Nonradioactive Murine Local Lymph Node Assay: Flow Cytometry Test Method Protocol (LLNA: BrdU-FC)

Revised Draft Background Review Document

March 2009

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List of Abbreviations and Acronyms

71	ACD	Allergic contact dermatitis
72	ACE	Acetone
73	AOO	Acetone: olive oil
74	BRD	Background Review Document
75	BrdU	Bromodeoxyuridine
76	BT	Buehler Test
77	CASRN	Chemical Abstracts Service Registry Number
78	Conc.	Concentration tested
79	CPSC	U.S. Consumer Product Safety Commission
80	DMF	Dimethylformamide
81	DMSO	Dimethyl sulfoxide
82	EC3	Estimated concentration needed to produce a stimulation index of three
83	ECVAM	European Centre for the Validation of Alternative Methods
84	eLLNA: BrdU-FC	Enhanced LLNA: BrdU-FC
85	EPA	U.S. Environmental Protection Agency
86	ESAC	ECVAM Scientific Advisory Committee
87	FDA	U.S. Food and Drug Administration
88	*FR*	*Federal Register*
89 90	GHS	United Nations Globally Harmonized System for the Labelling and Classification of Chemicals
91	GLP	Good Laboratory Practice
92	GP	Guinea pig
93	GPMT	Guinea Pig Maximization Test
94	HCA	Hexyl cinnamic aldehyde
95	HMT	Human Maximization Test
96	HPTA	Human Patch Test Allergen
97 98	ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
99	IR	Information requested
100	ISO	International Standards Organization
101	IWG	Immunotoxicity Working Group
102	JaCVAM	Japanese Center for the Validation of Alternative Methods
103	K_{ow}	Octanol-water partition coefficient
104	LNC	Lymph node cells
105	LLNA	Local Lymph Node Assay

106	LLNA: BrdU-FC	LLNA with detection of bromodeoxyuridine incorporation by flow
107		cytometry
108	MEK	Methyl ethyl ketone
109	MeSH	Medical Subject Headings
110	Min	Minimal
111	Mod	Moderate
112	MW	Molecular weight
113	NA	Not available
114	NC	Not calculated
115	NICEATM	National Toxicology Program Interagency Center for the Evaluation of
116		Alternative Toxicological Methods
117	NIEHS	National Institute of Environmental Health Sciences
118	NT	Not tested
119	NTP	National Toxicology Program
120	OECD	Organisation for Economic Co-operation and Development
121	OPPTS	Office of Prevention, Pesticides and Toxic Substances
122	Res	Result
123	SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
124	S.D.	Standard Deviation
125	SI	Stimulation Index
126	SLS	Sodium lauryl sulfate
127	TG	Test Guideline
128	U.S.	United States
129	Unk	Unknown
130	Veh.	Vehicle
131	vs.	Versus
132	w/v	Weight to volume ratio

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Preface

219

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

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

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

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

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

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

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

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

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

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

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

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

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

233   Toxicology Program Interagency Center for the Evaluation of Alternative Methods

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

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

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

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

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

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

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

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

242   NICEATM and ICCVAM provided the draft BRD and draft recommendations to an

243   international independent scientific peer review panel for their consideration at a public

244   meeting on March 4-6, 2008. A report of the Panel meeting was subsequently published on

245   the NICEATM-ICCVAM website[2]. Both the Panel and ICCVAM concluded that more

246   information was needed before a recommendation on the usefulness and limitations of each

247   of the three test methods could be made. The Panel recommended that NICEATM obtain

248   additional existing data that was not available to the Panel and reanalyze the performance of

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[2] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel08.htm

249   each non-radioactive LLNA method. NICEATM subsequently obtained additional data and

250   prepared updated BRDs. ICCVAM also prepared revised draft test method recommendations

251   based on the revised BRDs. This revised draft BRD addresses the validation database for the

252   LLNA: BrdU-FC.

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

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

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

256   comments received from the public and the Scientific Advisory Committee for Alternative

257   Toxicological Methods, and then finalize the BRDs and test method recommendations. These

258   will then be forwarded to Federal agencies for their consideration and acceptance decisions

259   where appropriate.

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

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

262   contributing to the preparation of this BRD, including the following staff from the

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281   **Executive Summary**

282   **Background**

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

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

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

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

287   skin reaction characterized by redness, swelling, and itching that can result from contact with a

288   sensitizing chemical or product. The recommendation was based on a comprehensive evaluation

289   that included an independent scientific peer review panel (Panel) assessment of the validation

290   status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are

291   available at the National Toxicology Program Interagency Center for the Evaluation of

292   Alternative Toxicological Methods (NICEATM)-ICCVAM website

293   (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf). The LLNA was

294   subsequently incorporated into national and international test guidelines for the assessment of

295   skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test

296   Guideline 429 [OECD 2002]; International Organization for Standardization [ISO] 10993-10:

297   Tests for Irritation and Sensitization [ISO 2002]; U.S. Environmental Protection Agency [EPA]

298   Health Effect Testing Guidelines on Skin Sensitization [EPA 2003]).

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

300   activities related to the LLNA for evaluation by ICCVAM and NICEATM (Available at

301   http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). One of the

302   nominated activities was an assessment of the validation status of non-radioactive alternatives to

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

304   "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in

305   the original and this revised background review document (BRD) was compiled by ICCVAM

306   and NICEATM in response to this nomination. The BRD provides a comprehensive review of

307   available data and information regarding the usefulness and limitations of one of these methods,

308   the LLNA with detection of bromodeoxyuridine (BrdU) incorporation by flow cytometry

309   (referred to hereafter as the LLNA: BrdU-FC).

310   **Revisions to the LLNA: BrdU-FC Evaluation**

311   NICEATM and ICCVAM convened an independent international scientific peer review panel

312   meeting on March 4-6, 2008. The Panel reviewed the draft BRD and commented on the extent to

313   which it supports the draft ICCVAM test method recommendations on the usefulness and

314   limitations of the LLNA: BrdU-FC. Both ICCVAM and the Panel concluded that they needed
315   more information before they could make a recommendation on the usefulness and limitations of
316   the LLNA: BrdU-FC.[3] The Panel requested individual animal data and evaluations of both intra-
317   and interlaboratory reproducibility. The Panel recommended that NICEATM obtain additional
318   data and reanalyze the performance of the LLNA: BrdU-FC method. In response, NICEATM
319   obtained additional LLNA: BrdU-FC data, which were used to update the evaluation as
320   described below. These data include:

321   • LLNA: BrdU-FC data from multiple studies with 2-mercaptobenzothiazole (MBT)
322   using different vehicles. These data were submitted in a response to a request for an
323   explanation for the discordant results for MBT. The new data indicate a vehicle
324   dependent response in the LLNA: BrdU-FC for identifying a positive result with
325   MBT. Results of the retests of MBT demonstrated positive results when tested in
326   dimethyl sulfoxide (DMSO) or dimethylformamide (DMF), but MBT gave negative
327   results in DaAE (DMSO: acetone: ethanol; 4:3:3). Revisions for the new data are
328   detailed in **Section 5.0** and **Appendix D**.

329   • Data from studies for sodium lauryl sulfate (SLS) using an enhanced LLNA: BrdU-
330   FC protocol (eLLNA: BrdU-FC). The eLLNA: BrdU-FC includes an assessment of
331   immunophenotypic markers to distinguish sensitizers from irritants, reportedly to
332   reduce the incidence of false positive results. SLS was used as a positive control in
333   DMSO tests; 2/5 animals exhibited ear swelling >25%, indicating that SLS induced
334   an irritation response. These new data are described in **Sections 5.0** and **6.0** with
335   details in **Appendix D**.

336   • New EC3 results were obtained from four tests each in LLNA: BrdU-FC for hexyl
337   cinnamic aldehyde (HCA) and 2,4-dinitrochlorobenzene (DNCB). These new data
338   demonstrated intralaboratory reproducibility within the range of acceptability for both
339   substances as described in the ICCVAM LLNA Performance Standards. These data
340   are detailed in **Section 7.0** and **Appendix D**.

341   **Test Method Protocol**
342   The protocol in this draft BRD has not been revised from the January 2008 draft BRD. The
343   LLNA: BrdU-FC was developed by MB Research Labs (2001). The traditional LLNA assesses
344   cell proliferation by measuring the incorporation of radioactivity into the deoxyribonucleic acid
345   (DNA) of dividing lymph node cells. In contrastLLNA: BrdU-FC uses flow cytometry to assess
346   cell proliferation by measuring the incorporation of the thymidine analog BrdU into the DNA of
347   dividing lymphocytes. A stimulation index (SI) is the ratio of the mean BrdU incorporation into

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

348   the lymph nodes of mice in the test substance group to the mean BrdU incorporation into the

349   lymph nodes of mice in the vehicle group. An SI value greater than or equal to three identifies a

350   substance as a sensitizer. Other than the procedure for measuring lymph node cell proliferation,

351   the protocol for the LLNA: BrdU-FC is similar to that of the traditional LLNA (Dean et al. 2001;

352   ICCVAM 1999). As noted above, the eLLNA: BrdU-FC includes enhancements for substances

353   with SI ≥ 3 that include an assessment of immunophenotypic markers to distinguish sensitizers

354   from irritants.

355   **Test Method Accuracy**

356   The accuracy evaluation in this draft BRD has been revised from the January 2008 draft BRD to

357   reduce the number of equivocal substances based on new data for MBT, and to include revisions

358   to the reference data for the traditional LLNA and human data. The accuracy of the LLNA:

359   BrdU-FC and the eLLNA: BrdU-FC was assessed using data submitted by MB Research Labs

360   (2007) for up to 45 substances. Of these 45 substances, 37 had LLNA: BrdU-FC, traditional

361   LLNA, and guinea pig data. Forty-two substances had LLNA: BrdU-FC, traditional LLNA, and

362   human data. Two of the 45 substances (equivocal substances) produced divergent results when

363   tested at least twice in the traditional LLNA and/or in the LLNA: BrdU-FC. To account for the

364   equivocal substances, two separate accuracy analyses were conducted. In one, only the

365   substances with unequivocal LLNA: BrdU-FC results were evaluated; in the other, the two

366   equivocal substances were included by using the more conservative result (i.e., by using the

367   positive responses) for both substances.

368   When the LLNA: BrdU-FC was compared to the traditional LLNA (excluding the two equivocal

369   substances), the LLNA: BrdU-FC had an accuracy of 95% (41/43), a false positive rate of 7%

370   (1/15), and a false negative rate of 4% (1/28).[4] Including the two equivocal substances resulted in

371   an accuracy for the LLNA: BrdU-FC of 93% (42/45), a false positive rate of 13% (2/16), and a

372   false negative rate of 3% (1/29).[4]

373   When the eLLNA: BrdU-FC was compared to the traditional LLNA, accuracy was 88% (38/43),

374   the false positive rate was 7% (1/15), and false negative rate was 14% (4/28). Using the

375   traditional LLNA as the reference classification, two nonsensitizers and two sensitizers were

376   identified incorrectly. However, the two substances identified by the eLLNA: BrdU-FC as

377   nonsensitizers (ethylene glycol dimethacrylate and sodium lauryl sulfate) were identified as

378   nonsensitizers by guinea pig skin sensitization tests also. SLS is also considered a nonsensitizer

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[4] The one false negative substance is aniline, which did not generate a strongly positive result in the
    traditional LLNA (EC3 = 48%, maximum SI = 3.6 at 50% in acetone: olive oil).

379   based on human data (i.e., human maximization test), but ethylene glycol dimethacrylate is
380   considered a sensitizer based on its inclusion as a human patch test kit allergen. Including the
381   two equivocal substances resulted in an accuracy for the eLLNA: BrdU-FC of 87% (39/45), a
382   false positive rate of 13% (2/16), and a false negative rate of 14% (4/29).

Test Method Reliability – Intralaboratory Reproducibility

384   The intralaboratory reproducibility has been revised to include new data for HCA and DCNB
385   that were not available for evaluation in the January 2008 draft BRD. Intralaboratory
386   reproducibility for the LLNA: BrdU-FC and the eLLNA: BrdU-FC outcomes were assessed with
387   a coefficient of variation (CV) analysis of SI values. For the SI values of 25% HCA, the positive
388   control substance, tested in various vehicles, the CVs ranged from 30.1% to 52.6%. EC3 results
389   were obtained from four tests each in LLNA: BrdU-FC for HCA and DNCB. These data
390   demonstrated intralaboratory reproducibility within the range of acceptability for both substances
391   as described in the ICCVAM LLNA Performance Standards.

Test Method Reliability – Interlaboratory Reproducibility

393   Nothing has been added to the interlaboratory reproducibility section since the January 2008
394   draft BRD. Interlaboratory reproducibility for the LLNA: BrdU-FC and the eLLNA: BrdU-FC
395   could not be addressed because data were only available from one laboratory.

Animal Welfare Considerations

397   The animal welfare considerations in this draft BRD have not changed from the January 2008
398   draft BRD. The LLNA: BrdU-FC and the eLLNA: BrdU-FC will use the same number of
399   animals as the traditional LLNA. However, because the traditional LLNA cannot be conducted
400   in some institutions because it involves radioactivity, availability and use of the nonradioactive
401   LLNA: BrdU-FC and the eLLNA: BrdU-FC test methods may further reduce use of the guinea
402   pig test methods. Such a reduction could reduce animal use and increase refinement as pain and
403   distress are avoided in the LLNA procedure.

Test Method Transferability

405   The test method transferability considerations in this draft BRD have not changed from the
406   January 2008 draft BRD. The transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC
407   is expected to be similar to that of the traditional LLNA. Unlike the traditional LLNA, the
408   LLNA: BrdU-FC and the eLLNA: BrdU-FC will not require facilities, equipment, and licensing
409   permits for handling radioactive materials. The level of training and expertise needed to conduct
410   the LLNA: BrdU-FC and the eLLNA: BrdU-FC should be similar to that needed for the

411 traditional LLNA except that proficiency in flow cytometry is required for the nonradioactive
412 test methods.

413 **ICCVAM Revised Draft Recommendations**
414 ICCVAM has developed draft recommendations for the LLNA: BrdU-FC with regard to its
415 usefulness and limitations, test method protocol, and future studies to further characterize its
416 usefulness and limitations. These recommendations appear in a separate document, *Draft*
417 *ICCVAM Test Method Recommendations, Non-radioactive Murine Local Lymph Node Assay:*
418 *Flow Cytometry Test Method Protocol (LLNA: BrdU-FC).*

1.0   Introduction

1.1   Public Health Perspective

Allergic contact dermatitis (ACD) is a frequent occupational health problem. According to the U.S. Department of Labor Bureau of Labor Statistics, ACD resulted in 980 lost workdays in 2005.[5]

ACD develops in two phases, induction and elicitation. The induction phase occurs when a susceptible individual is exposed topically to a skin-sensitizing substance. During induction, the substance passes through the epidermis, where it forms a hapten complex with dermal proteins. The Langerhans cells, the resident antigen-presenting cells in the skin, process the hapten complex. The processed hapten complex then migrates to the draining lymph nodes. Antigen presentation to T-lymphocytes follows, which leads to the clonal expansion of these cells. At this point, the individual is sensitized to the substance (Basketter et al. 2003; Jowsey et al. 2006). Studies have shown that the magnitude of lymphocyte proliferation correlates with the extent to which sensitization develops (Kimber and Dearman 1991, 1996).

The elicitation phase occurs when the individual is topically exposed to the same substance again. As in the induction phase, the substance penetrates the epidermis, is processed by the Langerhans cells, and is then presented to circulating T-lymphocytes. The T-lymphocytes are then activated, which causes release of cytokines and other inflammatory mediators. This release produces a rapid dermal immune response that can lead to ACD (ICCVAM 1999; Basketter et al. 2003; Jowsey et al. 2006).

1.2   Historical Background for the Murine Local Lymph Node Assay (LLNA)

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that LLNA is a valid substitute for currently accepted guinea pig (GP) test methods to assess the ACD potential of many, but not all, types of substances. ICCVAM based its recommendation on a comprehensive evaluation that included an assessment of the validation status of the LLNA by an independent scientific peer review panel (Panel). The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)/ICCVAM website (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf).

