1134   sensitizers. Discordant results are first discussed using the traditional LLNA as the reference

1135   test (**Section 6.6.1**) and then discordant results for SI ≥ 2.0, the single optimized alternative

1136   decision criterion, are discussed using the traditional LLNA, GP, and human outcomes as

1137   references (**Section 6.6.2**).

1138   *6.6.1   Discordant Results Using Alternative Decision Criteria Compared with the*

1139   *Traditional LLNA*

1140   **Table 6-8** shows how the number and identity of discordant substances changes with the

1141   alternate decision criteria when using the most prevalent outcome for the substances with

1142   multiple tests. Using SI ≥ 2.0 as the decision criterion resulted in three nonsensitizers in the

1143   traditional LLNA (i.e., chlorobenzene, hexane, and salicylic acid) being misclassified as

1144   sensitizers in the LLNA: DA. Also, methyl methacrylate, a sensitizer in the traditional

1145   LLNA, was misclassified as a nonsensitizer in the LLNA: DA. As the SI decision criterion

1146   was further reduced to SI ≥ 1.5 and SI ≥ 1.3, two additional substances, 1-bromobutane and

1147   methyl salicylate were also misclassified as sensitizers but methyl methacrylate was no

1148   longer incorrectly classified as a nonsensitizer by the LLNA: DA when compared to

1149   traditional LLNA results. In addition, using SI ≥ 1.3 also misclassified nickel (II) chloride as

1150   a sensitizer in the LLNA: DA compared to the traditional LLNA. Increasing the SI cutoff to

1151   values greater than three increased the number of sensitizers that were misclassified as

1152   nonsensitizers. At SI ≥ 5.0, 19 substances were discordant. As **Table 6-8** shows, all 19

1153   substances were sensitizers in the LLNA but misclassified as nonsensitizers in the LLNA:

1154   DA.

1155   Use of a statistical test (i.e., ANOVA or *t*-test) to identify sensitizers misclassified two

1156   sensitizers in the traditional LLNA (i.e., 2-mercaptobenzothiazole and methyl methacrylate)

1157   as nonsensitizers in the LLNA: DA and five nonsensitizers (i.e., 1-bromobutane,

1158   chlorobenzene, hexane, salicylic acid, and sulfanilamide) as sensitizers. Use of summary

1159   statistics (i.e., ≥95% CI, ≥2 SD or ≥3 SD) generally misclassified nonsensitizers in the

1160   traditional LLNA as sensitizers in the LLNA: DA. Specifically, using ≥3 SD of vehicle

1161   control mean misclassified five nonsensitizers as sensitizers: 1-bromobutane, chlorobenzene,

1162   hexane, nickel (II) chloride, and propylparaben. Using treatment group absorbance ≥2 SD of

1163   vehicle control mean misclassified the same five substances as sensitizers, as well as methyl

1164   salicylate and salicylic acid. Using the treatment group absorbance ≥95% CI of vehicle

1165   control mean misclassified all the nonsensitizers misclassified as sensitizers in the LLNA:

1166   DA when using either ≥3 SD or ≥2 SD of vehicle control mean, as well as four additional

1167   substances: diethyl phthalate, dimethyl isophthalate, isopropanol, and lactic acid. In some

1168   instances, use of summary statistics (i.e., ≥95% CI, ≥2 SD or ≥3 SD) misclassified sensitizers

1169   in the traditional LLNA as nonsensitizers in the LLNA: DA. Using ≥3 SD of vehicle control

1170   mean misclassified four traditional LLNA sensitizers as LLNA: DA nonsensitizers: butyl

1171   glycidyl ether, ethyl acrylate, methyl methacrylate, and propyl gallate. Using treatment group

1172   absorbance ≥2 SD of vehicle control mean only misclassified ethyl acrylate and propyl

1173   gallate as nonsensitizers in the LLNA; DA compared to the traditional LLNA and using the

1174   treatment group absorbance ≥95% CI did not misclassify any traditional LLNA sensitizers as

1175   LLNA: DA nonsensitizers.

1176   *6.6.2*   *Discordant Results for Accuracy Analysis Using a Single Optimized Alternative*

1177   *Decision Criteria (SI ≥ 2.0)*

1178   When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional

1179   LLNA, and GP data based on an SI ≥ 2.0, the LLNA: DA classified three substances (i.e.,

1180   chlorobenzene, salicylic acid, and methyl methacrylate) differently compared with the

1181   classification of the traditional LLNA (**Table 6-9**). Chlorobenzene and salicylic acid were

1182   classified as sensitizers in the LLNA: DA and as nonsensitizers by both the traditional LLNA

1183   and GP outcomes. Methyl methacrylate was classified as a nonsensitizer in the LLNA: DA

1184   and as a sensitizer by both the traditional LLNA and GP outcomes. In contrast, benzalkonium

1185   chloride, ethyl acrylate, ethylene glycol dimethacrylate, resorcinol, and sodium lauryl sulfate

1186   were identified as sensitizers by the LLNA: DA similar to the traditional LLNA but as

1187   nonsensitizers based on GP outcomes. Nickel (II) chloride was identified as a nonsensitizer

1188   by the LLNA: DA similar to the traditional LLNA but as a sensitizer based on GP outcomes.

1189   There are few commonalities among these substances with regard to chemical class, physical

1190   form, molecular weight, peptide reactivity (see **Appendix B** for physico-chemical

1191   information), EC3 range (based on traditional LLNA, see **Table 3-1**) and potential for skin

1192   irritation (**Appendix C**) as follows:

1193   • Chlorobenzene is a halogenated hydrocarbon compound and salicylic acid is a phenol
1194   and carboxylic acid; methyl methacrylate is a carboxylic acid; benzalkonium chloride
1195   is an amine (onium compound), ethyl acrylate and ethylene glycol dimethacrylate are
1196   carboxylic acids, resorcinol is a phenol, and SLS is an alcohol, sulfur, and lipid
1197   compound.

1198   • Chlorobenzene exists as a liquid and salicylic acid exists as a solid in its physical
1199   state; methyl methacrylate is a liquid; resorcinol and SLS are solids and ethyl acrylate
1200   and ethylene glycol dimethacrylate are liquids, whereas benzalkonium chloride can
1201   exist in both a solid and liquid physical state.

1202   • Chlorobenzene has a molecular weight of 113 g/mol and salicylic acid has a
1203   molecular weight of 138 g/mol; methyl methacrylate has a molecular weight of 100
1204   g/mol; the other five discordant substances have varying molecular weights that range
1205   from 100 g/mol for ethyl acrylate, 110 g/mol for resorcinol, 171 g/mol for
1206   benzalkonium chloride, and 198 g/mol for ethylene glycol dimethacrylate to 288
1207   g/mol for SLS.

1208   • All the discordant substances are soluble in water.

1209   • Chlorobenzene has minimal peptide reactivity; the peptide reactivity for resorcinol is
1210   identified as minimal, and that for ethyl acrylate and ethylene glycol dimethacrylate is
1211   high; peptide reactivity data for salicylic acid, methyl methacrylate, benzalkonium
1212   chloride and SLS is not available.

1213   • Methyl methacrylate is identified as a sensitizer by the traditional LLNA (EC3 =
1214   90%); benzalkonium chloride (EC3 = 0.1%), ethyl acrylate (EC3 = 32.8%), ethylene
1215   glycol dimethacrylate (EC3 = 28%), resorcinol (6.3%) and SLS (EC3 = 8.1%) are
1216   identified as sensitizers by the traditional LLNA.