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[5] Available at http://www.bls.gov/IIF

449   ICCVAM recommended that the LLNA be considered for regulatory acceptance or other
450   nonregulatory applications for assessing the ACD potential of substances, while
451   acknowledging that some testing situations would still require the use of traditional GP test
452   methods (ICCVAM 1999, Sailstad et al. 2001). The LLNA was subsequently incorporated
453   into national and international test guidelines for the assessment of skin sensitization
454   (Organisation for Economic Co-operation and Development [OECD] Test Guideline 429
455   [OECD 2002]; International Standards Organization [ISO] 10993-10: Tests for Irritation and
456   Sensitization [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effect
457   Testing Guidelines on Skin Sensitization [EPA 2003]).

458   On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally
459   nominated for evaluation by ICCVAM and NICEATM several activities related to the LLNA
460   (Available at
461   http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). The
462   requested activities included an assessment of the validation status of nonradioactive
463   alternatives to the current version of the LLNA (traditional LLNA) (ICCVAM 1999, Dean et
464   al. 2001), which uses radioactivity to detect sensitizers. ICCVAM and NICEATM compiled
465   the information in this background review document (BRD) in response to this nomination.
466   The BRD provides a comprehensive review of available data and information regarding the
467   usefulness and limitations of one of these methods, the LLNA with detection of
468   bromodeoxyuridine (BrdU) (LLNA: BrdU-FC). ICCVAM and its Immunotoxicity Working
469   Group (IWG) evaluated this method in draft test method recommendations based on the BRD
470   evaluation. An independent international scientific peer review panel (Panel) reviewed the
471   BRD in March 2008 to evaluate the extent to which the information contained in the BRD
472   supported the draft recommendations. The Panel concluded that additional information was
473   needed to evaluate the method, including original animal data, quantitative data for the
474   method, and an evaluation of interlaboratory reproducibility. NICEATM gathered the
475   additional information and produced this revised draft BRD for review by the Panel.

476   ICCVAM will consider the conclusions and recommendations of the Panel, along with
477   comments received from the public and SACATM, when developing the final BRD and final
478   recommendations on the usefulness and limitations of each nonradioactive alternative LLNA
479   test methods that is being considered.

480   **1.3   The LLNA: BrdU-FC**
481   The LLNA: BrdU-FC was developed by MB Research Labs (2001) as a nonradioactive
482   alternative to the current version of the traditional LLNA. While the traditional LLNA

483   assesses cellular proliferation by measuring the incorporation of radioactivity into the

484   deoxyribonucleic acid (DNA) of dividing lymph node cells, the LLNA: BrdU-FC assesses

485   the same endpoint by measuring the incorporation of the thymidine analog BrdU, which is

486   detected and quantified with a flow cytometer. Routine measurements of ear swelling are

487   also included as a measure of excessive local irritation when evaluating results. Additional

488   endpoints (i.e., immunophenotypic markers such as B220 and CD69) are incorporated in an

489   enhanced LLNA: BrdU-FC protocol (eLLNA: BrdU-FC) to further distinguish irritants from

490   sensitizers.

491   This document provides:

492   • A comprehensive summary of the LLNA: BrdU-FC test method protocol

493   • Identification of the substances used in the validation of the test method and the
494   test results

495   • The performance characteristics (accuracy and reliability) of the test method

496   • Animal welfare considerations

497   • Other considerations relevant to the usefulness and limitations of this test method
498   (e.g., transferability and cost of the test method)

499   ## 2.0   LLNA: BrdU-FC Test Method Protocol

500   The protocol in this draft BRD has not been revised from the January 2008 draft BRD. The
501   LLNA: BrdU-FC protocol (see **Figure 2-1** and **Appendix A**) follows the ICCVAM-
502   recommended protocol for the traditional LLNA (ICCVAM 1999; Dean et al. 2001) with the
503   exception of the method used to assess lymphocyte proliferation. To evaluate excessive skin
504   irritation when determining the highest dose level, as is recommended in the ICCVAM
505   LLNA protocol, the LLNA: BrdU-FC includes a quantitative assessment of potential dermal
506   irritation by measuring ear thickness with a digital micrometer at three separate timepoints
507   (once each on Days 1 [prior to dosing], 3, and 6).

508   In the traditional LLNA, the test substance is administered on three consecutive days. Forty-
509   eight hours after the final application of the test substance, ^{3}H-methyl thymidine or ^{125}I-
510   fluorodeoxyuridine (in phosphate-buffered saline; 250 µL/mouse) is injected into the tail
511   vein. This same dosing schedule is followed in the LLNA: BrdU-FC, but 200 µL per mouse
512   of BrdU is administered intraperitoneally rather than intravenously (see **Appendix A** for the
513   rationale for the route of administration and amount of BrdU). Five hours after BrdU
514   administration, lymph nodes are excised and processed. Measurement of the total number of
515   lymphocytes and the total number of cells with incorporated BrdU in the lymph node
516   preparation is described in **Appendix A**.

517   **Figure 2-1 Strategy for Using the LLNA: BrdU-FC to Detect Irritants vs. Sensitizers**
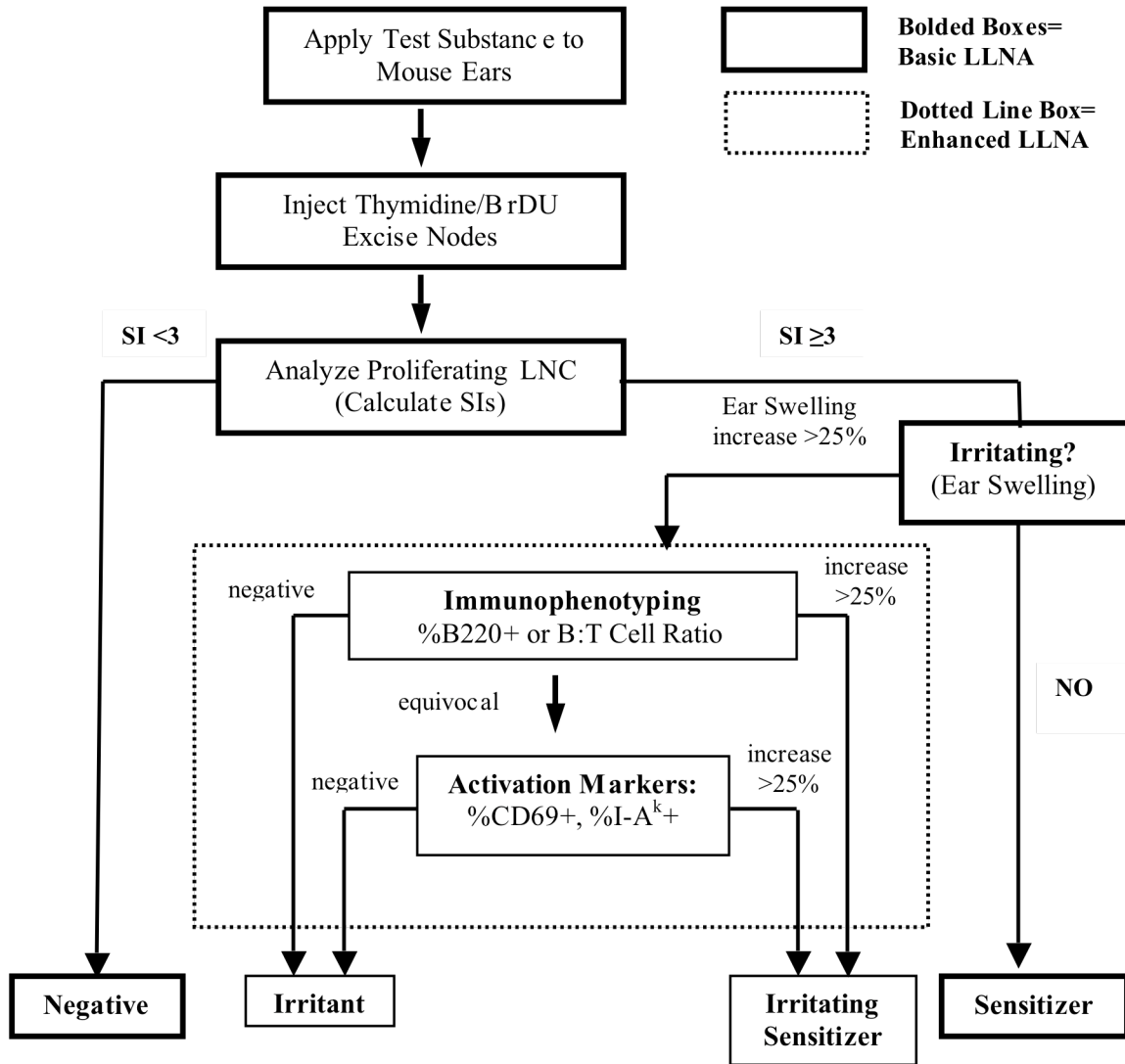
518

519   Abbreviations: B = B lymphocyte; BrdU = bromodeoxyuridine; LLNA = murine local lymph node assay;
520   LNC = lymph node cells; SI = stimulation index; T = T lymphocyte
521   The shaded box shows that the enhancements of immunophenotyping and measurement of activation markers
522   are used when SI ≥ 3 and mouse ear swelling ≥ 25% (i.e., the enhanced LLNA: BrdU-FC protocol [eLLNA:
523   BrdU-FC]).
524

524   As mentioned above, the eLLNA: BrdU-FC incorporates immunophenotypic endpoints,
525   which are evaluated sequentially using the criteria described in **Section 2.1**, to distinguish
526   irritants from dermal sensitizers when a stimulation index (SI) ≥ 3 is recorded. For mice
527   exhibiting ear swelling >25%, the first-tier endpoints include determination of the percentage
528   of B lymphocytes (B220+) or the B lymphocyte to T lymphocyte ratio (B:T cell ratio) in the
529   isolated lymph node cells of the treated mice. B220 is an isoform of a transmembrane protein
530   expressed on B lymphocytes that assists in the activation of the cells. Allergen-treated mice
531   have shown a preferential increase in the percentage of B220+ cells compared with irritant-
532   treated mice (Gerberick et al. 2002). An increase of more than 25% for B220+ cells or a B:T
533   cell ratio greater than 1.25 indicates that a substance is an irritating sensitizer. If the
534   percentage of B220+ cells or the B:T cell ratio increases by less than 25%, then the substance
535   is classified as an irritant. However, a second tier of immunophenotypic measurements can
536   be used to reconcile outcomes in which the B220+ cells or the B:T cell ratio produce a
537   borderline response. In those instances, an increase of greater than 25% in IA^K+ cells (B-
538   lymphocytes) or CD69 (T-lymphocytes) indicates an irritating sensitizer.

539   NICEATM has requested but not obtained a detailed protocol from MB Research Labs to
540   describe the specific procedures used to quantify the immunophenotypic endpoints.

2.1   Decision Criteria

542   Like the traditional LLNA, the LLNA: BrdU-FC uses an SI value to distinguish skin
543   sensitizers from nonsensitizers. The SI in the LLNA: BrdU-FC is the ratio of the mean
544   number of lymph node cells with incorporated BrdU from mice in each of the test substance
545   dose groups to the mean number of lymph node cells with incorporated BrdU from mice in
546   the vehicle control group. The formula is:

547
$$SI = \frac{\text{Mean number of BrdU - labeled cells in the treatment group}}{\text{Mean number of BrdU - labeled cells in the vehicle control group}}$$

548   An SI ≥ 3 is the threshold for labeling a substance as a sensitizer. This same SI threshold is
549   used in the traditional LLNA.

550   The eLLNA: BrdU-FC allows further evaluation of substances that produce SI values ≥3   in
551   order to distinguish between sensitizers and irritants. As detailed in **Figure 2-1**, if mouse ear
552   swelling exceeds 25% for substances with an SI ≥ 3, then an evaluation of the first set of
553   immunophenotypic markers is conducted (i.e., percentage of B220+ cells or the calculation
554   of the B:T cell ratio). If the percentage of B220+ cells increases less that 25% above control
555   values or the B:T cell ratio is <1.25, then the substance is classified as an irritant. If the

556   percentage of B220+ cells increases more than 25% above control values or the B:T cell ratio
557   is >1.25, then the substance is classified as an irritating sensitizer. If the increase in the
558   percentage of B220+ cells or the B:T cell ratio is equivocal (i.e., at least one mouse has ear
559   swelling >25% and the percentage of B220+ cells or the B:T cell ratio is significantly
560   elevated or is greater than 25% above control values), then an evaluation of the second set of
561   immunophenotypic markers is conducted (i.e., percentage of either IA^K+ cells or CD69+
562   cells). If the percentage of IA^K+ cells or CD69+ cells is >25% above control values, then the
563   substance is classified as a sensitizer. If the percentage of IA^K+ cells or CD69+ cells is <25%
564   above control values, then the substance is classified as an irritant.

565   **3.0   LLNA: BrdU-FC Validation Database**

566   To evaluate the performance of the LLNA: BrdU-FC and the eLLNA: BrdU-FC in
567   comparison to the traditional LLNA, MB Research Labs tested a total of 48 substances (MB
568   Research Labs 2007) (**Appendix B**). Traditional LLNA data were identified by NICEATM
569   for 45 of the 48 substances (**Table 3-1**). Traditional LLNA data were not identified for 4-
570   aminophenol HCl, chlorpromazine with ultraviolet radiation (chlorpromazine +UVR), and
571   croton oil; therefore, they are not included in this evaluation. Forty of the 45 substances
572   previously tested in the traditional LLNA were considered in the original evaluation of the
573   LLNA by ICCVAM (ICCVAM 1999). The traditional LLNA data for the five remaining
574   substances (cobalt chloride, diphenylcyclopropenone, fluorescein isothiocyanate, isopropyl
575   myristate, and linalool) were identified from Ryan et al. (2000), Basketter et al. (2006),
576   Gerberick et al. (2005), and Schneider and Akkan (2004). Of these 45 substances, 28 were
577   classified by the traditional LLNA as skin sensitizers and 17 were classified as
578   nonsensitizers. As shown by the EC3 values (i.e., calculated concentration that corresponds
579   to SI=3) in **Table 3-1**, the 28 sensitizers were representative of a full range of sensitization
580   responses (i.e., weak to strong sensitizers).

581   **Appendix B** provides information on the physicochemical properties (e.g., peptide reactivity,
582   octanol-water partition coefficient), Chemical Abstracts Service Registry Number, and
583   chemical class for each substance tested. When available, chemical class information was
584   retrieved from the National Library of Medicine's ChemIDplus[®] database. If chemical class
585   information was not located, they were assigned for each test substance using a standard
586   classification scheme, based on the National Library of Medicine Medical Subject Headings
587   (MeSH[®]) classification system (http://www.nlm.nih.gov/mesh/meshhome.html). A substance
588   could be assigned to more than one chemical class; however, no substance was assigned to
589   more than three classes. Chemical class information is presented only to provide an
590   indication of the variety of structural elements present in the structures that were evaluated in
591   this analysis. Classification of substances into chemical classes is not intended to represent
592   the impact of structure on biological activity with respect to sensitization potential. **Table 3-1**
593   shows that 23 chemical classes are represented by the 45 substances included in this
594   evaluation. Fifteen substances are classified in more than one chemical class. The classes
595   with the highest number of substances are carboxylic acids (12 substances) and amines
596   (seven substances).

597

598

599   **Table 3-1   Traditional LLNA EC3 Values and Chemical Classification of Substances**
600   **Tested in the LLNA: BrdU-FC (Sorted by EC3 Value)**

Substance Name	Chemical Class[1]	Traditional LLNA EC3[2]	No.[3]
Oxazalone	Heterocyclic compounds	0.003	5
Benzoyl peroxide	Carboxylic acids	0.01[5]	2
Tetrachlorosalicylanilide	Amides; Amines	0.04	1
2, 4-DNCB	Hydrocarbon, Halogenated; Nitro compounds; Hydrocarbons, Cyclic	0.05	15
Diphenylcyclopropenone	Hydrocarbons, Cyclic	0.05	1
Benzalkonium chloride	Onium compounds	0.10	1
4-Phenylenediamine	Amines	0.11	6
Potassium dichromate	Inorganic chemical, Chromium compounds, Potassium compounds	0.17	12
Copper chloride	Inorganic chemicals	0.4	1
Formaldehyde	Aldehydes	0.5	6
Cobalt chloride	Inorganic chemicals, Metals	0.6[3]	2
Isoeugenol	Carboxylic acids	1.5	47
2-Mercaptobenzothiazole	Heterocyclic compounds	1.7[6]	1
Ethylenediamine	Amines	2.2	1
Diethylenetriamine	Amines	3.3	1
Benzocaine	Carboxylic acids	3.4	1
Trimellitic anhydride	Anhydrides; Carboxylic acids	4.7	2
Resorcinol	Phenols	6.3	1
Sodium lauryl sulfate	Alcohols; Sulfur compounds; Lipids	8.1[6]	5
Citral	Hydrocarbons, Other	9.2	6
Hexyl cinnamic aldehyde	Aldehydes	9.7	21
Eugenol	Carboxylic acids	10	11
Ethylene glycol dimethacrylate	Carboxylic acids	28[7]	1
Linalool	Hydrocarbons	30	1
Isopropyl myristate	Lipids	44	1
Aniline	Amines	48	3
Pyridine	Heterocyclic compounds	72	1
Xylene	Hydrocarbons, Cyclic	96[5]	1
4-Aminobenzoic acid	Carboxylic acids	NA	NA
Benzoic acid	Carboxylic acids	NA	NA
Chlorobenzene	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA	NA
Glycerol	Alcohols; Carbohydrates	NA	NA
Hexane	Hydrocarbons, Acyclic	NA	NA
Hydrocortisone	Polycyclic compounds	NA	NA

Substance Name	Chemical Class[1]	Traditional LLNA EC3[2]	No.[3]
Isopropanol	Alcohols	NA	NA
Lactic acid	Carboxylic acids	NA	NA
6-Methylcoumarin	Heterocyclic compounds	NA	NA
Methyl salicylate	Phenols; Carboxylic acids	NA	NA
Nickel chloride	Inorganic chemicals	NA	NA
Propylene glycol	Alcohols	NA	NA
Propylparaben	Phenols; Carboxylic acids	NA	NA
Salicylic acid	Phenols; Carboxylic acids	NA	NA
Sulfanilimide	Amides; Sulfur compounds; Amines	NA	NA
Tween 80	Alcohols	NA	NA

601   Abbreviations: LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurement of
602   bromodeoxyuridine incorporation; EC3 = Estimated concentration needed to produce a stimulation index
603   (SI) = 3; NA = Not applicable, since maximum SI < 3
604   [1] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs,
605   developed by the National Library of Medicine (http://www.nlm.nih.gov/mesh/meshhome.html)
606   [2] Average EC3 values from the NICEATM LLNA database. All tests use acetone:olive oil (4:1) as the vehicle
607   unless otherwise noted.
608   [3] Number of traditional LLNA studies from which the EC3 data were obtained
609   [4] Vehicle= Dimethyl sulfoxide
610   [5] Vehicle = acetone/dibutyl phthalate (50:50)
611   [6] Vehicle not reported
612   [7] Vehicle = Dimethylformamide
613   [8] Vehicle = Methyl ethyl ketone
614

615

615 **4.0   Reference Data**

616 The reference data for the traditional LLNA used for the accuracy evaluation described in

617 **Section 6.0** were obtained from ICCVAM (1999), Ryan et al. (2000), Basketter et al. (1999,

618 2006), Gerberick et al. (2005), or Schneider and Akkan (2004). No traditional LLNA data were

619 identified for three substances: 4-aminophenol HCl, chlorpromazine +UVR, and croton oil;

620 therefore, they are not included in this evaluation. An independent quality assurance contractor

621 for the National Toxicology Program (NTP) audited the traditional LLNA data provided in

622 ICCVAM (1999). Audit procedures and findings are presented in the quality assurance report

623 on file at the National Institute of Environmental Health Sciences (NIEHS). The audit supports

624 the conclusion that the transcribed test data in the submission were accurate, consistent, and

625 complete as compared to the original study records. A similar audit of the traditional LLNA

626 data in Ryan et al. (200l), Schneider and Akkan (2004), Gerberick et al. (2005), and Basketter

627 et al. (2006) has not been possible, but copies of original data have been requested.

628 The reference data for the GP tests (Guinea Pig Maximization Test [GPMT] or Buehler Test

629 [BT]) and human tests (Human Maximization Test [HMT], Human Patch Test Allergen

630 [HPTA], or other human data) were obtained from Poole et al. (1970), Opdyke (1976a,

631 1976b), Gad et al. (1986), Gerberick et al. (1992, 2005), Kimber and Basketter (1997),

632 ICCVAM (1999), Rasanen et al. (1999), Basketter et al. (2000, 2003), Kwon et al. (2003),

633 and Schneider and Akkan (2004).

634 Neither GP nor human data could be located for four substances:

635 • croton oil

636 • chlorpromazine +UVR

637 • 4-aminophenol HCl

638 • fluorescein isothiocyanate

639 No GP data could be located for seven substances:

640 • diphenylcyclopropenone

641 • hexane

642 • hydrocortisone

643 • linalool

644 • pyridine

645 • xylene

646 • isopropyl myristate.

647 Additionally, no human data could be located for chlorobenzene or trimellitic anhydride.

648 **5.0   Test Method Data and Results**

649 Traditional LLNA data were identified by NICEATM for 45 of the 48 substances. Of these

650 45 substances, 37 had LLNA: BrdU-FC, traditional LLNA, and GP data. Forty-two

651 substances had LLNA: BrdU-FC, traditional LLNA, and human data. Two of the 45

652 substances produced discordant results when tested at least twice in the traditional LLNA

653 and/or in the LLNA (equivocal substances): BrdU-FC (i.e., benzocaine in both tests and

654 salicylic acid in the LLNA: BrdU-FC test). Data initially submitted for 2-

655 mercaptobenzothiazole (MBT) indicated that it produced equivocal results in the LLNA:

656 BrdU-FC, but results of retests that were subsequently provided to NICEATM demonstrated

657 this variability was likely due variations in the vehicle tested. MBT produced positive results

658 when tested in dimethyl sulfoxide (EC3 = 4.1% in DMSO; max SI = 8.0 at 25% MBT) or

659 when tested in dimethylformamide (EC3 = 22% in DMF; max SI = 3.3 at 25% MBT); MBT

660 (up to 25%) gave negative results in DaAE (DMSO: acetone: ethanol at a ratio of 4:3:3

661 parts; max SI = 1.3 at 10% MBT). Sodium lauryl sulfate (SLS) was used as a positive control

662 in DMSO tests (SI = 3.0–4.7 at 25% SLS; 2/5 animals exhibited ear swelling >25%,

663 indicating that SLS induced an irritation response).

664 All test results were obtained using the protocol in **Appendix A.** The LLNA: BrdU-FC

665 results for 48 substances are included in **Appendix C**. All substances were also evaluated in

666 the eLLNA: BrdU-FC protocol (only substances with SI ≥ 3 and mouse ear swelling ≥ 25%

667 were evaluated with the additional immunophenotypic markers included in the eLLNA: FC-

668 BrdU). In order to hide their identities during testing, test substances were not coded.

669   ## 6.0   Test Method Accuracy

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

671   BRD to reduce the number of equivocal substances based on new data for MBT, and to

672   include revisions to the reference data for the traditional LLNA and human data. A critical

673   component of a formal evaluation of the validation status of a test method is an assessment of

674   the accuracy of the proposed tested method when compared to the current reference test

675   method (ICCVAM 2003). Additional comparisons should also be made against any available

676   human data or experience from testing or accidental exposures. This aspect of assay

677   performance is typically evaluated by calculating:

678   - *Accuracy* (concordance): the proportion of correct outcomes (positive and
679   negative) of a test method

680   - *Sensitivity*: the proportion of all positive substances that are classified as positive

681   - *Specificity*: the proportion of all negative substances that are classified as negative

682   - *False positive rate*: the proportion of all negative substances that are incorrectly
683   identified as positive

684   - *False negative rate*: the proportion of all positive substances that are incorrectly
685   identified as negative

686   An accuracy analysis for the LLNA: BrdU-FC was conducted using data on 45 substances

687   tested by MB Research Labs (2007); these substances had also been tested in the traditional

688   LLNA. Thirty-seven of these substances had LLNA: BrdU-FC, traditional LLNA, and GP

689   data while 42 substances had LLNA: BrdU-FC, traditional LLNA, and human data. To

690   account for the substances that produced equivocal results in the LLNA: BrdU-FC (see

691   **Section 5.0**) two separate analyses were conducted: 1) only the substances with unequivocal

692   LLNA: BrdU-FC results were evaluated, and 2) the two equivocal substances were included

693   by using the more conservative result (i.e., positive) for both substances. Including the two

694   equivocal substances resulted in a net gain of one correctly identified sensitizer and one false

695   positive result when comparing the LLNA: BrdU-FC to the traditional LLNA, guinea pig,

696   and human results.

697   ## 6.1   LLNA: BrdU-FC Database Analysis

698   ### 6.1.1   Accuracy vs. the Traditional LLNA

699   Based on the available data, when compared to the traditional LLNA (excluding the two

700   equivocal substances) the LLNA: BrdU-FC had an accuracy of 95% (41/43), a sensitivity of

701   96% (27/28), a specificity of 93% (14/15), a false positive rate of 7% (1/15), and a false

702   negative rate of 4% (1/28) (**Table 6-1**).

703   Including the two equivocal substances resulted in an accuracy for the LLNA: BrdU-FC of

704   93% (42/45), a sensitivity of 97% (28/29), a specificity of 88% (14/16), a false positive rate

705   of 12% (2/16), and a false negative rate of 3% (1/29) (**Table 6-1**).

706   *6.1.2   Accuracy vs. Guinea Pig Data*

707   When the accuracy statistics for the LLNA: BrdU-FC and the traditional LLNA were

708   compared when GP results served as the reference data, the LLNA: BrdU-FC had a lower

709   accuracy rate (74% [26/35] vs. 81% [29/36]), lower sensitivity (84% [16/19] vs. 90%

710   [17/19]), and lower specificity (63% [10/16] vs. 71% [12/17]) compared with the traditional

711   LLNA. The LLNA: BrdU-FC also had a higher false positive rate (38% [6/16] vs. 29%

712   [5/17]) and a higher false negative rate of (16% [3/19] vs. 11% [2/19]) than the traditional

713   LLNA (**Table 6-1**).

714   Including the two equivocal substances resulted in only a slight reduction in overall

715   performance for the LLNA: BrdU-FC (e.g., accuracy reduced to 73% [27/37] from 74%

716   [26/35]) when compared to GP results (**Table 6-1**).

717   *6.1.3   Accuracy vs. Human Data*

718   When substances with only comparative LLNA: BrdU-FC data, traditional LLNA data, and

719   human outcomes were evaluated, the LLNA: BrdU-FC had similar accuracy (72% [29/40]

720   vs. 73% [30/41]), similar specificity (61% [8/13] vs. 64% [9/14]), and the same sensitivity

721   (78% [21/27]) as the traditional LLNA when using human sensitization outcomes as the

722   reference data. Similarly, the LLNA: BrdU-FC had a false positive rate (39% [5/13] vs. 36%

723   [5/14]) that was similar to the traditional LLNA, and the same false negative rate (22%

724   [6/27]) as the traditional LLNA, when each was compared to human sensitization outcomes.

725   Including the two equivocal substances resulted in a slight reduction in test method accuracy

726   for the LLNA: BrdU-FC (accuracy was reduced from 72% [29/40] to 71% [30/42]) when

727   compared to human sensitization outcomes (**Table 6-1**).

728   **Table 6-1   Evaluation of the Performance of the LLNA: BrdU-FC In Predicting Skin-Sensitizing Potential**

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: BrdU-FC vs. Traditional LLNA	43	95	41/43	96	27/28	93	14/15	7	1/15	4	1/28	96	27/28	93	14/15
LLNA: BrdU-FC vs. Traditional LLNA*	45	93	42/45	97	28/29	88	14/16	13	2/16	3	1/29	93	28/30	93	14/15
Substances with LLNA: BrdU-FC, Traditional LLNA, and GP Data															
LLNA: BrdU-FC vs. Traditional LLNA	35	94	33/35	96	21/22	92	12/13	8	1/13	4	1/22	95	21/22	92	12/13
LLNA: BrdU-FC vs. Traditional LLNA*	37	92	34/37	96	22/23	86	12/14	14	2/14	4	1/23	92	22/24	92	12/13
LLNA: BrdU-FC vs. GP[3]	35	74	26/35	84	16/19	63	10/16	37	6/16	16	3/19	73	16/22	77	10/13
LLNA: BrdU-FC vs. GP[3]*	37	73	27/37	85	17/20	59	10/17	41	7/17	15	3/20	71	17/24	77	10/13
Traditional LLNA vs. GP[3]	35	80	28/35	90	17/19	69	11/16	31	5/16	10	2/19	77	17/22	85	11/13
Traditional LLNA vs. GP[3]*	37	81	30/37	90	18/20	71	12/17	29	5/17	10	2/20	78	18/23	86	12/14
Substances with LLNA: BrdU-FC, Traditional LLNA, and Human Data															
LLNA: BrdU-FC vs. Traditional LLNA	40	95	38/40	96	25/26	93	13/14	7	1/14	4	1/26	96	25/26	93	13/14
LLNA: BrdU-FC vs. Traditional LLNA*	42	93	39/42	96	26/27	87	13/15	13	2/15	4	1/27	93	26/28	93	13/14
LLNA: BrdU-FC vs. Human[4]	40	72	29/40	78	21/27	61	8/13	39	5/13	22	6/27	81	21/26	57	8/14
LLNA: BrdU-FC vs. Human[4]*	42	71	30/42	79	22/28	57	8/14	43	6/14	21	6/28	79	22/28	57	8/14
Traditional LLNA vs. Human[4]	41	73	30/41	78	21/27	64	9/14	36	5/14	22	6/27	81	21/26	60	9/15
Traditional LLNA vs. Human[4]*	42	74	31/42	79	22/28	64	9/14	36	5/14	21	6/28	81	22/27	60	9/15

729   Abbreviations: LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurement of bromodeoxyuridine incorporation; GP = Guinea pig
730   skin sensitization outcomes; LLNA = Local Lymph Node Assay; No. = Number
731   * Includes 2 additional substances that produced divergent results when tested in the LLNA: BrdU-FC. In order to include these substances in the analysis,
732   they were assigned the more conservative classification (i.e., sensitizer).
733   [1] N = Number of substances included in this analysis
734   [2] The data on which the percentage calculation is based
735   [3] GP refers to outcomes obtained by studies conducted using either the Guinea Pig Maximization Test or the Buehler Test.
736   [4] *Human* refers to outcomes obtained by studies conducted using the Human Maximization Test or the inclusion of the test substance in a Human Patch Test
737   Allergen Kit.

738   **6.2   eLLNA: BrdU-FC Database Analysis**

739   *6.2.1   Accuracy vs. the Traditional LLNA*

740   A separate accuracy analysis was conducted for the eLLNA: BrdU-FC. As noted in **Section 2.0**,

741   only substances with SI ≥ 3 and mouse ear swelling ≥ 25% are evaluated with the additional

742   immunophenotypic markers included in the eLLNA: FC-BrdU. The results of the eLLNA:

743   BrdU-FC were the same as those for the LLNA: BrdU-FC with the exception of ethylene glycol

744   dimethacrylate, benzalkonium chloride, and sodium lauryl sulfate. These substances, which were

745   classified as sensitizers by the LLNA: BrdU-FC, were identified as irritants (i.e., nonsensitizers)

746   by the eLLNA: BrdU-FC. Since the traditional LLNA incorrectly identified two of these

747   substances (ethylene glycol dimethacrylate and sodium lauryl sulfate) as sensitizers, the

748   concordance of the eLLNA: BrdU-FC with the traditional LLNA was decreased (compared to

749   the LLNA: BrdU-FC without the immunophenotypic endpoints). Thus, based on the

750   43 substances with unequivocal eLLNA: BrdU-FC and traditional LLNA results, the eLLNA:

751   BrdU-FC decreased the accuracy (88% [38/43] vs. 95% [41/43]) and sensitivity (86% [24/28] vs.

752   96% [27/28]) and increased the false negative rate (14% [4/28] vs. 4% [1/28]) relative to the

753   LLNA: BrdU-FC (compare **Table 6-2** with **Table 6-1**). The specificity rates (93% [14/15]) and

754   the false positive rates (7% [1/15]) were the same for the eLLNA: BrdU-FC vs. the traditional

755   LLNA compared to the LLNA: BrdU-FC vs. the traditional LLNA.

756   Including the two equivocal substances resulted in an accuracy for the eLLNA: BrdU-FC of 87%

757   (39/45), a sensitivity of 86% (25/29), a specificity of 88% (14/16), a false positive rate of 13%

758   (2/16), and a false negative rate of 14% (4/29) (**Table 6-2**).