1217   • Chlorobenzene has low irritancy potential assumed based on clinical literature while
1218   salicylic acid is an irritant at 20% in mice; methyl methacrylate is a nonirritant in GP;

1219	benzalkonium chloride and SLS have been found to be skin irritants based on results
1220	in mice, rabbits, or humans and ethyl acrylate, ethylene glycol dimethacrylate, and
1221	resorcinol are considered nonirritants based on studies in humans or GP (**Table 6-9**).

1222   When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional

1223   LLNA, and human outcomes based on an SI ≥ 2.0, the LLNA: DA classified three substances

1224   (i.e., hexane, salicylic acid, and methyl methacrylate) differently compared with the

1225   classification of the traditional LLNA (**Table 6-10**). Hexane and salicylic acid were

1226   classified as sensitizers in the LLNA: DA and as nonsensitizer by both the traditional LLNA

1227   and human outcomes. In contrast, methyl methacrylate was identified as a nonsensitizer by

1228   the LLNA: DA but as a sensitizer based on traditional LLNA and human outcomes.

1229   Isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide were all classified as

1230   nonsensitizers by the LLNA: DA and the traditional LLNA but as sensitizers based on human

1231   outcomes (**Table 6-10**). In contrast, SLS was classified as a sensitizer by the LLNA: DA and

1232   traditional LLNA but as a sensitizer based on human outcomes. In instances where the

1233   substances were discordant in the LLNA: DA compared to the traditional LLNA, the

1234   discordant substances were tested at the same maximum concentration. There are few

1235   commonalities among these substances with regard to chemical class, physical form,

1236   molecular weight, peptide reactivity (see **Appendix B** for physico-chemical information),

1237   EC3 range (based on traditional LLNA, see **Table 3-1**) and potential for skin irritation

1238   (**Appendix C**):

1239   • Hexane is an acyclic hydrocarbon compound and salicylic acid is a phenol and

1240   carboxylic acid; methyl methacrylate is a carboxylic acid; isopropanol is an alcohol,

1241   nickel (II) chloride is a metal, propylparaben is a phenol compound, and

1242   sulfanilamide is sulfur compound; SLS is an alcohol, sulfur, and lipid compound.

1243   • Hexane is a liquid and salicylic acid is a solid; methyl methacrylate is a liquid;

1244   isopropanol is a liquid while nickel (II) chloride, propylparaben, and sulfanilamide

1245   are solids; SLS is a solid.

1246   • Hexane has a molecular weight of 86 g/mol; methyl methacrylate has a molecular

1247   weight of 100 g/mol; the other discordant substances have varying molecular weights

| 1248 | that range from 60 g/mol for isopropanol, 130 g/mol for nickel (II) chloride, 172 |
| 1249 | g/mol for sulfanilamide, and 180 g/mol for propylparaben to 288 g/mol for SLS. |

1250 • Hexane, salicylic acid, isopropanol, methyl methacrylate, nickel (II) chloride,
1251   sulfanilamide, and SLS are soluble in water; propylparaben is not.

1252 • Hexane, isopropanol, propylparaben, and sulfanilamide have minimal peptide
1253   reactivity; peptide reactivity information for salicylic acid methyl methacrylate nickel
1254   (II) chloride SLS is not available.

1255 • Methyl methacrylate is identified as a sensitizer by the traditional LLNA (EC3 =
1256   90%) as is SLS (EC3 = 8.1%).

1257 • Hexane has been found to be an irritant at 100% in humans as has salicylic acid in
1258   mice; isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide are
1259   considered to be nonirritants based on studies in rabbits, GP, or humans; SLS has
1260   been found to be a skin irritants based on results in mice, rabbits, or humans (**Table**
1261   **6-10**).

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1263 **Table 6-8   Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA**
1264 **Based on the Most Prevalent Outcome 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
3-Aminophenol (3.2%)					-	-	-	-	-	-			
p-Benzoquinone (0.01%)					-	-	-						
1-Bromobutane (-)	+	+	+	+								+	+
Butyl glycidyl ether (30.9%)				-	-								
Chlorobenzene (-)	+	+	+	+							+	+	+
Cinnamic aldehyde (1.9%)					-								
Citral (9.2%)					-	-							
Cobalt chloride (0.6%)					-	-							
Diethyl maleate (3.6%)					-	-	-						
Diethyl phthalate (-)		+											
Dimethyl isophthalate (-)		+											
Ethyl acrylate (32.8%)			-	-	-	-							
Ethylene glycol dimethacrylate (28%)					-	-							
Formaldehyde (0.5)					-								
Hexane (-)	+	+	+	+							+	+	+
Imidazolidinyl urea (24%)					-								
Isopropanol (-)		+											
Lactic acid (-)		+											

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%)	-				-	-	-	-	-	-			
Methyl methacrylate (90%)	-		-	-	-	-	-	-	-	-	-		
Methyl salicylate (-)		+	+									+	+
Nickel (II) chloride (-)		+	+	+									+
Nickel (II) sulfate hexahydrate (4.8%)					-	-	-	-	-	-			
Phenyl benzoate (13.6%)					-	-							
Propyl gallate (0.320%)			-	-	-								
Propylparaben (-)		+	+	+									
Resorcinol (6.3%)					-	-							
Salicylic acid (-)	+	+	+								+	+	+
Sulfanilamide (-)	+												
Sodium lauryl sulfate (8.1%)					-	-	-	-					
Trimellitic anhydride (4.7%)					-								

1265 Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by
1266 Daicel Chemical Industries, Ltd. based on ATP Content; SD = standard deviation; SI = stimulation index.
1267 [1]Compared to the traditional LLNA; traditional LLNA result in parentheses are "-" for nonsensitizers and EC3 (%) for sensitizers.
1268 [2]LLNA: DA outcomes are indicated by "+" for sensitizer results and "-" for nonsensitizer results.
1269 [3]Analysis of variance assessed differences of group means when substances were tested at multiple doses or *t*-test when substances were tested at
1270   one dose. The ATP data were log-transformed prior to statistical analysis. Significance by analysis of variance at p < 0.05 was further tested by
1271   Dunnett's test.
1272 [4]The mean ATP of at least one treatment group was outside the 95% CI for the mean ATP of the vehicle control group.
1273 [5]The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.
1274 [6]The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

1275 **Table 6-9   Discordant Results for the LLNA: DA (Using SI ≥ 2.0 for Sensitizers)**
1276 **Compared to Traditional LLNA and GP Reference Data[1]**

Substance Name	Vehicle[2]	LLNA: DA[3]	Traditional LLNA[3]	Guinea Pig Studies[4]	Skin Irritant?
Benzalkonium chloride	AOO ACE[5]	+ (6.7, 2.5%)	+ (11.1, 2%)[6]	-	Irritant at 2% and 1% ACE (mice)
Ethyl acrylate	AOO	+ (4.3, 50%)[7]	+ (4.0, 50%)	-	Nonirritant at 0.3 M (GP)
Ethylene glycol dimethacrylate	MEK	+ (4.5, 50%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)
Resorcinol	AOO	+ (4.3, 25%)[5]	+ (10.4, 50%)	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate	DMF	+ (3.4, 10%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Chlorobenzene	AOO	+ (2.4, 25%)	- (1.7, 10%)[5]	-	No data. Low irritancy potential assumed based on clinical literature.
Salicylic acid	AOO	+ (2.0, 25%)	- (2.4, 25%)	-	Irritant at 20% aq. (mice)
Methyl methacrylate	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 M (GP)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)