759 **Table 6-2 Evaluation of the Performance of the eLLNA: BrdU-FC[1] In Predicting Skin-Sensitizing Potential**

Comparison	N	Accuracy %	No.[2]	Sensitivity %	No.[2]	Specificity %	No.[2]	False Positive Rate %	No.[2]	False Negative Rate %	No.[2]	Positive Predictivity %	No.[2]	Negative Predictivity %	No.[2]
eLLNA: BrdU-FC vs. Traditional LLNA	43	88	38/43	86	24/28	93	14/15	7	1/15	14	4/28	96	24/25	78	14/18
eLLNA: BrdU-FC vs. Traditional LLNA*	45	87	39/45	86	25/29	88	14/16	13	2/16	14	4/29	93	25/27	78	14/18
Substances with eLLNA: BrdU-FC, Traditional LLNA, and GP Data															
eLLNA: BrdU-FC vs. Traditional LLNA	35	86	30/35	82	18/22	92	12/13	8	1/13	18	4/22	95	18/19	75	12/16
eLLNA: BrdU-FC vs. Traditional LLNA*	37	84	31/37	83	19/23	86	12/14	14	2/14	17	4/23	91	19/21	75	12/16
eLLNA: BrdU-FC vs. GP	35	83	29/35	84	16/19	81	13/16	19	3/16	16	3/19	84	16/19	81	13/16
eLLNA: BrdU-FC vs. GP*	37	81	30/37	85	17/20	77	13/17	23	4/17	15	1/18	81	17/21	81	13/16
Traditional LLNA vs. GP	35	80	28/35	90	17/19	69	11/16	31	5/16	10	2/19	77	17/22	85	11/13
Traditional LLNA vs. GP*	37	81	30/37	90	18/20	71	12/17	29	5/17	10	2/20	78	18/23	86	12/14
Substances with eLLNA: BrdU-FC, Traditional LLNA, and Human Data															
eLLNA: BrdU-FC vs. Traditional LLNA	40	88	35/40	85	22/26	93	13/14	7	1/14	15	4/26	96	22/23	77	13/17
eLLNA: BrdU-FC vs. Traditional LLNA*	42	86	36/42	85	23/27	87	13/15	13	2/15	15	4/27	92	23/25	77	13/17
eLLNA: BrdU-FC vs. Human[3]	40	70	28/40	70	19/27	69	9/13	31	4/13	30	8/27	83	19/23	53	9/17
eLLNA: BrdU-FC vs. Human[3]*	42	69	29/42	71	20/28	64	9/14	36	5/14	29	8/28	80	20/25	53	9/17
Traditional LLNA vs. Human[3]	41	73	30/41	78	21/27	64	9/14	36	5/14	22	6/27	81	21/26	60	9/15
Traditional LLNA vs. Human[3]*	42	74	31/42	79	22/28	64	9/14	36	5/14	21	6/28	82	22/27	60	9/15

760 Abbreviations: eLLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurement of bromodeoxyuridine incorporation enhanced with
761 immunophenotypic endpoints; GP = Guinea pig skin sensitization outcomes obtained using either the Guinea Pig Maximization Test or the Buehler Test;
762 LLNA = Murine local lymph node assay; N = Number of substances included in this analysis; No. = Number
763 * Includes 3 additional substances that produced divergent results when tested in the LLNA: BrdU-FC. In order to include these substances in the analysis, they
764 were assigned the more conservative classification (i.e., sensitizer)
765 [1] The results of the eLLNA: BrdU-FC were the same as those for the LLNA: BrdU-FC with the exception of ethylene glycol dimethacrylate and sodium lauryl
766 sulfate, which were classified as irritants rather than sensitizers.
767 [2] The data on which the percentage calculation is based.
768 [3] Human refers to outcomes obtained by studies conducted using the Human Maximization Test or the inclusion of the test substance in a Human Patch Test
769 Allergen Kit.

770   ***6.2.2   Accuracy vs. Guinea Pig Data***

771   The concordance of the eLLNA: BrdU-FC with GP data was greater than the concordance of

772   LLNA: BrdU-FC data to GP data) because ethylene glycol dimethacrylate and sodium lauryl

773   sulfate were classified as nonsensitizers in both eLLNA: BrdU-FC and GP tests. These

774   substances were classified as sensitizers by the LLNA: BrdU-FC. For the 35 substances with

775   eLLNA: BrdU-FC, GP, and traditional LLNA data, the eLLNA: BrdU-FC protocol improved the

776   performance of the LLNA: BrdU-FC (compare **Table 6-2** with **Table 6-1**). Accuracy increased

777   to 83% (29/35) from 74% (26/35); specificity increased to 81% (13/16) from 63% (10/16); and

778   the false positive rate decreased from 38% (6/16) to 19% (3/16). The sensitivity (84% [16/19])

779   and the false negative rates (16% [3/19]) were the same for the LLNA: BrdU-FC and the

780   eLLNA: BrdU-FC.

781   As in the LLNA: BrdU-FC, including the two equivocal substances resulted in only a slight

782   reduction in overall performance for the eLLNA: BrdU-FC (accuracy reduced from 83% [29/35]

783   to 81% [30/37]) when compared to GP results (**Table 6-2**).

784   ***6.2.3   Accuracy vs. Human Data***

785   When the substances with comparative eLLNA: BrdU-FC data, traditional LLNA data, and

786   human outcomes were evaluated, the eLLNA: BrdU-FC had similar accuracy, sensitivity, and

787   false negative rates to the LLNA: BrdU-FC. The accuracy for the eLLNA: BrdU-FC (in

788   reference to human data) was slightly decreased to 70% (28/40) from 72% (29/40) for LLNA:

789   BrdU-FC; the sensitivity decreased to 70% (19/27) from 78% (21/27); and the false negative rate

790   increased from 22% (6/27) to 30% (8/27). The specificity for the eLLNA: BrdU-FC increased to

791   69% (9/13) from 61% (8/13); and the false positive rate decreased to 31% (4/13) from 39%

792   (5/13) for LLNA: BrdU-FC.

793   Including the two equivocal substances did not change overall performance for the eLLNA:

794   BrdU-FC (e.g., accuracy remained 69% [29/42]) when compared to human sensitization

795   outcomes (**Table 6-2**).

796

6.3   Accuracy Analysis Based on ICCVAM Draft Performance Standards

796

797   ICCVAM has proposed test method performance standards for the LLNA (ICCVAM 2009)

798   These test method performance standards are proposed to evaluate the performance of LLNA

799   test methods that incorporate specific protocol modifications to measure lymphocyte

800   proliferation compared to the traditional LLNA. As shown in **Table 6-3**, 13 of the 18 minimum

801   reference substances have been tested in the LLNA: BrdU-FC and the eLLNA: BrdU-FC. Eight

802   substances were sensitizers, and five substances were nonsensitizers. Two substances, 2-

803   mercaptobenzothiazole (sensitizer, mean EC3 = 2.5%) and salicylic acid (nonsensitizer),

804   produced equivocal results in the LLNA: BrdU-FC and the eLLNA: BrdU-FC. The LLNA:

805   BrdU-FC and the eLLNA: BrdU-FC results for the remaining 11 substances were consistent with

806   those of the traditional LLNA.

807   Three of the four optional reference substances included in the ICCVAM LLNA performance

808   standards were also tested in the LLNA: BrdU-FC. Ethylene glycol dimethacrylate and sodium

809   lauryl sulfate, two nonsensitizers, were both false positives in the LLNA: BrdU-FC. They were

810   also false positives in the traditional LLNA. However, when tested in the eLLNA: BrdU-FC,

811   ethylene glycol dimethacrylate and sodium lauryl sulfate were identified as irritants rather than

812   sensitizers. The third optional reference substance, sulfanilamide (false negative in the traditional

813   LLNA), also produced a false negative result when tested in either the LLNA: BrdU-FC or the

814   eLLNA: BrdU-FC.

815   **Table 6-4** shows the EC3 range of substances tested in the LLNA: BrdU-FC based on the overall

816   database of 45 substances in comparison to that of substances from list of minimum reference

817   standards in the revised draft ICCVAM LLNA performance standards substances list. The table

818   reveals that, although not all of the draft ICCVAM performance standards reference substances

819   have been tested in the LLNA: BrdU-FC, the EC3 range of those tested is similar to that for

820   substances on the draft performance standards list. In general, there is a proportionally increased

821   number of substances tested in the LLNA: BrdU-FC in each of the categories included in the

822   table.

823

823 **Table 6-3   Evaluation of the Performance of the LLNA: BrdU-FC When Compared to the**
824 **ICCVAM Performance Standards Reference Substances (Sorted by Ascending**
825 **Traditional LLNA EC3 Value)[1]**

Name	ICCVAM Draft LLNA Performance Standards[1]				LLNA: BrdU-FC[2]		
	Result	EC3 (%)	N	Vehicle	Result	EC3 (%)	Vehicle
5-Chloro-2-methyl-4-isothiazolin-3-one	+	0.009	1	DMF	NT	NT	IR
2,4-Dinitrochlorobenzene	+	0.049	15	AOO	+	0.01-*0.09*	AOO
4-Phenylenediamine	+	0.11	10	AOO	+	0.45	IR
4-Methylaminophenol sulfate	+	0.8	1	DMF	NT	NT	IR
Isoeugenol	+	1.5	49	AOO	+	NR	IR
2-Mercaptobenzothiazole	+	2.5	2	AOO	+	4.1	DMSO
Cobalt chloride	+	0.6	2	DMSO	+	1	L92
Citral	+	9.8	6	AOO	+	2	DaAE
Hexyl cinnamic aldehyde	+	9.7	22	AOO	+	6-16	AOO
Eugenol	+	10.1	11	AOO	+	13.2	IR
Phenyl benzoate	+	13.6	3	AOO	NT	NT	IR
Cinnamic alcohol	+	21	1	AOO	NT	NT	IR
Imidazolidinyl urea	+	24	1	DMF	NT	NT	IR
Chlorobenzene	-	NA	1	AOO	-	NA	IR
Isopropanol	-	NA	1	AOO	-	>50%	IR
Lactic acid	-	NA	2	DMSO	-	NA	IR
Methyl salicylate	-	NA	10	AOO	-	NA	IR
Salicylic acid	**-**	NA	1	***AOO***	**+/-**	***NA***	***IR***
Ethylene glycol dimethylacrylate	FP	28	1	MEK	+[3]	40.0	IR
Sodium lauryl sulfate	FP	8.1	5	DMF	+[3]	4.8	DMSO
Nickel sulfate	FN	NA	2	DMF	NT	NT	IR
Sulfanilamide	FN	NA	1	DMF	-	>50%	IR

826 ***Bolded italic text*** highlights discordant LLNA: BrdU-FC vs. traditional LLNA test results.

827 Abbreviations: AOO = acetone and olive oil; DaAE = DMSO, acetone, and ethanol; DMF = dimethylformamide;
828 DMSO = dimethyl sulfoxide; FN = false negative; FP = false positive; LLNA: BrdU-FC = Murine local lymph node
829 assay with flow cytometry measurement of bromodeoxyuridine incorporation; IR = Information requested; L92 =
830 1% pluronic acid L92 surfactant in water; NA = Not applicable (stimulation index < 3); NR = Not reported; NT =
831 Not tested; + = Sensitizer; - = Nonsensitizer; +/- = equivocal compounds that were not included in contingency table
832 evaluations.
833 [1] From Revised Draft ICCVAM Performance Standards for the LLNA (available:
834   http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm)
835 [2] From MB Research Labs (2007)
836 [3] Classified by the LLNA: BrdU-FC as an irritant but not a sensitizer using an enhanced LLNA: BrdU-FC with
837   immunophenotypic endpoints (i.e., the eLLNA: BrdU-FC).
838

838   **Table 6-4   Characteristics of the Substances Tested in the LLNA: BrdU-FC vs. the**
839   **ICCVAM Performance Standards Substances List[1]**

EC3 range (%)	No. Chems	Solid/ Liquid	Actual EC3 Range (%)[2]	Human Data	Peptide Reactivity (High/Mod/ Min/Unk)[3]
<0.1	**4**	**4/0**	**0.0034-0.05**	**4**	**3/1/0/0**
	2	1/1	0.009-0.05	2	0/1/0/1
≥0.1 to <1	**5**	**4/1**	**0.1-0.53**	**4**	**2/1/0/2**
	2	2/0	0.11-0.8	2	1/0/0/1
≥1 to <10	**9**	**4/5**	**1.53-9.9**	**9**	**1/0/2/6**
	5	2/3	1.6-9.9	5	1/0/1/3
≥10 to <100	**8**	**1/7**	**10.1-95.8**	**8**	**1/0/1/6**
	4	3/1	10.1-24	4	0/1/0/3
Negative	**19**	**12/7**	**NC**	**18**	**0/0/0/19**
	5	2/3	NC	3	0/0/2/3
Overall	**45**	**25/20**	**0.0034-95.8**	**43**	**7/2/3/33**
	18	10/8	0.009-24	16	2/2/3/11

840   **Bolded text** represents characteristics of the LLNA: BrdU-FC database.
841   Abbreviations: Chems = Chemicals; EC3 = Estimated concentration needed to produce a stimulation index of 3; NC
842   = Not calculated because maximum stimulation index < 3.0; No. = Number; Min = Minimal; Mod = Moderate;
843   SI = Stimulation index; Unk = Unknown
844   [1]   From Revised Draft ICCVAM Performance Standards for the LLNA (available:
845   http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). Includes the 18 "required" substances for
846   testing
847   [2]   Based on traditional LLNA studies for substances in the LLNA: BrdU-FC database (bold values) and the draft
848   ICCVAM LLNA performance standards substances
849   [3]   Data obtained from Gerberick et al. (2007)

850   **6.4   Discordant Results**

851   The number of substances that yielded different sensitizer/nonsensitizer classifications in the

852   LLNA: BrdU-FC and the reference methods (i.e., GP tests, human tests) were compared to the

853   number of discordant results in the traditional LLNA. Substances were evaluated to identify

854   commonalities among the discordant substances. The effect of testing with different vehicles

855   could not be evaluated because the submission from MB Research Labs did not identify the

856   vehicle used for each test substance. NICEATM has requested this information, and MB

857   Research Labs has agreed to supply it as soon as possible.

858   When analyses were restricted to the 35 substances with unequivocal LLNA: BrdU-FC,

859   traditional LLNA, and GP data, the LLNA: BrdU-FC classified two substances differently

860   compared with the traditional LLNA (**Table 6-5**). The LLNA: BrdU-FC identified Tween[®] 80 (a

861   liquid surfactant, MW = 1310 g/mol) as a sensitizer, while the traditional LLNA classified it as a

862   nonsensitizer. Conversely, in the LLNA: BrdU-FC, aniline (a liquid, MW = 93 g/mol) was

863   negative (SI value, concentrations tested, and vehicle used were not available), but it was

864   positive in the traditional LLNA (SI=3.6@ 50% aniline in AOO). Note that Tween[®] 80 is a

865   sensitizer in humans, indicating that the traditional LLNA underpredicted the sensitization

866   potential in humans, and that the positive response in the LLNA: BrdU-FC agrees with the

867   human outcome.