1277 Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous ; DMF = *N,N*-
1278 dimethylformamide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local
1279 lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.
1280 "+" = Sensitizer.
1281 "-" = Nonsensitizer.
1282 [1]Data source indicated in **Appendix C.**
1283 [2]Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.
1284 [3]Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum
1285 concentration tested, unless otherwise noted.
1286 [4]Based on studies using either the human maximization test, inclusion of the test substance in a human patch
1287 test allergen kit and/or published clinical case studies/reports.
1288 [5]Benzalkonium chloride tested in AOO vehicle in LLNA: DA and ACE vehicle in traditional LLNA.
1289 [6]Highest SI occurred at concentration 1%.
1290 [7]Highest SI occurred at concentration 25%.
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**Table 6-10   Discordant Results for the LLNA: DA (Using SI ≥ 2.0 for Sensitizers)
Compared to Traditional LLNA and Human Reference Data[1]**

Substance	Vehicle[2]	LLNA: DA[3]	Traditional LLNA[3]	Human Outcomes[4]	Skin Irritant?
Hexane	AOO	+ (2.3, 100%)	- (2.2, 100%)	- (0/25 at 100%)	Irritant at 100% (humans)
Salicylic acid	AOO	+ (2.0, 25%)	- (2.4, 25%)	-	Irritant at 20% aq. (mice)
Sodium lauryl sulfate	DMF	+ (3.4, 10%)	+ (8.9, 20%)	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Isopropanol	AOO	- (1.97, 50%)	- (1.7, 50%)[5]	+ (case study at 0.001%)	Negative at 100% (rabbits)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)
Propylparaben	AOO	- (1.3, 25%)	- (1.4, 25%)[6]	+ (HMT)	Nonirritant at 10% (GP)
Sulfanilamide	DMF	- (0.9, 50%)[7]	- (1.0, 50%)[8]	+	Nonirritant at 25% (humans)
Methyl methacrylate	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 M (GP)

1293   Abbreviations: aq. = aqueous; AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; GP = guinea
1294   pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel
1295   Chemical Industries, Ltd. based on ATP content; SI = stimulation index.
1296   "+" = Sensitizer.
1297   "-" = Nonsensitizer.
1298   [1]Data source indicated in **Appendix C.**
1299   [2]Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.
1300   [3]Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum
1301   concentration tested, unless otherwise noted.
1302   [4]Based on studies using either the human maximization test, inclusion of the test substance in a human patch
1303   test allergen kit and/or published clinical case studies/reports.
1304   [5]Highest SI occurred at concentration 10%.
1305   [6]Highest SI occurred at concentration 5%.
1306   [6]Highest SI occurred at concentration 25%.
1307   [6]Highest SI occurred at concentration 10 and 25%.

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1309   **6.7   Accuracy Analysis Using Multiple Alternative Decision Criteria**

1310   As detailed in **Section 6.5,** the accuracy of the LLNA: DA when using a number of

1311   alternative decision criteria was evaluated using the traditional LLNA as the reference test.

1312   Compared to the traditional LLNA (SI ≥ 3.0), the best overall performance (i.e., accuracy of

1313   91% [40/44] and sensitivity of 97% [31/32]) was achieved using the decision criterion of

1314   SI ≥ 2.0 (**Table 6-6**). The SI ≥ 2.0 also produced a false positive rate of 25% (3/12) and a

1315   false negative rate of 3% (1/32) (**Table 6-6**). Increasing the SI decision criterion to SI ≥ 2.5

1316   decreased the false positive rate to 0% (0/12) but increased the false negative rate to 13%

1317   (4/32). The SI ≥ 2.0 produced one false negative result for the substance methyl methacrylate

1318   (EC3 = 90%). Upon evaluating the LLNA: DA test data for methyl methacrylate, the

1319   maximum SI achieved was 1.81 at 100%. Thus, decreasing the SI decision criterion to

1320   SI ≥ 1.7 decreased the false negative rate to 0% (0/32). The 0% false positive rate using

1321   SI ≥ 2.5 and the 0% false negative rate using SI ≥ 1.7 prompted an evaluation using two

1322   decision criteria for LLNA: DA results: one criterion to classify substances as sensitizers

1323   (i.e., SI ≥ 2.5) and one criterion to classify substances as nonsensitizers (SI ≤ 1.7).

1324   It should be noted that this analysis was based on the same strategy for combining results as

1325   that described in **Section 6.5** for the substances tested multiple times (i.e., the

1326   sensitizer/nonsensitizer outcome for each substance using the most prevalent outcome).

1327   **Section 7.3** details the reproducibility of substances tested multiple times and indicates that,

1328   there were no instances of false positive results for nonsensitizers (i.e., SI ≥ 2.5). Among the

1329   80 tests that produced a maximum SI ≥ 2.5, 0% (0/80) were nonsensitizers (i.e., produced a

1330   false positive result). See **Section 7.3** for more details regarding these results.

1331   **6.8   Discordant Results for Accuracy Analysis Using Multiple Alternative**

1332   **   Decision Criteria**

1333   While optimum false positive and false negative rates can be achieved using these two

1334   different decision criteria, a range of SI values (i.e., 1.7 < SI < 2.5) now exists for which the

1335   correct classification is not definitive (i.e., there is a chance for false positives or false

1336   negatives for substances in this range). Chemical class, physical form, molecular weight,

1337   peptide reactivity (see **Appendix B** for physico-chemical properties), traditional LLNA EC3

1338   range (**Table 3-1**), or potential for skin irritation (**Appendix C**) were examined to identify

1339   commonalities among the substances that produced SI values between 1.7 and 2.5 in an

1340   attempt to identify similar characteristics among these substances that could be used to

1341   correctly classify such substances.

1342   Ten substances produced SI values between 1.7 and 2.5 (**Table 6-11**). Five of the 10

1343   substances are nonsensitizers (i.e., chlorobenzene, hexane, isopropanol, methyl salicylate,

1344   salicylic acid) and five are sensitizers (i.e., 3-aminophenol, cobalt chloride, 2-

1345   mercaptobenzothiazole, methyl methacrylate, nickel [II] sulfate hexahydrate) based on

1346   traditional LLNA results. Among the five nonsensitizers, six chemical classes are

1347   represented; two substances are classified as carboxylic acids (i.e., salicylic acid and methyl

1348   salicylate [also a phenol]), one substance is a halogenated and cyclic hydrocarbon (i.e.,

1349   chlorobenzene), one substance is an acyclic hydrocarbon (i.e., hexane), and one substance is

1350   an alcohol (i.e., isopropanol). Other characteristics of the nonsensitizers (based on traditional

1351   LLNA data) include:

1352   •   Four substances are liquids (i.e., chlorobenzene, hexane, isopropanol, and
1353   methyl salicylate) and one substance is a solid (i.e., salicylic acid).

1354   •   Molecular weights range from 60 g/mol for isopropanol, 86 g/mol for hexane,
1355   113 g/mol for chlorobenzene, 138 g/mol for salicylic acid to 152 g/mol for
1356   methyl salicylate.

1357   •   All five substances are soluble in water.

1358   •   The peptide reactivity for chlorobenzene, hexane, isopropanol, and methyl
1359   salicylate is minimal; peptide reactivity information for salicylic acid is not
1360   available.

1361   •   Hexane, methyl salicylate, and salicylic acid are considered irritants based on
1362   data in either mice or humans and isopropanol is considered negative based on
1363   data in rabbits; irritancy data for chlorobenzene is not available but irritancy
1364   potential is assumed to be low based on clinical literature (**Table 6-11**).

1365   Among the five sensitizers, five chemical classes are represented; one substance is a

1366   carboxylic acid (i.e., methyl methacrylate), two substances are metals (i.e., nickel [II] sulfate

1367   hexahydrate and cobalt chloride), one substance is a phenol (i.e., 2-aminophenol [also an

1368   amine]), and one substance is a heterocyclic compound (i.e., 2-mercaptobenzothiazole).