868 **Table 6-5   Discordant Results with Respect to Traditional LLNA and Guinea Pig**
869   **Reference Data[1]**

Substance Name	Vehicle[2]	LLNA: BrdU-FC[3]	Traditional LLNA[3]	Guinea Pig Studies[4]	Skin Irritant?
Benzalkonium chloride	ACE	+	+ 11.1, 2%[5]	-	Irritant at 2% (mice)
Copper chloride	DMSO	+	+ 13.8, 5%[6]	-	Nonirritant at 0.25% (GP)
Resorcinol	AOO	+	+ 10.4, 50%	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate	DMF	+ 3.0, 25%	+ 8.9, 20%	-	Irritant at 20% (rabbits)
Ethylene glycol dimethacrylate	MEK	+	+ 7, 50%	-	Nonirritant at 1% (GP)
Tween 80	AOO	+	- NR	-	Nonirritant at 25% (humans)
Aniline	AOO	-	+ 3.6, 100%[7]	+	Negative at 100% (GP)
4-Aminobenzoic acid	AOO	-	- 1.6, 10%[8]	+	Irritant at 25% (humans)
Nickel chloride	DMSO	-	- 2.4, 5%	+	Negative at ≤ 0.15% (GP)

870 Abbreviations: LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurements of
871 bromodeoxyuridine; Traditional LLNA = Murine local lymph node assay using radioactivity to detect sensitizers;
872 GP = Guinea pig; NA = Not available; SI = Stimulation index; + = Sensitizer; - = Nonsensitizer
873 [1] Data sources are listed in **Appendix C1**.
874 [2] Vehicles apply to tests for the traditional LLNA; ACE = acetone; AOO = acetone: olive oil;
875   DMF = dimethylformamide; DMSO = dimethyl sulfoxide; MEK = methyl ethyl ketone. Vehicle information was
876   generally not reported for LLNA: BrdU-FC, except for sodium lauryl sulfate, for which the vehicle was DMSO.
877 [3] The numbers under the + or - calls are the highest SI and the maximum concentration tested. The results of the
878   eLLNA: BrdU-FC were the same as those for the LLNA: BrdU-FC with the exception of benzalkonium chloride,
879   ethylene glycol dimethacrylate, and sodium lauryl sulfate, which were classified as irritants rather than sensitizers.
880 [4] From ICCVAM (1999) and based on studies using either the Guinea Pig Maximization Test or the Buehler Test.
881 [5] Highest SI occurred at a concentration of 1%.
882 [6] Highest SI occurred at a concentration of 2.5%.
883 [7] Highest SI occurred at a concentration of 50%.
884 [8] Highest SI occurred at a concentration of 5%.
885

886 When compared to the outcomes of GP tests, the LLNA: BrdU-FC misclassified nine substances;

887 the eLLNA: BrdU-FC misclassified six substances; and the traditional LLNA misclassified

888 seven substances. The LLNA: BrdU-FC and the traditional LLNA had six discordant substances

889 in common.

890 Benzalkonium chloride, copper chloride, resorcinol, ethylene glycol dimethacrylate, and sodium

891 lauryl sulfate were incorrectly classified as sensitizers (compared with the GP results) by the

892   LLNA: BrdU-FC and the traditional LLNA. No commonalities were identified for these five

893   substances. They represent seven different chemical classes: onium compounds, phenols,

894   inorganics, alcohols, carboxylic acids, organic sulfur compounds, and lipids. There are four

895   solids and one liquid, ranging in molecular weight from 99 to 288, with octanol-water partition

896   coefficients ranging from 1.0 to 1.7. One substance, ethylene glycol dimethacrylate, is

897   considered highly peptide reactive.

898   Nickel chloride (a solid, MW = 130 g/mol) and 4-Aminobenzoic acid (a solid carboxylic acid,

899   MW = 137 g/mol) were incorrectly classified as nonsensitizers by the LLNA: BrdU-FC and the

900   traditional LLNA. Both of the BrdU-FC tests misclassified aniline (a liquid amine, MW = 93

901   g/mol) as a nonsensitizer, but the traditional LLNA did not. The eLLNA: BrdU-FC protocol

902   classified benzalkonium chloride, ethylene glycol dimethacrylate, and sodium lauryl sulfate as

903   irritants.

904   When analyses were restricted to the 40 substances with unequivocal LLNA: BrdU-FC,

905   traditional LLNA, and human outcomes, the discordant substances for the LLNA: BrdU-FC, the

906   eLLNA: BrdU-FC, and traditional LLNA were the same as that for the set of 34 substances with

907   unequivocal LLNA: BrdU-FC, traditional LLNA, and GP outcomes (**Table 6-4**). As described

908   earlier in this section, the LLNA: BrdU-FC and the traditional LLNA classified two substances

909   differently (Tween® 80 and aniline).

910   When comparing to the outcomes of human tests, both the LLNA: BrdU-FC and the traditional

911   LLNA misclassified 11 substances (**Table 6-6**). Ten of the 11 discordant substances

912   misclassified by the LLNA: BrdU-FC were also misclassified by the traditional LLNA. Of these

913   10 substances, five were misclassified as sensitizers (copper chloride, isopropyl myristate,

914   linalool, sodium lauryl sulfate, and xylene) and the other five (isopropanol, nickel chloride,

915   propylene glycol, propylparaben, and sulfanilamide) were misclassified as nonsensitizers by both

916   methods. Among the five false positives, three are liquids and two are solids; they range in

917   molecular weight from 99 to 288 g/mol, with octanol-water partition coefficients that range from

918   1.7 to 3.9. One substance, isopropyl myristate, is considered minimally peptide reactive. Peptide

919   reactivity data on the other substances could not be located.

920   No commonalities were noted among the five human sensitizers that were misclassified as

921   nonsensitizers by both LLNA: BrdU-FC and traditional methods. The five substances represent

922   alcohols, amides, amines, carboxylic acids, phenols, sulfur compounds, and inorganic chemicals

923   (some of the substances could fit in more than one chemical class). Three are solids and two are

924   liquids, with molecular weights ranging from 60 to 180, and octanol-water partition coefficients

925   ranging from 0.3 to 3.0. Four of the false negative substances are considered minimally peptide

926 reactive. The eLLNA: BrdU-FC protocol also misclassified these same five sensitizing

927 substances as nonsensitizers. Both of the BrdU-FC tests misclassified aniline, but the traditional

928 LLNA did not.

929 **Table 6-6   Discordant Results with Respect to Human Outcomes[1]**

Substance Name	Vehicle[2]	LLNA: BrdU-FC[3]	Traditional LLNA[3]	Human Call[4]	Skin Irritant?
Copper chloride	DMSO	+	+ 13.8, 2.5%	-	Nonirritant at 0.25% (GP)
Isopropyl myristate	AOO	+	+ 3.4, 100%	-	Negative at 100% (rabbits)
Linalool	AOO	+	+ 8.3, 100%	-	Mild Irritant at 100% (rabbits)
Sodium lauryl sulfate	DMF	+ 4.7, 25%	+ 8.9, 20%	-	Irritant at 20% (rabbits)
Xylene	AOO	+	+ 3.1, 100%	-	Irritant at 100% (humans)
Tween 80	AOO	+	- NR	+	Nonirritant at 25% (humans)
Aniline	AOO	-	+ 3.6, 100%[5]	+	Negative at 100% (GP)
Isopropanol	AOO	-	- 1.7, 50%[6]	+	Negative at 100% (rabbits)
Nickel chloride	DMSO	-	- 2.4, 5%	+	Negative at ≤ 0.15% (GP)
Propylene glycol	Water	-	- 1.6, 100%	+	Nonirritant at 25% (humans)
Propylparaben	AOO	-	- 1.4, 25%[7]	+	Nonirritant at 10% (GP)
Sulfanilimide	DMF	-	- 1, 50%[6]	+	Nonirritant at 25% (humans)

930 Abbreviations: LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurements of

931 bromodeoxyuridine; Traditional LLNA = Murine local lymph node assay using radioactivity to detect sensitizers;

932 + = Sensitizer; - = Nonsensitizer; NR = Not reported

933 [1] Data sources are listed in **Appendix C1**.

934 [2] Vehicles apply to tests for the traditional LLNA; AOO = acetone: olive oil; DMF = dimethylformamide; DMSO =

935 dimethyl sulfoxide. Vehicle information was generally not reported for LLNA: BrdU-FC, except for sodium

936 lauryl sulfate, for which the vehicle was DMSO.

937 [3] The numbers under the + or - calls are the highest SI and the maximum concentration tested.

938 [4] Outcomes obtained by studies conducted with the human maximization test or the inclusion of the test substance

939 in a human patch test allergen kit

940 [5] Highest SI occurred at a concentration of 50%.

941 [6] Highest SI occurred at a concentration of 10%.

942 [7] Highest SI occurred at a concentration of 5%.

943 **7.0   LLNA: BrdU-FC Reliability**

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

951   For an evaluation of intralaboratory reproducibility, the only available data on multiply tested
952   substances in the LLNA: BrdU-FC is for hexyl cinnamic aldehyde (HCA). However,
953   interlaboratory reproducibility could not be assessed because the test results were generated in
954   one laboratory. The HCA test results for the LLNA: BrdU-FC are amenable to intralaboratory
955   reproducibility analyses only for the SI values for HCA because only one concentration was
956   tested multiple times. The initial data submission did not include EC3 values for HCA; however,
957   data were submitted later that included EC3 results for two positive controls, HCA and 2,4-
958   dintrochlorobenzene.

959   Presumably, there are additional data that could be used to analyze intralaboratory
960   reproducibility for multiply tested substances in the LLNA: BrdU-FC based on the equivocal
961   classifications assigned to benzocaine and salicylic acid (see **Section 5.0**). These data have been
962   requested but not obtained.

963   **7.1   Intralaboratory Reproducibility – SI**

964   The intralaboratory reproducibility has been revised to include new data for HCA and 2,4-DCNB
965   that were not available for evaluation in the January 2008 draft BRD. MB Research Labs
966   provided SI data for multiple tests of HCA in different vehicles. The SI values reported for 2 to
967   26 tests of 25% HCA in each of six vehicles were used to calculate a coefficient of variation
968   (CV) for the assessment of intralaboratory variability. As shown in **Table 7-1**, the CVs ranged
969   from 30% to 53%. The intralaboratory reproducibility of the traditional LLNA was not assessed
970   by CV analysis of SI values (ICCVAM 1999).

971 **Table 7-1   Reproducibility of Hexyl Cinnamic Aldehyde (25% w/v) Tested**
972 **by LLNA: BrdU-FC in Different Vehicles**

Vehicle	N	Mean SI	SD	CV (%)	N for SI<3
Dimethylacetamide:Acetone: Ethanol (DAE 433)	5	13	6.2	46	0
Acetone:Olive Oil (4:1) (AOO)	19	11	5.5	51	0
Dimethyl sulfoxide (DMSO)	26	6.7	3.4	52	2
N,N-Dimethylformamide	4	8.7	4.6	53	0
Ethanol:Water (50%/50%)	4	15	6.3	41	0
Acetone	2	21	6.4	30	0

973 Abbreviations: CV = Coefficient of variation; N = number of tests conducted; SD = Standard deviation;
974 SI = Stimulation index; w/v = Weight-to-volume ratio
975
976 MB Research Labs subsequently provided EC3 results from four tests each in LLNA: BrdU-FC

977 for HCA and 2,4-DNCB. As shown in **Table 7-2** the intralaboratory reproducibility of the EC3

978 values ranged from 8-16% for HCA and from 0.03-0.06% for 2,4-DNCB. It should be noted that

979 these values are within the range of acceptability for reproducibility as described in the

980 ICCVAM LLNA Performance Standards.

981

982 **Table 7-2   Intralaboratory Reproducibility – EC3 Results for Positive Controls in the**
983 **LLNA: BrdU-FC**

Test Substance (Vehicle)	Test 1	Test 2	Test 3	Test 4	Acceptable Range[1]
HCA (AOO)	15%	16%	13%	8.4%	5-20%
DNCB (AOO)	0.06%	0.03%	0.05%	0.03%	0.03-0.10%

984 Abbreviations: AOO = Acetone:olive oil (4:1); DNCB = 2,4-Dinitrochlorobenzene; HCA = Hexyl cinnamic
985 aldehyde; EC3 = Estimated concentration necessary to produce a stimulation index of 3
986 [1] ICCVAM LLNA Performance Standards (http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm)

987   **8.0   Data Quality**

988   MB Research Labs stated that, while most of the LLNA: BrdU-FC and the eLLNA: BrdU-FC

989   data evaluated were not generated in complete compliance with Good Laboratory Practice (GLP)

990   guidelines, their facilities routinely conduct GLP-compliant studies and they have an accredited

991   quality assurance unit. In response to a request for the original data, MB Research Labs indicated

992   that resources were not available to extract these data or to determine which of the individual

993   tests were conducted in compliance with GLPs. MB Research Labs staff members did check the

994   reported data for consistency with the raw data, but the data has not been independently audited.

995   **9.0   Other Scientific Reports and Reviews**

996   All available data for the LLNA: BrdU-FC and the eLLNA: BrdU-FC test methods provided by

997   MB Research have been presented and discussed in the above sections. No other relevant data or

998   scientific reviews of the LLNA: BrdU-FC and the eLLNA: BrdU-FC were identified in online

999   literature search of entries in MEDLINE and SCOPUS (last updated December 10, 2007).

1000   **10.0   Animal Welfare Considerations**

1001   The animal welfare considerations in this draft BRD have not changed from the January 2008

1002   draft BRD. The LLNA: BrdU-FC and the eLLNA: BrdU-FC test methods will require the same

1003   number of animals as the traditional LLNA. However, because the traditional LLNA uses

1004   radioactivity and, accordingly, its use might be restricted due to the complications associated

1005   with handling radioactive materials (e.g., storage, disposal) use of a nonradioactive alternative to

1006   the traditional LLNA, such as the LLNA: BrdU-FC or the eLLNA: BrdU-FC could further

1007   reduce the number of guinea pigs used to assess skin sensitization.

1008   **10.1   Rationale for the Use of Animals**

1009   The rationale for the use of animals in the LLNA: BrdU-FC and the eLLNA: BrdU-FC is the

1010   same as that for the traditional LLNA: there are no valid and accepted nonanimal ways to

1011   determine the potential of substances and products to produce skin sensitization, except for

1012   situations in which human studies could be conducted ethically and meet regulatory safety

1013   assessment requirements. The most detailed information about the induction and regulation of

1014   immunological responses are available for mice (ICCVAM 1999).

1015

1015   **10.2   Basis for Determining the Number of Animals Used**

1016   The number of animals used for the experimental, vehicle, and positive control groups is based

1017   on the number of animals specified in the ICCVAM-recommended traditional LLNA protocol

1018   (ICCVAM 1999; Dean et al. 2001).

1019   **10.3   Reduction Considerations**

1020   A further reduction of 40% (15 vs. 25) could be achieved by using a limit dose version of the

1021   LLNA: BrdU-FC, when dose-response information is not needed for hazard identification. In

1022   such an approach, only the highest soluble dose of test substances that does not induce systemic

1023   toxicity or excessive local irritation would be administered, and the two lower dose groups

1024   would not be used. Additional reductions could be achieved by testing more substances

1025   concurrently, so that the same vehicle and positive control group could be used for multiple

1026   substances, thereby reducing the number of animals by 10, or 40%, for each additional substance

1027   (15 vs. 25).

1028   # 11.0 Practical Considerations

1029   Several issues are taken into account when assessing the practicality of an alternative to an

1030   existing test method. In addition to performance evaluations of alternative test methods,

1031   necessary laboratory equipment and supplies, required levels of personnel training, labor costs,

1032   and the time required to complete the test method must be assessed and compared to the existing

1033   test method. The time, personnel cost, and effort required to conduct the proposed test method(s)

1034   must be considered reasonable when compared to those of the test method it is intended to

1035   replace.

1036   **11.1   Transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC**

1037   The test method transferability considerations in this draft BRD have not changed from the

1038   January 2008 draft BRD. Test method transferability addresses the ability of a method to be

1039   accurately and reliably performed by multiple laboratories (ICCVAM 2003), including both

1040   those experienced in the particular type of procedure and those with less or no experience in the

1041   procedure. It would be expected that the transferability of the LLNA: BrdU-FC and the eLLNA:

1042   BrdU-FC would be similar to that of the traditional LLNA because the protocols of the two

1043   methods (except for the detection of lymphocyte proliferation and immunophenotypic

1044   measurements) are identical. However, without interlaboratory reproducibility data, the extent of

1045   transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC cannot be definitively

1046   assessed.

1047   **11.2   Facilities and Major Fixed Equipment Required to Conduct the LLNA: BrdU-FC**
1048   **and the eLLNA: BrdU-FC**

1049   Unlike the traditional LLNA, the LLNA: BrdU-FC and the eLLNA: BrdU-FC will not require

1050   facilities, equipment, and licensing permits for handling radioactive materials. However, the

1051   LLNA: BrdU-FC does require access to a flow cytometer to assess lymphocyte proliferation. A

1052   flow cytometer is not routinely included in many laboratories, and a new flow cytometer can cost

1053 $100,000 or more. The remaining requirements (e.g., animal care facilities) are the same for the

1054   two methods.

1055   **11.3   LLNA: BrdU-FC Training Considerations**

1056   The level of training and expertise needed to conduct the LLNA: BrdU-FC and the eLLNA:

1057   BrdU-FC should be similar to the traditional LLNA, although the LLNA: BrdU-FC and the

1058   eLLNA: BrdU-FC require that users operate a flow cytometer instead of a scintillation counter

1059   and be able process flow cytometric data.

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APPENDIX A

LLNA: BrdU-FC Test Method Submission from MB Research Labs

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MB RESEARCH LABORATORIES
STANDARD PROTOCOL
5650A-06

1.0   TITLE OF STUDY: LOCAL LYMPH NODE ASSAY IN MICE (LLNA)

2.0   OBJECTIVE: To determine the sensitizing potential of topically applied test substances. This LLNA protocol, modified using flow cytometry analysis, is designed to be an alternative assay for the Buehler Guinea Pig Sensitization Assay defined in the ICCVAM report (63 CFR 37405-6, July 10, 1998) and the LLNA as defined in EPA OPPTS 870.2600, Final Guideline (March 2003), and OECD Test Guideline 429, effective April 2002.

3.0   TEST ARTICLE:

   3.1:   Source: All test articles will be supplied by the sponsor. Prior to initiation of the study, the sponsor should provide test article characterization to the Study Director that should include, if technically feasible, the name and quantities of unknown contaminants and impurities. Refer to section:13.3.3 of this protocol for additional information.

   3.2:   Label: Each test article will be identified by source, name, and/or code number, date of receipt at MB Research, and MB Project Number.

   3.3:   Storage: The test article will be stored at room temperature and humidity unless otherwise specified by the Sponsor.

   3.4:   Hazards: Based on the information provided by the Sponsor, appropriate routine safety precautions will be exercised in the handling of the test article.

   3.5:   Vehicle: As necessary, a suitable vehicle will be added to the test article to generate dilutions of the test article. The vehicle will be AOO (acetone:olive oil, 4:1) unless otherwise directed by the Sponsor. When a vehicle or diluent other than AOO will be used, it must be one that does not elicit any significant toxic effects and does not substantially alter the chemical or toxicological properties of the test article. Solubility testing and the use of vehicles other than AOO will be documented in the raw data.

4.0   GENERAL TEST SYSTEM PARAMETERS:

			IRRITATION PRESCREEN	QUANTITATIVE IRRITATION TEST (if needed)	MAIN TEST
4.1:	Animal Requirements:				
4.1.1:	Total Number of Animals	:	6	at least 12	at least 25
4.1.2:	Number of Groups	:	at least 5, including one (1) control group receiving vehicle alone and (1) positive control group, **plus** at least three (3) test groups receiving consecutive concentrations		
4.1.3:	No. Animals/Group	:	at least 5 (all female)		
4.1.4:	Species/Strain	:	CBA/J or CBA/JHsd		
4.1.5:	Age	:	8-12 weeks old at study initiation (age matched +/- one week)		

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

4.2:   Justification of Species/Strain and Number of Animals:

  4.2.1:   Species/Sex: LLNA uses female mice, the preferred experimental gender and species where there is the most detailed information available about the induction and regulation of immunological responses. The test guidelines specify females until gender-specific differences in the LLNA response are shown not to exist.

  4.2.2:   Strain: The protocol utilizes young adult (8-12 week old) female CBA strain mice. Female CBA/J, CBA/JHsd, or CBA/Ca strain mice are acceptable (as per OECD and EPA test guidelines) for use in the assay since, in several inter-laboratory validation studies, they displayed comparable responses. The source and strain used will be indicated in the study report.

  4.2.3:   Number of Animals:

    4.2.3.1. Irritation Prescreen: The 6 mouse prescreen is the minimum number needed to determine if the test article has dermal irritation properties at the highest attainable concentration (maximum solubility) in the vehicle

    4.2.3.2. Quantitative Irritation Test: The optional Quantitative Irritation Test (12 mice) is used when irritation is present and the maximum acceptable dosing concentrations need to be determined.

    4.2.3.3. Main Test: The minimum number of animals in the definitive test is 25, in 5 groups of 5 mice each. Occasionally, especially when dermal irritation is present, an additional 1-2 treatment groups (5-10 mice) may be necessary. For specialty vehicles or formulations, a naïve group or a second vehicle group may be needed. The LLNA permits the reduction of animals required to assess the contact sensitizing activity of test substances compared to studies involving the use of guinea pigs. The minimum number per group recommended by ICCVAM and the EPA-OPPTS 870.2600 and OECD #429 test guidelines is five mice.

4.3:   Husbandry:

  4.3.1:   Equilibration: The test animals will be conditioned to the housing facilities for at least five (5) days prior to study initiation.

  4.3.2:   Housing: Animals will be housed individually in suspended cages which conform to the size recommendations in the Guide for the Care and Use of Laboratory Animals DHEW (NIH). Absorbent white paper bedding, placed beneath the cage, will be changed at least two to three times per week. The animal room, reserved exclusively for mice, is temperature controlled and is equipped with a 12-hour light/dark cycle. Temperature and humidity will be continuously recorded using automatic recording devices.

  4.3.3:   Food: Fresh PMI (Diet #5001) will be available at all times.

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

4.3.4: Water will be available at all times.

    4.3.4.1: Analysis of Water and Acceptable Levels of Contaminants: Analysis of water is performed approximately four (4) times per year and results are compared against a list of acceptable levels of contaminants as provided by the water testing laboratory.

4.4: Control of Bias: From the available pool of animals, healthy female (must be nulliparous and non-pregnant) mice of the same age specified herein will be assigned to groups using standard accepted methods of randomization. The method for attaining the random numbers will be recorded in the study file.

    4.4.1: Pre-study Body Weights: At the initiation of the study, the weight variation of test animals will not exceed ± 20 percent of the mean body weight.

4.5: Identification:

    4.5.1: Cage: Each cage will be identified by a cage tag indicating the date of dosing, test article identification, MB project number, dose level, number and sex of animals.

    4.5.2: Animal: Each animal will be identified by an indelible tail mark corresponding to the numbers documented on the data collection forms.

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

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5.0 EXPERIMENTAL DESIGN:

5.1:   Introduction: The LLNA determines the sensitization potential of a test substance by measuring the proliferation of lymphocytes in the auricular lymph nodes draining the site of exposure (ears). Lymphocyte proliferation will be measured by determining the incorporation of bromodeoxyuridine (BrdU) using a flow cytometer, a method shown to be equivalent to ^{3}T-thymidine-based measurements of lymphocyte proliferation.

5.2:   Summary of Experimental Design:

LLNA PROTOCOL	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7+
7+ DAYS	T BW, ET	T	T ET	---	---	BrdU, BW, ET	P
						Sacrifice	

T   =   Topical application of test substance, vehicle or control

BrdU   =   At 5 hrs pre-sacrifice (t = −5 hrs), systemic administration of BrdU in PBS (200 µl per mouse; i.p.); at sacrifice, excision and processing of each mouse lymph node set (on an individual animal basis); preparation of a single-cell suspension of Lymph Node Cells (LNC).

BW   =   Body Weight

ET   =   Ear Thickness measured (digital micrometer or Peacock Dial thickness gauge) within 24 hrs pre-test, on Day 3 prior to dosing and pre-sacrifice on Day 6.

P   =   Post In-life phase, ex-vivo flow cytometry procedures performed, measurement of BrdU incorporation into lymph node cells (LNC) analyzed, and Stimulation Index (SI) and other parameters calculated.

5.3:   Solvent/Vehicle Selection and Preparation: When preparing solutions, a suitable solvent vehicle will be selected from the following list (in order of preference) or according to instructions from the Sponsor. The default vehicle is AOO 4:1 (see Section 3.5). If AOO 4:1 is not useful as the vehicle, the secondary vehicles in section 5.3.1 will be investigated for solubility of the test article(s). Alternatively, a suitable vehicle may be chosen by the Sponsor in Section 13.1.2, based on the Sponsor's historical irritation and solubility data.

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

5.3.1: Optional LLNA Vehicles:

4:1 v/v Acetone/Olive oil (AOO)
Dimethyl sulfoxide (DMSO)
4:3:3 DMSO:Acetone:Ethanol (D_SAE 433)
Acetone
N,N-Dimethylformamide (DMF)
Dimethylacetamide (DMA)
4:3:3 Dimethylacetamide:Acetone:Ethanol (D_aAE 433)
Ethanol (50%, 95%, or 100%)
Methyl ethyl ketone (MEK), aka 2-Butanone
Ultra Pure Petrolatum
Propylene glycol (PG)

The preferred vehicle AOO is prepared by adding 4 parts (ml) of acetone for every 1 part (ml) olive oil. Wholly aqueous vehicles are to be avoided as per test guidelines. The vehicle will be labeled with description of contents, date of preparation, expiration date/condition, storage/handling and the name/initials of the technician.

5.3.2: Vehicle Preference: Where possible the following vehicles should be used for the LLNA (in order of preference): AOO > Dimethylsulfoxide > D_SAE-433 (= D_aAE) > Acetone. If AOO is not to be used (or if a "clinically relevant solvent" or the "commercial formulation" into which the test article is added is to be used), the Sponsor will indicate this vehicle in section 13.1.2 of the SPONSOR REQUEST section of this protocol.

5.4: Positive Control: The moderate sensitizer alpha-hexyl cinnamic aldehyde (HCA, supplied by MB) at 25% or 50% in AOO (or suitable vehicle) will be used as the positive control as indicated by Sponsor in section 13. Additional positive controls such as the strong sensitizer 2,4-dinitrochlorobenzene (DNCB, supplied by MB) at 0.1% in AOO (or suitable vehicle) may be added at the Sponsor's option, especially if optional immunophenotyping endpoints are to be added to the study. These chemicals have produced consistent responses in the LLNA with the solvents AOO or D_aAE 433. Other positive, negative, naïve, or irritant controls may be added by the Sponsor, in consultation with the Study Director (see section 13.1.2).

5.5: Negative Control: There is no suggested negative control for the LLNA. A negative control substance or a naïve control group may be added as an additional group at the option of the Sponsor and at additional expense (see section 13.1.2). All test article groups will be compared to their respective vehicle control group.

5.6: Test Solution Preparation:

5.6.1: Safety: Safety glasses and gloves must be worn during solution preparation. If the test substance, vehicle and/or control are known to present an inhalation hazard, all procedures must be carried out in a fume hood.

5.6.2: Test Article Preparation: The sample preparation will be documented in the raw data. Fresh test substance solutions/suspensions will be prepared on each treatment day. Substances of low solubility can be mixed using a mechanical agitator or using a magnetic stirrer. Heat above 38°C will not be used unless the substance is known to be heat stable.

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

5.6.3: Test Article Concentrations: The test article is normally assayed at three to five consecutive concentrations from within the following range:

100%, 50%, 25%, 10%, 5%, 2.5%, 1.0%, 0.5%, 0.25%, etc. (w/v for solids, v/v for liquids)

The Sponsor will indicate doses to be used in Section 13.3.2 based upon previous experience or studies (if available), structure activity analysis, dermal irritation and solubility. Optimal test concentrations will be prepared based upon the maximum solubility of the test article in the vehicle, while avoiding overt or severe systemic toxicity or local irritation. In the event of no such support data, a Quantitative Irritation Test (see 5.6.3.2) may be required in place of or in addition to an Irritation Prescreen (see 5.6.3.1) to determine irritation and solubility thresholds.

5.6.3.1: Irritation Prescreen: An initial irritation test (ear swelling; edema) will be performed using 100%, 50% and 25% of test article (or the 3 highest concentrations obtainable in chosen vehicle). Six mice (two per concentration) will be used and the prescreen will be conducted under identical conditions as the main study, except for the assessment of lymph node proliferative activity. If no irritation is observed (ear swelling <25%), then these concentrations will be used in the main study. If significant irritation (≥25% increase in ear swelling) is observed, a Quantitative Irritation Test (see 5.6.3.2) should be performed.

5.6.3.2: Quantitative Irritation Test: If irritation (ear swelling; edema) is encountered in the Irritation Prescreen (5.6.3.1), an expanded Quantitative Irritation Test should be performed using 12 additional mice (either 4 test article concentrations at n = 3 mice, or 6 test article concentrations at n = 2 mice); see section 13.1.2. If all doses tested are irritating, additional irritation tests at decreasing concentrations of test article should be performed until the irritation threshold (maximum non-irritating dose) is determined.

5.7: Topical Application:

5.7.1: Safety: Gloves must be worn during this operation.

5.7.2: Application: Each group of mice will be treated by topical application of a different selected concentration of the test substance to the dorsum of both ears one time per day for three consecutive days. Control mice will be treated with the vehicle alone. The application volume (25 µl per ear) will be administered using a positive displacement pipette and will be spread over the entire dorsal surface of the ear. The time of dosing will be recorded.

5.8: Administration and incorporation of BrdU in vivo: Five days after the first topical application of test article, all mice will be injected intraperitoneally with 200 µl of a 5-Bromo-2'-deoxy-Uridine solution (BrdU, 15 mg/ml in PBS).

5.9: Observations:

5.9.1: Dermal Reactions: All mice will be observed once daily for signs of local irritation at the application site.

5.9.2: Systemic: At a minimum, mice will be observed once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations will be systematically recorded, with records maintained for each individual mouse.

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

5.9.3: Body Weights: Body weights will be recorded pre-test and prior to BrdU injection. Significant weight loss[1] (2 g or more) will be noted and addressed.

5.9.4: Ear Swelling: Both ears of each animal will be observed for edema and/or erythema, and ear thickness measurements will be taken on Day 1 (pre-dose), Day 3 (at approximately 48 hours after the first dose), and on Day 6, using a thickness gauge (digital micrometer or Peacock Dial thickness gauge).

5.10: Post Mortem Lymph Node Extraction:

5.10.1: Sacrifice: Animals showing severe and enduring signs of distress and pain, or animals in a moribund condition and not expected to survive until the next observation interval will be humanely sacrificed using CO_2. On the day of study termination, five (5) hours after the injection of BrdU, the surviving mice will be euthanized by asphyxiation with CO_2.

5.10.2: Excision and Preparation of Lymph Node Cells: Following sacrifice, all of the draining auricular lymph nodes from each mouse will be excised and combined. On an individual animal basis, single cell suspensions of lymph node cells (LNC) will be prepared from the collected lymph nodes by gentle disaggregation, and erythrocytes will be lysed and removed from the LNC suspension.

5.11: Optional Immunophenotyping Cell Treatment: An aliquot of the cells will be stored at 4°C in storage media for up to 72 hours for optional flow cytometry analysis of immunophenotype or surface marker expression, e.g., %B220+, %CD3+, %CD69+, and/or %I-A^x+ cells, as per MB SOP vol. VII.D.1.

5.12: Fixation of Cells: An aliquot of the LNC will be preserved with a suitable buffer or alcohol and will be stored at $-20^{\circ}C$ until further processed. Storage should not exceed two months.

5.13: Determination of Cell Proliferation:

5.13.1: Enumeration of Cells: After propidium iodide staining, a single-cell suspension of LNC will be analyzed using a Becton-Dickinson flow cytometer specifically programmed for propidium iodide staining to enumerate nucleated cells in the lymph node cell suspension. The total number of cells per mouse will be calculated from the values obtained by multiplying by the appropriate dilution factors.