1369   Other characteristics of the substances that are classified as sensitizers by the traditional

1370   LLNA include:

1371   •   Four substances are solids (i.e., 3-aminophenol, cobalt chloride, 2-
1372   mercaptobenzothiazole, and nickel [II] sulfate hexahydrate) and one substance
1373   is a liquid (i.e., methyl methacrylate).

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- Molecular weights range from 100 g/mol for methyl methacrylate, 109 g/mol for 3-aminophenol, 130 g/mol for cobalt chloride, 155 g/mol for nickel (II) sulfate hexahydrate to 167 g/mol for 2-mercaptobenzothiazole.

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- 2-Mercaptobenzothiazole is insoluble in water; the other four substances are soluble in water.

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- The peptide reactivity for 2-mercaptobenzothiazole is high and that for 3-aminophenol is minimal; peptide reactivity data for the three other substances is not available.

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- The EC3 values for the five substances identified as sensitizers by the traditional LLNA are: 0.6% for cobalt chloride, 1.7% for 2-mercaptobenzothiazole, 3.2% for 3-aminophenol, 4.8% for nickel [II] sulfate hexahydrate, and 90% for methyl methacrylate.

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- All five substances are considered nonirritants based on available GP data (**Table 6-11**).

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1388 **Table 6-11   Discordant Results for the LLNA: DA When Multiple Decision Criteria**
1389 **are Used[1]**

Substance[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Skin Irritant?
Chlorobenzene	AOO	2.4, 25%	- (1.7, 25%)[5]	No data. Low irritancy potential assumed based on clinical literature.
Hexane	AOO	2.3, 100%	- (2.2, 100%)	Irritant at 100% (humans)
Isopropanol	AOO	1.97, 50%[5]	- (1.7, 50%)[5]	Negative at 100% (rabbits)
Methyl salicylate	AOO	1.77, 25%[5]	- (2.9, 20%)	Irritant at 10% AOO (mice)
Salicylic acid	AOO	2.0, 25%	- (2.4, 25%)	Irritant at 20% aq. (mice)
3-Aminophenol (3.2%) (2 LLNA: DA tests)	AOO	2.4, 10% and 1.8, 10%[6]	+ (5.7, 10%)	Nonirritant at 5% (GP)
Cobalt chloride (0.6%)	DMSO	2.0, 5%	+ (7.2, 5%)	Negative at ≤ 0.5% (GP)
2-Mercaptobenzothiazole (1.7%)	DMF	2.0, 50%[5]	+ (8.6, 10%)	Nonirritant at 10% (GP)
Methyl methacrylate (90%)	AOO	1.8, 100%	+ (3.6, 100%)	Nonirritant at 3 M (GP)
Nickel (II) sulfate hexahydrate (4.8%) (2 LLNA: DA tests)	DMSO	2.1, 10% and 2.2, 5%[7]	+ (3.1, 5%)	Nonirritant at 0.15% (GP); Irritant at 10% (humans)

1390 Abbreviations: AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO =
1391 dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local
1392 lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content.
1393 "+" = Sensitizer.
1394 "-" = Nonsensitizer.
1395 [1]Data source indicated in **Appendix C.**
1396 [2]Numbers in parentheses are EC3 values (concentrations needed to produce a stimulation index [SI] of three)
1397 for substances that are sensitizers in the traditional LLNA (see **Table 3-1)**.
1398 [3]Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.
1399 [4]Numbers indicated are highest SI and maximum concentration tested; highest SI is at maximum concentration
1400 tested, unless otherwise noted.
1401 [5]Highest SI occurred at concentration 10%.
1402 [6]Highest SI occurred at concentration 3%.
1403 [7]Highest SI occurred at concentration 2.5%.

1404   **7.0   LLNA: DA Test Method Reliability**

1405   An assessment of test method reliability (intralaboratory repeatability and intra- and inter-

1406   laboratory reproducibility) is an essential element of any evaluation of the performance of an

1407   alternative test method (ICCVAM 2003). Repeatability refers to the closeness of agreement

1408   between test results obtained within a single laboratory when the procedure is performed on

1409   the same substance under identical conditions within a given time period (ICCVAM 1997,

1410   2003). Intralaboratory reproducibility refers to the extent to which qualified personnel within

1411   the same laboratory can replicate results using a specific test protocol at different times.

1412   Interlaboratory reproducibility refers to the extent to which different laboratories can

1413   replicate results using the same protocol and test substances, and indicates the extent to

1414   which a test method can be transferred successfully among laboratories. With regard to the

1415   LLNA: DA test method, there are no known intralaboratory repeatability studies, which was

1416   also the situation with the traditional LLNA.

1417   The reproducibility evaluation in this revised draft BRD has been updated from the January

1418   2008 draft BRD to include an interlaboratory reproducibility evaluation and a reproducibility

1419   analysis using separate SI criteria to identify sensitizers and nonsensitizers. The available

1420   LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility

1421   analyses. The evaluation of a single decision criterion in **Section 6.6** showed that SI ≥ 2.0

1422   was the SI value that produced the lowest false negative rate among the alternative decision

1423   criteria evaluated (i.e., 3% [1/32]) when the traditional LLNA was the reference test (**Table**

1424   **6-6**). **Appendix F** describes the evaluation of reproducibility for the decision criterion of SI ≥

1425   2.0 to identify sensitizers, which was evaluated in **Section 6.6**.The evaluation of multiple

1426   decision criteria in **Section 6.7** evaluated SI ≥ 2.5 as the decision criterion for classifying

1427   substances as sensitizers when used with a decision criterion of SI ≤ 1.7 to identify

1428   nonsensitizers. Thus, this section provides an assessment of reproducibility for the decision

1429   criterion of SI ≥ 2.5 to identify sensitizers.

1430   **7.1   Intralaboratory Reproducibility**

1431   Idehara et al. (2008) evaluated intralaboratory reproducibility of EC3 values for the LLNA:

1432   DA using two substances (isoeugenol and eugenol) that were each tested in three different

1433   experiments (**Table 7-1**). The data indicate CVs of 21% and 11% for isoeugenol and

1434   eugenol, respectively. The authors state that for both compounds the EC3 values appeared to

1435   be close and that for each test substance the SI values for the same concentration were fairly

1436   reproducible (Idehara et al. 2008). NICEATM also determined the intralaboratory

1437   reproducibility of EC2.5 values (estimated concentrations needed to produce a stimulation

1438   index of 2.5) for the same set of data. The results for EC2.5 indicate slightly larger

1439   intralaboratory variability compared to EC3 results with CVs of 33% and 13% for isoeugenol

1440   and eugenol, respectively.

1441   **Table 7-1   Intralaboratory Reproducibility of EC3 and EC2.5 Values Using the**
1442   **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%**
EC2.5	**0.82%**	**1.37%**	**0.75%**
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC2.5: 1.46% ± 0.48% and 33% 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%**
EC2.5	**4.33%**	**3.59%**	**2.87%**
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC2.5: 4.23% ± 0.57% and 13% CV*			

1443   Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC2.5 = estimated concentration
1444   needed to produce a stimulation index of 2.5; EC3 = estimated concentration needed to produce a stimulation
1445   index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd.
1446   based on ATP content.
1447   [1]Based on results discussed in Idehara et al. 2008; the number per group was not specified.
1448   [2]Mean stimulation index value ± standard deviation.
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1450   **7.2   Interlaboratory Reproducibility**

1451   Furthermore, data were submitted to NICEATM (**Appendix D**) from a two-phased

1452   interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the

1453   first phase of the interlaboratory validation study, a blinded test of 12 substances was

1454   conducted in 10 laboratories. Three substances (i.e. 2,4-dinitrochlorobenzene, hexyl cinnamic

1455   aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances

1456   were randomly assigned to subsets of three of the 10 laboratories **(Table 7-2)**. In each

1457   laboratory, each substance was tested one time at three different concentrations. The dose

1458   levels for each substance were predetermined (i.e., the participating laboratories did not

1459   determine their own dose levels for testing). Nine substances are sensitizers and three

1460   substances are nonsensitizers according to the traditional LLNA. Six substances are

1461   ICCVAM-recommended LLNA performance standards reference substances: cobalt chloride,

1462   2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl

1463   salicylate.

1464   The second phase of the interlaboratory validation study was designed to determine the

1465   reason for inconsistencies obtained from the two metals dissolved in DMSO (i.e., cobalt

1466   chloride and nickel (II) sulfate hexahydrate) and thus to further evaluate the reliability of the

1467   LLNA: DA for testing metallic salts using DMSO as a vehicle. Five coded substances (two

1468   of the five substances were unique to the second phase of the interlaboratory validation

1469   study) were tested in seven laboratories **(Table 7-3)**. One substance (i.e. hexyl cinnamic

1470   aldehyde) was tested in all seven laboratories. The remaining four substances (i.e., cobalt

1471   chloride, nickel (II) sulfate hexahydrate, lactic acid, and potassium dichromate) were

1472   randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the

1473   substance one time at three different dose levels. Again, the dose levels for each substance

1474   were predetermined. Of the two substances not previously tested in the first phase of the

1475   interlaboratory validation study (i.e., lactic acid and potassium dichromate), one is a

1476   nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively.

1477   In addition, lactic acid is an ICCVAM-recommended LLNA performance standards

1478   reference substance.

1479   The LLNA: DA test results from the two-phased interlaboratory validation studies are

1480   amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive)

1481   or nonsensitizer (negative) classification, and EC2.5 values. Analyses of interlaboratory

1482   reproducibility were performed using a concordance analysis for the qualitative results

1483   (sensitizer vs. nonsensitizer) (**Section 7.2.1**) and a CV analysis for the quantitative results

1484   (EC2.5 values) (**Sections 7.2** and **7.3**).

1485   **Table 7-2   Substances and Allocation for the First Phase of the Interlaboratory**
1486   **Validation Study for the LLNA: DA**

Substance[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitro-chlorobenzene (+)	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		

1487   Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local
1488   lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content.
1489   [1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
1490   [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%)
1491   of the interlaboratory validation study.
1492

1492 **Table 7-3** **Substances and Allocation for the Second Phase of the Interlaboratory**
1493 **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

1494 Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay
1495 modified by Daicel Chemical Industries, Ltd. based on ATP content.
1496 [1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
1497 [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%)
1498 of the interlaboratory validation study.
1499

1500 *7.2.1 Interlaboratory Reproducibility – Qualitative Results*

1501 The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances

1502 that were tested during the first phase of the LLNA: DA interlaboratory validation study is

1503 shown in **Table 7-4** for SI ≥ 2.5. In a qualitative comparison of LLNA: DA calls (i.e.,

1504 sensitizer/nonsensitizer), ten substances tested in either three or 10 laboratories had

1505 consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those

1506 substances. There were two discordant substances (i.e., 3-aminophenol and nickel (II) sulfate

1507 hexahydrate) for which interlaboratory concordance was 67% (2/3). One of the three

1508 laboratories that tested 3-aminophenol reported SI ≥ 2.5, at the highest dose tested (i.e., SI =

1509 2.83 at 10%) and two laboratories did not achieve SI ≥ 2.5 at any dose tested (**Appendix D**).

1510 One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum

1511 SI = 1.52, while the other two laboratories produced an SI ≥ 2.5 at all three doses tested

1512 (**Appendix D**). Notably, when analyzing the dose response curves for the 3 tests performed

1513 for nickel (II) sulfate in the first phase of the two-phased interlaboratory validation study,

1514 only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative

1515 to increase in concentration). Since the evaluation of interlaboratory reproducibility for the

1516 traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there

1517   were no traditional LLNA concordance data for comparison with the LLNA: DA

1518   concordance data from the first phase of the interlaboratory validation study.

1519   **Table 7-4**   **Qualitative Results for the First Phase of the Interlaboratory Validation**
1520   **Studies for the LLNA: DA (SI ≥ 2.5)**

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**

1521   Bolded substances did not achieve 100% interlaboratory concordance.
1522   Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP
1523   content; SI = stimulation index
1524   [1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
1525   [2](+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests.
1526   [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%)
1527   of the interlaboratory validation study.
1528   [4]Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.
1529   [5]Insufficient dose response.
1530

1531   The qualitative (positive/negative) interlaboratory concordance analysis for the five

1532   substances that were tested during the second phase of the LLNA: DA interlaboratory

1533   validation study is shown in **Table 7-5**. In a qualitative comparison of LLNA: DA calls (i.e.,

1534   sensitizer/nonsensitizer), four substances (i.e., hexyl cinnamic aldehyde, lactic acid, nickel

1535   [II] sulfate hexahydrate, and potassium dichromate) tested in either four or seven laboratories

1536   had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those

1537   substances. There was one discordant substance (i.e., cobalt chloride) for which

1538   interlaboratory concordance was 75% (3/4). One of the four laboratories that tested cobalt

1539   chloride did not report a maximum SI ≥ 2.5 at any dose, while the other three laboratories

1540   produced an SI ≥ 2.5 at the highest dose tested. Cobalt chloride was also tested in the first

1541   phase of the interlaboratory validation study where interlaboratory concordance was 100%

1542   (3/3). Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility

1543   for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM

1544   1999), and therefore there were no traditional LLNA concordance data for comparison with

1545   the LLNA: DA concordance data from the second phase of the interlaboratory validation

1546   study.

1547   **Table 7-5   Qualitative Results for the Second Phase of the Interlaboratory**
1548   **Validation Study for the LLNA: DA (SI ≥ 2.5)**

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

1549   Bolded substance did not achieve 100% interlaboratory concordance.
1550   Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP
1551   content; SI = stimulation index.
1552   [1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
1553   [2](+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests.
1554   [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%)
1555   of the interlaboratory validation study.
1556

1557   *7.2.2   Interlaboratory Reproducibility – EC2.5 Values*

1558   The available quantitative (i.e., EC2.5 value) data for interlaboratory reproducibility analysis

1559   were obtained from the LLNA: DA results for ten sensitizers that were tested during the first

1560   and second phase of the LLNA: DA interlaboratory validation study. The equation used for

1561   calculating EC2.5 values for the positive results was modified based on the method of linear

1562   interpolation reported by Gerberick et al. (2004) for the EC3:

1563
$$EC2.5 = c + \left[\frac{(2.5 - d)}{(b - d)} \right] \times (a - c)$$

1564   where the data points lying immediately above and below the SI = 2.5 on the dose response

1565   curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For

1566   substances for which the lowest concentration tested resulted in an SI > 2.5, an EC2.5 value

1567   was extrapolated according to the equation:

1568
$$EC2.5_{ex} = 2^{\left\{\log_2(c) + \frac{(2.5-d)}{(b-d)} \times [\log_2(a) - \log_2(c)]\right\}}$$

1569   where the point with the higher SI is denoted with the coordinates of (a, b) and the point with

1570   the lower SI is denoted (c, d) (Gerberick et al. 2004).