5.14: Determination of BrdU Incorporation:

5.14.1: Acid Denaturation of DNA: DNA of LNC will be acid denatured so that the BrdU antibody can access and quantitatively interact with the BrdU that has been incorporated into the cellular DNA.

5.14.2: Neutralization of the Cellular Material: Samples will be neutralized by washing the cells with borate buffer (pH 8.5), or other comparable neutralization buffer.

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[1] Page updated 02/20/07 to clarify significant body weight changes

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STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

5.14.3: BrdU-specific Staining of Cells: Nuclei will be washed with a staining buffer and incubated with BrdU-specific fluorescent antibody conjugate. The nuclei will be washed with staining buffer and resuspended in PBS containing RNase A and the DNA-specific dye propidium iodide. Following at least a 30-minute incubation at room temperature, the total DNA content of the nuclei, as well as the percentage of nuclei staining positive for BrdU (i.e. percentage of proliferating lymphocytes), will be determined using flow cytometry.

5.15: Test Duration: The test duration of the in-life phase of the study is six (6) days.

6.0: FLOW CYTOMETRY:

6.1: Flow Cytometer: Flow Cytometry and all cell processing will be conducted according to MB Research Laboratories Standard Operating Procedures. Lymph node cell analyses will be performed using a Beckton Dickinson FACScan flow cytometer using 15 mW of power at 488 nm excitation wavelength. BD CellQuest ver. 3.3 acquisition software on a Macintosh G4 acquisition system will be used to capture and store data (List Mode Data files, as .LMD or .FCS) on a dedicated secure network drive. Data files will be analyzed using FlowJo for PC or CellQuest to determine appropriate analysis gate and % positive LNC populations.

7.0: DATA ANALYSIS AND CALCULATION OF STIMULATION INDEX (SI):

7.1: Data Analysis: For analysis of individual animal lymph node sets (right and left side draining local nodes), the proliferative response of lymph node cells (LNC) will be expressed as the total number of BrdU-positive lymphocytes per (individual animal) lymph node sets. The mean value of the total number of BrdU-positive cells and its associated standard deviation (S.D.) will be calculated for each group. The SI, i.e. the ratio of the mean BrdU incorporation into LNC of each test article group divided by that of the vehicle group, will be calculated for each test group according to Equation 1 below:

Equation 1:

$$ SI = \frac{\text{Mean \#BrdU+ cells in Treatment Group}}{\text{Mean \#BrdU+ cells in Vehicle Group}} $$

7.2: Equivocal Results: In the case where dose-related increases in cell proliferation (i.e., BrdU-positive cells) result in a SI that approaches but does not reach 3, the regulatory guidelines may warrant additional tests be performed using higher concentrations of the test substance (or in another vehicle) if possible. In such cases, the effect of the vehicle on the outcome should also be examined.

7.3: Statistical Analysis: For each test group, the individual SI values along with the mean SI and standard deviation will be calculated. If further statistical analysis is required by a regulatory agency to which the report will be submitted, the analysis will be performed only upon request by the sponsor.

8.0: DATA INTERPRETATION:

8.1: Interpretation: A substance will be regarded as a sensitizer in the LLNA if at least one concentration of the test article results in a 3-fold or greater increase in LNC proliferation relative to that of Control (Vehicle) lymph nodes, as indicated by an SI ≥3.0. The data should also be compatible with a biological dose response, although an allowance must be made, especially at high topical application concentrations, for local irritation, systemic toxicity or immunological suppression.

9.0: PROCESSING OF TISSUE:

9.1: Tissues: Other than the lymph nodes processed as above, no other tissues will be taken.

9.2: Optional Tissues: At the option of the Sponsor, the dosage site (ears) will be excised and preserved in 10% neutral formalin for H&E staining and histopathological evaluation at an additional cost. Other tissues or organs may be specified by the Sponsor to be isolated and preserved. Histopathology will be performed by W. Ray Brown, D.V.M., Ph.D., DACVP, Research Pathology Services, Inc., New Britain, PA.

10.0: REVISION OF THE PROTOCOL: Any amendment to or deviation from this protocol will be fully documented in the study file, including the reason for the change, the authority for said change and the date thereof.

11.0: RECORDS TO BE MAINTAINED:

11.1: Collection of Data: All data generated during the conduct of the in-life phase of this study will be recorded in ink on worksheets. All entries will be dated, initialed and verified by another person. Flow cytometry data files will be write-protected, backed-up and a copy stored off-site. The original computer-acquired data will be analyzed and histograms, dot plots and % positive LNC will be printed out for each animal and endpoint, and stored with the raw data.

11.2: Reports:

11.2.1: Draft Report: A draft report will be submitted to the sponsor prior to submission of the final report.

11.2.2: Final Report: Following approval by the sponsor of the draft report, the final report will be submitted and will include, but not be limited to:

- Species, strain, sex, number, age and source of test animals
- Equilibration, housing conditions during exposure and post-exposure, bedding material, room temperature and humidity, light/dark cycle, diet and water
- Method of random assignment
- Physical nature, purity, stability, and lot number of test article
- Justification for choice of solvent/vehicle
- Individual and test group data (i.e., mean and std. dev.) presented in tabular form
- Systemic signs and body weights for each group
- Description of adverse effects of treatment on the mice

- List of references cited in the report, including references to any published literature used in developing the protocol, performing the testing, making and interpreting observations and compiling and evaluating results

- The number of lymphocytes, the %BrdU+ cells and the #BrdU+ cells for each animal will be determined. The calculated Stimulation Index for each group (compared to its respective Vehicle Control group) will be presented in tabular form.

11.3: Retention of Data:

    11.3.1: Raw Data will be filed at MB Research by project number.

    11.3.2: Final Reports will be filed at MB Research by sponsor name and MB project number.

    11.3.3: Test Article: Any remaining test article will be returned to the sponsor upon submission of the study report.

    11.3.4: Tissues, cells, blocks & slides will be stored at MB Research and indexed by sponsor name and MB project number. The sponsor will be contacted to determine final disposition upon submission of the report.

12.0 GOOD LABORATORY PRACTICES:

12.1: This study will be conducted in accordance with the Good Laboratory Practices of the EPA, 40 CFR 160 and 792, FDA 21 CFR 58, and as specified in, The Testing of Chemicals, published by the Organization for Economic Cooperation & Development (OECD), 1997.

12.2: Protocol: MB Research will have on file a copy of this protocol, signed and dated by both the responsible MB Study Director and the Sponsor's authorized representative.

12.3: Quality Assurance: The Quality Assurance Unit will inspect at least one in-life phase of this study, audit the raw data and audit the report in accordance with the Standard Operating Procedures of MB and the applicable government regulations.

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

13.0 SPONSOR REQUEST:

13.1: The Sponsor requests that this protocol be implemented:

☐ As written   (or)   ☐ Modifications as per attached description of changes

13.1.1: Options:
Other Vehicle (see sections 3.5 & 5.3.2): _____

13.1.2: Options (at additional cost):

Quantitative Irritation Test conc's: _____%   _____%   _____%   _____%   _____%   _____%

Additional Vehicle (see sections 3.5 & 5.3.2): _____

Additional Controls (see 5.4 & 5.5):   ☐ 25% SLS & DMSO ☐ Naive ☐ Other: _____

Immunophenotyping: ☐ %B cells   ☐ %T cells   ☐ %I-A^x+ cells   ☐ %CD69+ cells

Optional additional Tissues (e.g., ears-see 9.1): _____

13.2: Will report be submitted to a regulatory agency?   ☐ No   ☐ Yes (agency): _____

13.3: Test Article: will be identified in the report and supporting documentation exactly as indicated below:

13.3.1: Identity: The test article is identified as follows: _____

_____

pH (when applicable): _____ Lot/Batch #: _____ CAS #: _____

13.3.2: Test Concentrations (%v/v or %w/v):_____%   _____%   _____% Additional concs.:_____

13.3.3: Characterization of the test article is required in support of data submissions and should include identity, strength, purity, composition, stability and uniformity. This data must be reviewed by the Study Director prior to study initiation and included in the final report. (EPA 40 CFR 160.105 and 792.105; FDA 21 CFR 58.105, OECD 6.2). This information is:
☐ provided   (or)   ☐ not available

13.3.4: Material Safety Data Sheet Supplied: ☐ Yes   ☐ No

13.3.5: DOT Hazardous Material: ☐ No   ☐ Yes (indicate DOT shipping Name) _____
EPA Hazardous Waste: ☐ No   ☐ Yes (indicate EPA Waste Number) _____

13.3.6: Shipping Instructions for Return of Residual Test Article: (Refer to Study Information Sheet for costs)
☐ UPS / Ambient temperature (no charge)   ☐ Express carrier / Ambient temperature
☐ Overnight carrier / Dry Ice   ☐ Overnight carrier / Ice packs

13.4: Authorization Statement: This protocol is authorized for implementation at MB. This study is necessary to estimate the toxic effects of the test compound. To the best of my knowledge and information, this test is not an unnecessary duplication of any previous studies.

13.4.1: Confidentiality: Study results and reports will be released only to the below named Sponsor representative unless other Sponsor representatives are identified below.

BY: _____   FOR: _____
        (signature)   (date)   (company Name)

        _____   _____
        (typed name)   (address)

        _____   _____
        (title)   (city)   (state)   (zip)

        _____   _____
        (email)   (phone)   (fax)

Additional Sponsor Representative:_____

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

MB RESEARCH LABS
PROTOCOL NO: 5650A-06
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14.0 MB RESEARCH ACKNOWLEDGMENT: Request for implementation of this protocol and receipt of the test article is acknowledged by MB Research.

14.1   Test Article Identity: _____

   14.1.1:   Date Received: _____

   14.1.2:   Physical Description: _____

   14.1.3:   Test Article Characterization:

      14.1.3.1:   ☐ Not supplied by Sponsor, or

      14.1.3.2:   ☐ Received and Reviewed by Study Director: _____

14.2: MB Project Number assigned to this study: _____

14.3: Animal Supplier: The Licensed USDA animal supplier is:_____

14.4: Proposed Study Dates:

   14.4.1: Experimental Start Date: _____

   14.4.2: Experimental Term Date: _____

   14.4.3: Study Completion Date (Submission of Report): Approximately 6-8 weeks following Experimental Term Date.

14.5: Approval: There are currently no suitable non-animal alternatives to this study as determined according to MB Research SOP Vol. III A. This protocol is designed to avoid or minimize discomfort. The procedures will be performed by personnel thoroughly trained in the humane care and use of laboratory animals. If pain does occur as a result of the nature of the test article being used, it will be addressed according to MB SOP Vol. III A. This protocol is approved for implementation at MB Research by the below named MB Study Director.

by: _____   date

Study Director

Testing Facility: MB Research Laboratories
                  1765 Wentz Road, P. O. Box 178
                  Spinnerstown, PA 18968

This protocol was originally reviewed by the Institutional Animal Care and Use Committee (IACUC) of MB Research on the date indicated below and found to comply with acceptable standards of animal welfare and humane care. The IACUC committee will review this protocol on an annual basis. This review will be documented in the IACUC minutes and included in the semi-annual report to the institutional official.

DATE:_____10/19/06 [1]_____

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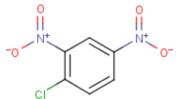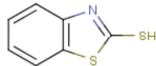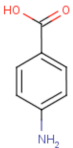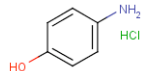
[1]Page revised 10/20/06 to reflect an updated IACUC review of this protocol.

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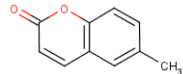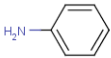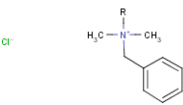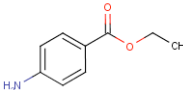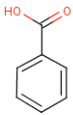
APPENDIX B
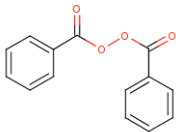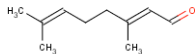
Physico-Chemical Properties Substances Tested Using the LLNA: BrdU-FC and the eLLNA: BrdU-FC Protocol

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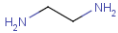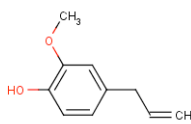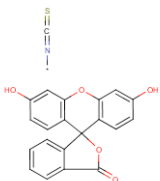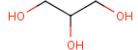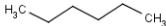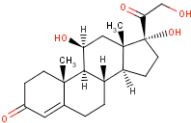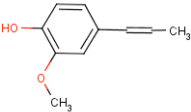
Substance Name	Synonyms	CASRN	Mol. Weight (g/mol)	Kow[1]	Peptide Reactivity[2]	Physical Form	Chemical Class[3]	Structure
2, 4-Dinitrochlorobenzene	Dinitrochlorobenzene; DNCB	97-00-7	203	2.27	High	Solid	Hydrocarbon, Halogenated; Nitro Compounds; Hydrocarbons, Cyclic	
2-Mercaptobenzothiazole	Captax	149-30-4	167	2.86	High	Solid	Heterocylic compounds	
4-Aminobenzoic acid	PABA	150-13-0	137	0.83	NA	Solid	Carboxylic Acids	
4-Aminophenol HCl	4-Hydroxyanilinium chloride	51-78-5	145	NA	NA	Solid	Amines; Phenols	
4-Phenylenediamine	p-Phenylenediamine	106-50-3	108	-0.39	NA	Solid	Amines	
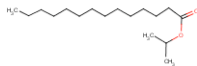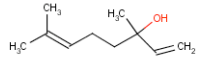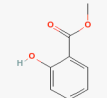
Substance Name	Synonyms	CASRN	Mol. Weight (g/mol)	Kow[1]	Peptide Reactivity[2]	Physical Form	Chemical Class[3]	Structure
6-Methylcoumarin	6-MC	92-48-8	160	2.15	Minimal	Solid	Heterocyclic Compounds	
Aniline	Benzenamine	62-53-3	93.1	1.56	NA	Liquid	Amines	
Benzalkonium chloride	Alkylbenzyldimethyl ammonium chloride; Germitol; Zephiral	8001-54-5	171	NA	NA	Solid/Liquid	Onium Compounds	
Benzocaine	Ethyl 4-aminobenzoate	94-09-7	165	1.8	NA	Solid	Carboxylic Acids	
Benzoic acid	Benzenecarboxylic acid Benzeneformic acid Benzenemethanoic acid Benzoate	65-85-0	212	1.87	NA	Solid	Carboxylic Acids	
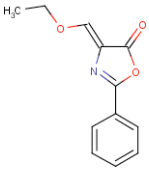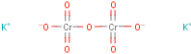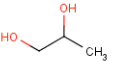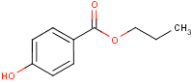
Substance Name	Synonyms	CASRN	Mol. Weight (g/mol)	Kow[1]	Peptide Reactivity[2]	Physical Form	Chemical Class[3]	Structure
Benzoyl peroxide	Dibenzoyl peroxide	94-36-0	242	3.46	High	Solid	Carboxylic acids	
Chlorobenzene	Phenyl chloride	108-90-7	113	2.64	Minimal	Liquid	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	
Chlorpromazine + UVR	NA	NA	NA	NA	NA	NA	Sulfur Compounds; Heterocyclic Compounds	NA
Citral	2,6-Octadienal, 3,7-dimethyl-	5392-40-5	152	3.45	NA	Liquid	Hydrocarbons, Other	
Cobalt chloride	Cobaltous chloride	7646-79-9	130	0.85	NA	Solid	Inorganic chemicals, Metals; Elements	