1571   The EC2.5 values from each laboratory were used to calculate CV values for each substance.

1572   The resulting values for the first and second phase of the interlaboratory validation study are

1573   shown in **Tables 7-6** and **7-7**, respectively. In the first phase of the interlaboratory validation

1574   study, CV values ranged from 26% (i.e., hexyl cinnamic aldehyde) to 133% (i.e., cobalt

1575   chloride) and the mean CV was 79% (**Table 7-6**). In the second phase of the interlaboratory

1576   validation study, CV values ranged from 20% (i.e., hexyl cinnamic aldehyde) to 92% (i.e.,

1577   cobalt chloride) and the mean CV was 62% (**Table 7-7**).

1578   The ICCVAM-recommended LLNA performance standards indicate that interlaboratory

1579   reproducibility should be evaluated with at least two sensitizing chemicals with well-

1580   characterized activity in the traditional LLNA. Acceptable reproducibility is attained when

1581   each laboratory obtains ECt values (estimated concentrations needed to produce a stimulation

1582   index of a specified threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and

1583   within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the

1584   interlaboratory validation study, five laboratories reported EC2.5 values outside the

1585   acceptance range indicated for 2,4-dinitrochlorobenzene; two of the five laboratories

1586   obtained EC2.5 values that were lower than the specified acceptance range (i.e., 0.025%) and

1587   three of the five laboratories obtained EC2.5 values that were higher than the specified

1588   acceptance range (i.e., 0.1%) (**Table 7-6**). For hexyl cinnamic aldehyde, all the laboratories

1589   obtained an EC2.5 value within the acceptance range (5% to 20%). In the second phase of the

1590   interlaboratory validation study, only hexyl cinnamic aldehyde was tested and all seven

1591   laboratories obtained EC2.5 values that were within the acceptance range indicated (**Table**

1592   **7-7**).

Table 7-6   EC2.5 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance[1]	Laboratory										Mean EC2.5 (%)	CV (%)
	1	2	3	4	5	6	7	8	9	10		
2,4-Dinitrochlorobenzene (+)	0.026 (11.97)	0.063 (9.23)	0.039 (9.96)	0.022 (8.53)	0.112 (7.86)	0.025 (15.14)	0.011 (13.18)	0.039 (12.60)	0.023 (10.89)	0.131 (4.71)	0.049	84
Hexyl cinnamic aldehyde (+)	8.473 (5.78)	9.414 (4.82)	11.402 (4.44)	7.900 (5.11)	14.594 (3.97)	10.759 (5.50)	6.778 (7.09)	7.032 (10.22)	12.530 (3.88)	9.135 (3.51)	9.802	26
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		6.418				6.469	11.525				8.137	36
3-Aminophenol (+)	5.471		NA					NA			5.471	NA
Dimethyl isophthalate (-)	NA		NA			NA					NA	NA
Isoeugenol (+)				0.657	5.191				0.874		2.240	114
Methyl salicylate (-)			NA			NA				NA	NA	NA
Formaldehyde (+)	0.393	1.105			4.179						1.892	106
Glutaraldehyde (+)	0.091	0.351			0.296						0.246	56
Cobalt chloride[2] (+)				0.822[3]		0.047		0.104			0.325	133
Nickel (II) sulfate hexahydrate (+)				NA[4]		0.352		IDR			0.352	NA

Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances (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 (i.e., 0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC2.5 values (estimated concentration needed to produce a stimulation index of 2.5) 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; IDR = insufficient dose response; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content. NA = not applicable.

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

1605 **Table 7-7** **EC2.5 Values from the Second Phase of the Interlaboratory Validation**
1606 **Study for the LLNA: DA**

Substance[1]	Laboratory							Mean	%CV
	11	12	13	14	15	16	17		
Hexyl cinnamic aldehyde (+)	**7.737 (4.47)**	**7.374 (5.71)**	**6.772 (5.41)**	**6.361 (7.60)**	**9.902 (3.92)**	**5.366 (8.42)**	**6.783 (6.45)**	**7.185**	**20**
Cobalt chloride[2] (+)	NA		4.111	1.202			0.699	2.004	92
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		NA		NA	NA
Potassium dichromate (+)	0.372	0.269			0.087		0.063	0.198	75

1607 Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance
1608 standards reference substances (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved.
1609 For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (i.e., 25%). None of the
1610 EC2.5 values (estimated concentrations needed to produce a stimulation index of 2.5) are outside of the acceptable range
1611 indicated in the ICCVAM-recommended LLNA performance standards (i.e., 5 - 20% for hexyl cinnamic aldehyde).
1612 Abbreviations: CV = coefficient of variation; NA = not applicable.
1613 [1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
1614 [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%)
1615 of the interlaboratory validation study.
1616

1617 The interlaboratory CV values for both the first and second phase of the interlaboratory

1618 validation study for the LLNA: DA EC2.5 values were higher than that for the traditional

1619 LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional

1620 LLNA reported CV values of 6.8 to 83.7% for five substances tested in five laboratories

1621 (**Table 7-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional

1622 LLNA and the LLNA: DA (i.e., hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and

1623 isoeugenol). All interlaboratory CV values for the LLNA: DA were greater than that for the

1624 traditional LLNA. The CV of 84% for 2,4-dinitrochlorobenzene was greater than the two CV

1625 values of 37.4% and 27.2% (which were calculated from five values each), reported by

1626 ICCVAM (1999). The CV of 26% and 20% for hexyl cinnamic aldehyde tested in the first

1627 and second phase of the LLNA: DA interlaboratory validation study, respectively, were both

1628 greater than the 6.8% reported by ICCVAM (1999). The CV of 114% for isoeugenol tested

1629 in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

1630

	1630	**Table 7-8**	**Interlaboratory Reproducibility of the EC3 for Substances Tested in the**

1630 **Table 7-8** **Interlaboratory Reproducibility of the EC3 for Substances Tested in the**
1631 **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
SLS	13.4	4.4	1.5	17.1	4.0	83.7

1632 Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a
1633 stimulation index of three; LLNA = murine local lymph node assay; SLS = sodium lauryl sulfate.
1634 [1]From ICCVAM 1999 report.

1635

1636 **7.3   Reproducibility for the LLNA: DA Accuracy Analysis Using Multiple**

1637   **Alternative Decision Criteria**

1638 **Section 6.7** details the accuracy analysis for the LLNA: DA (using the most prevalent

1639 outcome for substances with multiple tests) when using two decision criteria for LLNA: DA

1640 results: one criterion to classify substances as sensitizers (SI ≥ 2.5) and one criterion to

1641 classify substances as nonsensitizers (SI ≤ 1.7). SI ≥ 2.5 was evaluated for classifying

1642 sensitizers because it resulted in no false positives, and SI ≤ 1.7 was evaluated for classifying

1643 substances as nonsensitizers because it resulted in no false negatives, with respect to

1644 traditional LLNA data. This section evaluates reproducibility of the concordance with the

1645 traditional LLNA results by examining the frequency with which SI values in the validation

1646 database of 44 substances occurred in one of three SI categories. The three SI categories

1647 were:

1648   • SI ≤ 1.7 for classifying nonsensitizers

1649   • 1.7 < SI < 2.5, the range of uncertainty with respect to classification by the
1650   traditional LLNA

1651   • SI ≥ 2.5 to classify substances as sensitizers

1652   The validation database for the LLNA: DA consists of 123 tests of 44 substances. The

1653   maximum SI achieved by each test and the traditional LLNA outcome (sensitizer vs.

1654   nonsensitizer) were used to determine the frequency of the maximum SI. **Table 7-9** shows

1655   the proportion of sensitizers and nonsensitizers, according to the traditional LLNA for each

1656   SI category. Eighty-seven percent of the tests (27/31) that yielded SI ≤ 1.7 were for

1657   substances that were classified as nonsensitizers by the traditional LLNA; 13% of the tests

1658   (4/31) that yielded SI ≤ 1.7 were for substances that were classified as sensitizers by the

1659   traditional LLNA. Fifty-eight percent (7/12) of the tests that yielded 1.7 < SI < 2.5 were for

1660   substances that were classified as sensitizers by the traditional LLNA. Four tests produced SI

1661   values near either end of this range (i.e., SI = 1.7 or SI = 2.5). One of the 3-aminophenol

1662   studies and one of the methyl salicylate studies produced SI = 1.76 and 1.77, respectively,

1663   and the chlorobenzene test produced SI = 2.44. The remainder of the tests in this category,

1664   42% (5/12), were classified as nonsensitizers by the traditional LLNA. One hundred percent

1665   (80/80) of the tests that yielded SI ≥ 2.5 were for substances that were classified as

1666   sensitizers by the traditional LLNA and 0% (0/80) were classified as nonsensitizers.

1667   **Table 7-9   Frequency of Maximum SI for LLNA: DA Tests by Category and**
1668   **Traditional LLNA Outcome**

Classification Based on Traditional LLNA	Classification Concordance with Traditional LLNA[1]			
	Maximum SI ≤ 1.7	1.7 < Maximum SI < 2.5	Maximum SI ≥ 2.5	Total
Sensitizer	4 (13%)	7 (58%)	80 (100%)	91
Nonsensitizer	27 (87%)	5 (42%)	0 (0%)	32
Total	31	12	80	123

1669   Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified
1670   by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.
1671   [1]Numbers shown reflect number of tests. Includes all tests of substances that were tested multiple times.
1672   Percentage in parentheses reflects percentage of the total number of tests for each SI category.
1673

1674   The 123 tests evaluated in **Table 7-9** include multiple tests for 14 substances. For the 14

1675   substances, three to 18 tests were available. **Table 7-10** shows the proportion of the tests for

1676   each substance that produced SI values in each category. For the four nonsensitizers with

1677   multiple test results, there were 22 tests that produced SI ≤ 1.7 and two tests that produced an

1678   SI of between 1.7 and 2.5. For the 10 sensitizers with multiple test results, however, SI

1679   values occurred in all three SI categories. The results for nickel (II) sulfate hexahydrate were

1680   particularly variable: 50% (4/8) produced SI ≤ 1.7 (i.e., four tests with SI = 0.79, 1.24, 1.52,

1681   and 1.56), 25% (2/8) produced $1.7 < SI < 2.5$ (SI = 2.13 and 2.17), and 25% (2/8) produced

1682 $SI \geq 2.5$ (SI = 3.49 and 11.78). 3-Aminophenol produced SI values in two categories: 67%

1683   (2/3) of the tests had $1.7 < SI < 2.5$ (SI = 1.76 and 2.38), and 33% (1/3) of the tests had $SI \geq$

1684   2.5 (SI = 2.83). Cobalt chloride tests also produced SI values in two categories: 12.5% (1/8)

1685   of the tests had $1.7 < SI < 2.5$ (SI = 2.01) and seven of eight tests (i.e., 87.5%) produced $SI \geq$

1686   2.5 (SI = 2.54, 2.66, 3.64, 4.25, 5.06, 8.07, and 20.55). The multiple test results for the

1687   remaining seven traditional LLNA sensitizers were 100% concordant (**Table 7-10**).

1688   **Table 7-10   Concordance of LLNA: DA Tests for Substances with Multiple Tests by**
1689   **Maximum SI Category**

Substance	Concordance Among Multiple Tests[1]			Total
	Maximum SI ≤ 1.7	1.7 < Maximum SI < 2.5	Maximum SI ≥ 2.5	
Sensitizers[2]				
Abietic acid	0 (0%)	0 (0%)	4 (100%)	4
3-Aminophenol	0 (0%)	2 (67%)	1 (33%)	3
Cobalt chloride	0 (0%)	1 (12.5%)	7 (87.5%)	8
2,4-Dinitrochlorobenzene	0 (0%)	0 (0%)	11 (100%)	11
Formaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Glutaraldehyde	0 (0%)	0 (0%)	4 (100%)	4
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	18 (100%)	18
Isoeugenol	0 (0%)	0 (0%)	4 (100%)	4
Nickel (II) sulfate hexahydrate	4 (50%)	2 (25%)	2 (25%)	8
Potassium dichromate	0 (0%)	0 (0%)	5 (100%)	5
Nonsensitizers[2]				
Dimethyl isophthalate	4 (100%)	0 (0%)	0 (0%)	4
Isopropanol	10 (91%)	1 (9%)	0 (0%)	11
Lactic acid	5 (100%)	0 (0%)	0 (0%)	5
Methyl salicylate	3 (75%)	1 (25%)	0 (0%)	4

1690   Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified
1691   by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.
1692   [1]Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of
1693   tests for each substance.
1694   [2]According to traditional LLNA results.
1695

1696

1696 **8.0   LLNA: DA Data Quality**

1697 The data quality section in this revised draft BRD has been updated from the January 2008

1698 draft BRD to indicate that all of the studies included in this performance evaluation are based

1699 on individual animal data submitted to NICEATM in the form of original data and study

1700 records. Furthermore, since the January 2008 draft BRD was made available, manuscripts

1701 detailing the results for 31 substances evaluated in the intralaboratory study and 14

1702 substances evaluated in the two-phased interlaboratory validation have been published in the

1703 peer-reviewed literature (Idehara et al. 2008; Omori et al. 2008). Also, an independent audit

1704 has been conducted to confirm that the reported data from the intralaboratory validation

1705 study (i.e., assessment of 31 substances from Idehara et al. 2008) performed by Daicel

1706 Chemical Industries, Ltd. was the same as the data originally recorded (Idehara et al. 2008).

1707 The data from the two-phased interlaboratory validation study were not subjected to a formal

1708 audit, but the raw data were reportedly entered directly into formatted MS-Excel templates

1709 provided by the study management team prior to being used for analyses (Omori et al. 2007).

1710 In addition, data recently received for 14 substances evaluated in an intralaboratory

1711 validation study (Idehara, unpublished) were also not subjected to a formal audit. The

1712 intralaboratory assessment at Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara,

1713 unpublished), as well as the two-phased interlaboratory validation study (Omori et al. 2008),

1714 did not conduct their studies in compliance with Good Laboratory Practice guidelines,

1715 although all of the participating laboratories reportedly have this capability.

1716

1716   ## 9.0.   Other Scientific Reports and Reviews

1717   This section has been updated to include information on the intralaboratory validation study

1718   and the two-phased interlaboratory validation based on publication of the data since the

1719   January 2008 draft BRD. In addition, information is included on the regulatory acceptance of

1720   the LLNA: DA test method by the Japanese Center for the Validation of Alternative Methods

1721   (JaCVAM).

1722   Yamashita et al. (2005) describe the development of the LLNA: DA as an alternative non-

1723   radioisotope LLNA test method. The manuscript details the determination of an optimal

1724   dosing schedule and further compares SI values obtained from lymph node weights versus

1725   ATP content to determine an appropriate lymphocyte proliferation endpoint. The authors

1726   further assessed the intermediate precision and sensitivity/specificity of the LLNA: DA. In

1727   these experiments, four compounds (2,4-dinitrochlorbenzene, eugenol, α-hexyl cinnamic

1728   aldehyde, and methyl salicylate) were tested and no significant differences were noted in the

1729   SI levels generated from the LLNA: DA and the traditional LLNA. This study provided the

1730   basis for the expanded intralaboratory study of 31 substances analyzed by Daicel Chemical

1731   Industries, Ltd. (described in **Sections 6.0** and **7.0**) for which the data were published by

1732   Idehara et al. (2008).

1733   Idehara et al. (2008) summarize the LLNA: DA test method in terms of test substance dosing

1734   schedule, preparation of single cell suspensions of the auricular lymph nodes, measurement

1735   of ATP content, and explanation of statistical analyses employed. The authors further

1736   describe how the results correlate between ATP content and lymph node cell number, the test

1737   results (i.e., mean SI values and EC3) obtained for the 31 substances, the concordance of the

1738   LLNA: DA versus the traditional LLNA EC3, and the reproducibility of EC3 and SI values.

1739   Based on the details included in the manuscript, the authors conclude that the SI values

1740   obtained from measuring ATP content were similar to the traditional LLNA and therefore the

1741   LLNA: DA was a promising non-radioisotope modified test method for evaluating the skin

1742   sensitization potential of substances.

1743   Omori et al. (2008) describe the two-phased interlaboratory validation study used to evaluate

1744   the reliability and relevance of the LLNA: DA test method (see **Section 7.0**). They describe

1745   the organization and technology transfer of the test method between the laboratories, as well

1746   as test substance selection and allocation. They further describe the development of the

1747   LLNA: DA and the resulting standard protocol for the LLNA: DA interlaboratory study. The

1748   provide the interlaboratory data for analyzing both ATP content with regard to SI values and

1749   lymph node weight and discuss assay sensitivity and interlaboratory variability. Based on the

1750   data summarized in the manuscript, the authors conclude that in the first phase of the

1751   interlaboratory validation study, a large variation was observed for two substances (i.e.,

1752   cobalt chloride and nickel [II] sulfate hexahydrate) but in the second phase of the

1753   interlaboratory validation study this variation was small. The authors attributed the initial

1754   variation to application of DMSO as the solvent for the metallic salts and therefore, prior to

1755   the second phase of the interlaboratory validation study, included operation of LLNA: DA

1756   with DMSO in the technology transfer seminar. In conclusion, the authors view the LLNA:

1757   DA as a reliable test method for predicting skin sensitization potential of substances.

1758   Regarding the LLNA: DA test method, non-commission members of JaCVAM met on

1759   August 28, 2008 at the National Institute of Health Sciences, Tokyo, Japan, and endorsed the

1760   following statement: "Following the review of the results of the Ministry of Health, Labour

1761   and Welfare (MHLW)-funded validation study on the LLNA: DA coordinated by Japanese

1762   Society for Alternative to Animal Experiments, it is concluded that the LLNA: DA can be

1763   used for distinguishing between sensitizer and nonsensitizer chemicals within the context of

1764   the OECD testing guidelines No. 429 on skin sensitization: LLNA. The JaCVAM regulatory

1765   acceptance board has been regularly kept informed of the progress of the study, and this

1766   endorsement was based on an assessment of various documents, including, in particular, the

1767   report on the results from the study, and also on the evaluation supported by MHLW of the

1768   study prepared for the JaCVAM ad hoc peer review panel." JaCVAM has informed

1769   NICEATM-ICCVAM that in January 2009 they will submit the SPSF for recommendation of

1770   the LLNA: DA from the Japanese National Coordinator to OECD secretary. They will make

1771   clear that the SPSF was produced in collaboration with NICEATM-ICCVAM.

1772

1772   **10.0   Animal Welfare Considerations**

1773   This section of the draft BRD has not changed from the January 2008 draft BRD. The

1774   LLNA: DA will require the use of the same number of animals when compared to the

1775   updated ICCVAM LLNA protocol (Appendix A of ICCVAM 2009). However, since the

1776   traditional LLNA uses radioactive materials and as such its use might be restricted due to the

1777   complications associated with storage, use, and disposal, broader use of a non-radioactive

1778   alternative to the traditional LLNA, such as the LLNA: DA, could further reduce the number

1779   of guinea pigs that are used to assess skin sensitization.

1780   **10.1   Rationale for the Need to Use Animals**

1781   The rationale for the use of animals in the LLNA: DA is the same as the rationale for the

1782   traditional LLNA. There currently are no valid and accepted non-animal test methods to

1783   determine the ACD potential of substances and products, except for situations where human

1784   studies could be conducted ethically and where such studies would meet regulatory safety

1785   assessment requirements. Additionally, the most detailed information about the induction and

1786   regulation of immunological responses are available for mice (ICCVAM 1999).

1787   **10.2   Basis for Determining the Number of Animals Used**

1788   The number of animals used for the experimental, vehicle, and positive control groups is

1789   based on the number of animals specified in the updated ICCVAM LLNA protocol

1790   (Appendix A of ICCVAM 2009).

1791   **10.3   Reduction considerations**

1792   A further reduction of 40% (15 vs. 25) could be achieved by using a reduced version of the

1793   LLNA: DA, in cases where dose response information is not needed for hazard identification

1794   purposes. In such an approach, only the highest soluble dose of the test article that does not

1795   elicit toxicity would be administered, and the two lower dose groups would not be used.

1796   Additional reductions could be achieved by testing more substances concurrently, so that the

1797   same vehicle and positive control group could be used for multiple substances.

1798

1798 **11.0   Practical Considerations**

1799   This section of the draft BRD has not changed from the January 2008 draft BRD. Several

1800   issues are taken into account when assessing the practicality of using an alternative to an

1801   existing test method. In addition to performance evaluations, assessments of the laboratory

1802   equipment and supplies needed to conduct the alternative test method, level of personnel

1803   training, labor costs, and the time required to complete the test method relative to the existing

1804   test method are necessary. The time, personnel cost, and effort required to conduct the

1805   proposed test method(s) must be considered to be reasonable when compared to the existing

1806   test method it is intended to replace.

1807   **11.1   Transferability of the LLNA: DA**

1808   Test method transferability addresses the ability of a method to be accurately and reliably

1809   performed by multiple laboratories (ICCVAM 2003), including those experienced in the

1810   particular type of procedure as well as laboratories with less or no experience in the

1811   particular procedure. It would be expected that the transferability of the LLNA: DA would be

1812   similar to the traditional LLNA, since their test method protocols are experimentally similar.

1813   Notably, the test method developer does indicate that when the LLNA: DA test method is

1814   conducted, all the procedural steps from lymph node excision to the determination of ATP

1815   content should be performed without delay since ATP content decreases over time (Idehara

1816   et al. 2008; Omori et al. 2008).

1817   **11.2   Laboratories and Major Fixed Equipment Required to Conduct the LLNA:**
1818   **DA**

1819   Compared to the traditional LLNA, the LLNA: DA will not require laboratories, equipment,

1820   and licensing permits for handling radioactive materials. However, the LLNA: DA does

1821   require access to a luminometer capable of detecting light emission by ATP for the

1822   assessment of lymphocyte proliferation. The remaining requirements (e.g., animal care

1823   laboratories) are the same between the two methods.

1824

1824   **11.3   LLNA: DA Training Considerations**

1825   The level of training and expertise needed to conduct the LLNA: DA should be similar to the

1826   traditional LLNA, although the LLNA: DA includes an additional requirement that users

1827   operate a luminometer instead of a scintillation counter and be able process this data.

1828

1828 **12.0   References**

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1861   and butyl glycolates, and lactic acid, ammonium, calcium, potassium, sodium, and tea-

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