Substance Name	Synonyms	CASRN	Mol. Weight (g/mol)	Kow[1]	Peptide Reactivity[2]	Physical Form	Chemical Class[3]	Structure
Copper chloride	NA	1344-67-8	99.0	NA	NA	Solid	Inorganic chemicals, Elements	Cu—Cl
Croton oil	Croton resin	8001-28-3	NA	NA	NA	Liquid	Lipids	NA
Diethylenetriamine	1,2-Ethanediamine, N-(2-aminoethyl)-	111-40-0	103	0.29	NA	Liquid	Amines	
Diphenylcyclopropenone	2,3-Diphenylcyclopropenone	886-38-4	206	3.25	High	Solid	Hydrocarbons, Cyclic	
Ethylene glycol dimethacrylate	EGDMA	97-90-5	198	1.38	High	Liquid	Carboxylic Acids	
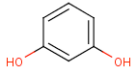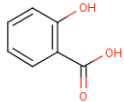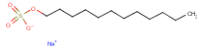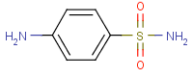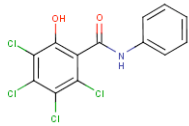
Substance Name	Synonyms	CASRN	Mol. Weight (g/mol)	Kow[1]	Peptide Reactivity[2]	Physical Form	Chemical Class[3]	Structure
Ethylenediamine	1,2-Diaminoethane	107-15-3	60.1	-2.04		Liquid	Amines	
Eugenol	2-Methoxy-4-(2-propenyl)phenol; Allylguaiacol	97-53-0	164	2.73		Liquid	Carboxylic Acids	
Fluorescein isothiocyanate	FITC	27072-45-3	389	3.32	High	Solid	Polycyclic Compounds; Isocyanates; Sulfur Compounds	
Formaldehyde	Formalin	50-00-0	30.0	0.35	Moderate	Liquid	Aldehydes	
Glycerol	Glycerin	56-81-5	92.1	0.05	Minimal	Liquid	Alcohols; Carbohydrates	
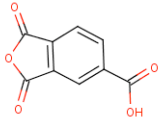
Substance Name	Synonyms	CASRN	Mol. Weight (g/mol)	Kow[1]	Peptide Reactivity[2]	Physical Form	Chemical Class[3]	Structure
Hexane	Hexyl hydride; n-Hexane	110-54-3	86.2	3.29	Minimal	Liquid	Hydrocarbons, Acyclic	
Hexyl cinnamic aldehyde	alpha-Hexylcinnamaldehyde; HCA	101-86-0	216	4.82	Minimal	Liquid	Aldehydes	
Hydrocortisone	11-beta-Hydrocortisone	50-23-7	362	1.16		Solid	Polycyclic Compounds	
Isoeugenol	2-Methoxy-4-propenylphenol; 4-Propenylguaiacol	97-54-1	164	2.65		Liquid	Carboxylic acids	
Isopropanol	Isopropyl alcohol, 2-Propanol	67-63-0	60.1	0.28	Minimal	Liquid	Alcohols	

Substance Name	Synonyms	CASRN	Mol. Weight (g/mol)	Kow[1]	Peptide Reactivity[2]	Physical Form	Chemical Class[3]	Structure
Isopropyl myristate	1-Methylethyl tetradecanoat	110-27-0	270	3.88	Minimal	Liquid	Lipids	
Lactic acid	2-Hydroxypropanoic acid	50-21-5	90.1	-0.65	Minimal	Solid	Carboxylic Acids	
Linalool	3,7-dimethylocta-,6-dien-3-ol	78-70-6	154	2.97		Liquid	Hydrocarbons	
Methyl salicylate	Oil of wintergreen; Methyl 2-hydroxybenzoate	119-36-8	152	2.6	Minimal	Liquid	Phenols; Carboxylic Acids	
Nickel chloride	Nickel dichloride	7718-54-9	129	NA		Solid	Inorganic chemicals, Elements	

Substance Name	Synonyms	CASRN	Mol. Weight (g/mol)	Kow[1]	Peptide Reactivity[2]	Physical Form	Chemical Class[3]	Structure
Oxazalone	4-Ethoxymethylene-2-phenyloxazol-5-one	15646-46-5	217	1.87	High	Solid	Heterocyclic Compounds	
Potassium dichromate	PDC; Dipotassium bichromate	7778-50-9	294	-3.59		Solid	Inorganic Chemical, Chromium Compounds; Potassium Compounds	
Propylene glycol	1,2-Dihydroxypropane; 1,2-Propanediol	57-55-6	76.1	0.43	Minimal	Liquid	Alcohols	
Propylparaben	4-Hydroxybenzoic acid, propyl ester; Propyl p-hydroxybenzoate	94-13-3	180	2.98	Minimal	Solid	Phenols; Carboxylic Acids	
Pyridine	Azabenzene	110-86-1	79.1	1.31	NA	Liquid	Heterocyclic Compounds	

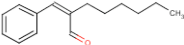| Substance Name	Synonyms	CASRN	Mol. Weight (g/mol)	Kow[1]	Peptide Reactivity[2]	Physical Form	Chemical Class[3]	Structure
Resorcinol	1,3-Dihydroxybenzene	108-46-3	110	1.03	Minimal	Solid	Phenols	
Salicylic acid	2-Hydroxybenzoic acid	69-72-7	138	1.03	NA	Solid	Phenols; Carboxylic Acids	
Sodium lauryl sulfate	Sodium dodecyl sulfate, SLS, SDS, Irium	151-21-3	288	1.69	NA	Solid	Alcohols; Sulfur Compounds; Lipids	
Sulfanilimide	4-Aminobenzenesulfonamide; p-Anilinesulfonamide; p-Sulfamidoaniline	63-74-1	172	0.4	Minimal	Solid	Amides; Sulfur Compounds; Amines	
Tetrachlorosalicylanilide	NA	7426-07-5	351	NA	Moderate	Solid	Amides; Amines	

Substance Name	Synonyms	CASRN	Mol. Weight (g/mol)	Kow[1]	Peptide Reactivity[2]	Physical Form	Chemical Class[3]	Structure
Trimellitic anhydride	4-Carboxyphthalic anhydride	552-30-7	192	1.95	Low	Solid	Anhydrides; Carboxylic Acids	
Tween 80	Polyethylene glycol sorbitan monooleate Polyoxyethylene sorbitan monooleate Polysorbate 80	9005-65-6	1310	NA	NA	Liquid	Alcohols	NA
Xylene	Dimethylbenzene	1330-20-7	107	3.16	NA	Liquid	Hydrocarbons, Cyclic	

Abbreviations: CASRN = Chemical Abstract Services Registry Number; g/mol = grams per mole; Mol. = Molecular; NA = Not available.

[1]Kow represents the estimated octanol-water partition coefficient (expressed on log scale) calculated by the Syracuse Research Corporation from the website: http://www.syrres.com/esc/est_kowdemo.htm.
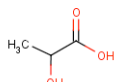
[2]Peptide reactivity data obtained from: Gerberick et al. 2007. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. Toxicol Sci 97:417-427.

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

APPENDIX C

Comparative LLNA: BrdU-FC, Traditional LLNA,
Guinea Pig Skin Sensitization, and Human Data

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Appendix C   Comparative Performance of the LLNA: BrdU-FC, Traditional LLNA, Guinea Pig, and Human Assays (in Alphabetic Order by Substance)

Substance Name	CASRN	LLNA Vehicle[1]	LLNA: BrdU-FC Call[2]	Trad. LLNA Call[2]	Guinea Pig Call[3]	Human Call[4]	Traditional LLNA Reference	Skin Irritant?	Skin Irritation Data Reference
4-Aminobenzoic acid	150-13-0	AOO	-	- 1.6 at 5%, 10%	+	-	Loveless et al. 1996	Irritant at 25% (humans)	Kligman 1966c
4-Aminophenol HCl	51-78-5	NA	+	NA	NA	NA	NA	Negative at ≤ 20% (GP)	Basketter and Scholes 1992c
Aniline	62-53-3	AOO	-	+ 3.6 at 50%, 100%	+	+	Basketter et al. 1991	Negative at 100% (GP)	Basketter et al. 2007g
Benzalkonium chloride	8001-54-5	ACE	+	+ 11.1 at 1%, 2%	-	+	Gerberick et al. 1992	Irritant at 2% ACE (mice)	Gerberick et al 2002
Benzocaine	94-09-7	AOO	+/-	+/- 7.6, 20%	+	+	Kimber et al. 1989b	Negative at ≤ 10% (GP)	Basketter and Scholes 1992c
Benzoic acid	65-85-0	DaAE (fc), ACE	- 2.5 at 5%, 25%	- 0.9 at 10%, 20%	-	-	Gerberick et al. 1992	NA	NA
Benzoyl peroxide	94-36-0	ACE	+	+ 31.4 at 5%, 10%	+	+	Kimber et al. 1998	NA	NA
Chlorobenzene	108-90-7	AOO	-	- 1.7 at 10%, 25%	-	NA	Gerberick et al. 2005	NR	Basketter et al. 1998
Chlorpromazine + UVR	NA	NA	+	NA	NA	NA	NA	NA	NA
Citral	5392-40-5	DaAE (fc), AOO	+ 14.1, 25%	+ 20.5, 20%	+	+	Basketter et al. 1991	Nonirritant @ 0.5% (GP)	Basketter et al. 2007g
Cobalt chloride	7646-79-9	L92 (fc), DMSO	+ 19.9, 5%	+ 7.2, 5%	+	+	Ikarashi et al. 1992	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992c
Copper chloride	7758-89-6	DMSO	+	+ 13.8 at 2.5%, 5%	-	-	Basketter and Scholes 1992	Nonirritant at 0.25% (GP)	Basketter and Scholes 1992c
Croton oil	8001-28-3	NA	+	NA	NA	NA	NA	NA	NA
Diethylenetriamine	111-40-0	AOO	+	+ 12.1, 10%	+	+	Gerberick et al. 2005	NA	NA
2, 4-Dinitrochlorobenzene	97-00-7	AOO	+ 8.0, 0.1%	+ 43.9, 0.25%	+	+	Kimber et al. 1995	Nonirritant at 0.1% (GP)	Basketter et al. 2007g

Substance Name	CASRN	LLNA Vehicle[1]	LLNA: BrdU-FC Call[2]	Trad. LLNA Call[2]	Guinea Pig Call[3]	Human Call[4]	Traditional LLNA Reference	Skin Irritant?	Skin Irritation Data Reference
Diphenylcyclopropenone	886-38-4	AOO	+	NR	NA	+	Basketter et al. 2000	NA	NA
Ethylene glycol dimethacrylate	97-90-5	MEK	+	+ 7.0, 50%	-	+	Gerberick et al. 2005	Nonirritant at 1% (GP)	Wahlberg and Boman 1985
Ethylenediamine	107-15-3	AOO	+	+ 6.1, 5%	+	+	Gerberick et al. 2005	Nonirritant at 2.5% (GP)	Basketter et al. 2007g
Eugenol	97-53-0	AOO	+	+ 17, 50%	+	+	Loveless et al. 1996	Mild Irritant at 100% (rabbits)	ECETOC #66, 1995
Fluorescein isothiocyanate	27072-45-3	Acetone /DBP	+	+ 16.6, 1.5%	NA	NA	Gerberick et al. 2005	NA	NA
Formaldehyde	50-00-0	DaAE (fc), AOO	+ 10.9, 2.5%	+ 11.9, 25%	+	+	Kimber et al. 1991b	Nonirritant at 2% (GP)	Basketter et al. 2007g
Glycerol	56-81-5	DMF	-	- 1.1 at 25%, 100%	-	-	Gerberick et al. 2005	NA	NA
Hexane	110-54-3	DaAE (fc), AOO	- 2.7, 100%	- 2.2, 100%	NA	-	Gerberick et al. 2005	Irritant at 100% (humans)	Kligman 1966c
Hexyl cinnamic aldehyde	101-86-0	AOO	+ 12.0, 25%	+ 20, 50%	+	+	Loveless et al. 1996	Mild Irritant at 100% (rabbits)	ECETOC #66, 1995
Hydrocortisone	50-23-7	NR	-	- 0.3 at 2.5%, 10%	NA	-	Scheider and Akkan 2004	Nonirritant at 25% (humans)	Kligman 1966c
Isoeugenol	97-54-1	AOO	+	+ 31, 5%	+	+	Basketter and Cadby 2004	Nonirritant at 5% (GP)	Basketter et al. 2007g
Isopropanol	67-63-0	AOO	-	- 1.7 at10%, 50%	-	+	Gerberick et al. 2005	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropyl myristate	110-27-0	AOO	+	+ 3.4, 100%	NA	-	Gerberick et al. 2005	Negative at 100% (rabbits)	ECETOC #66, 1995
Lactic acid	50-21-5	DMSO	-	- 2.2, 25%	-	-	Gerberick et al. 2005	Slightly irritating at 10% aq (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
Linalool	78-70-6	AOO	+	+ 8.3, 100%	NA	-	Ryan et al 2000	Mild Irritant at 100% (rabbits)	ECETOC #66, 1995
2-Mercaptobenzothiazole	149-30-4	DMF	+ 3.3, 25%	+ 8.6, 10%	+	+	Gerberick et al. 2005	Nonirritant at 10% (GP)	Basketter et al. 2007g

Substance Name	CASRN	LLNA Vehicle[1]	LLNA: BrdU-FC Call[2]	Trad. LLNA Call[2]	Guinea Pig Call[3]	Human Call[4]	Traditional LLNA Reference	Skin Irritant?	Skin Irritation Data Reference
6-Methylcoumarin	92-48-8	DaAE (fc), ACE	- 0.7 at 5%, 25%	- 0.9 at 10%, 20%	-	-	Gerberick et al. 1992	Mild Irritant at 100% (rabbits)	Opdyke 1976b
Methyl salicylate	119-36-8	AOO	-	- 2.9, 20%	-	-	Kimber et al. 1995	Irritant at 10% AOO (mice)	Gerberick et al 2002
Nickel chloride	7718-54-9	DMSO	-	- 2.4, 5%	+	+	Basketter and Scholes 1992	Negative at ≤ 0.15% (GP)	Basketter and Scholes 1992c
Oxazalone	15646-46-5	DaAE (fc); AOO	+ 14.3, 0.025%	+ 59, 0.05%	+	+	Loveless et al. 1996	NA	NA
4-phenylenediamine	106-50-3	AOO	+	+ 26.4, 1%	+	+	Gerberick et al. 2004	Nonirritant at 0.5% (GP)	Basketter et al. 2007g
Potassium dichromate	7778-50-9	L92 (fc), DMSO	+ 4.9, 0.5%	+ 33.6, 0.5%	+	+	Kimber et al. 1991b	Nonirritant at 0.15% (GP)	Basketter et al. 2007g
Propylene glycol	57-55-6	Water	-	- 1.6, 100%	-	+	Gerberick et al. 2005	Nonirritant at 25% (humans)	Kligman 1966c
Propylparaben	94-13-3	AOO	-	- 1.4 at 5%, 25%	-	+	Gerberick et al. 2005	Nonirritant at 10% (GP)	Basketter and Scholes 1992c
Pyridine	110-86-1	AOO	+	+ 3.9, 100%	NA	+	Gerberick et al 2005	NA	NA
Resorcinol	108-46-3	AOO	+	+ 10.4, 50%	-	+	Basketter et al. 2007d	Nonirritant at 15% (humans)	Kligman 1966c
Salicylic acid	69-72-7	AOO	-	- 2.5, 25%	-	-	Gerberick et al 2005	Irritant at 20% (mice)	Gerberick et al. 2002
Sodium lauryl sulfate	151-21-3	DMSO (fc), DMF	+ 4.7, 25%	+ 8.9, 20%	-	-	Loveless et al. 1996	Irritant at 20% (rabbits)	ECETOC #66, 1995
Sulfanilimide	63-74-1	DMF	-	- 1 at 10%, 50%	-	+	Gerberick et al 2005	Nonirritant at 25% (humans)	Kligman 1966c
Tetrachlorosalicylanilide	1154-59-2	DaAE (fc), ACE	+ 5.8, 0.1%	+ 18, 1%	+	+	Gerberick et al 2005	NA	NA
Trimellitic anhydride	552-30-7	AOO	+	+ 4.6, 25%	+	NA	Gerberick et al 2005	Negative at ≤ 10% (GP)	Basketter and Scholes 1992c
Tween 80	9005-65-6	AOO	+	NR	-	+	Basketter et al 2000	Nonirritant at 25% (humans)	Kligman 1966c
Xylene	1330-20-7	AOO	+	+ 3.1, 100%	NA	-	Basketter et al 1996	Irritant at 100% (humans)	Kligman 1966c

Abbreviations: CASRN = Chemical Abstract Services Registry Number; GP = guinea pig; LLNA = local lymph node assay; LLNA: BrdU-FC = murine local lymph node assay with flow cytometry measurements of bromodeoxyuridine; Trad. LLNA = murine local lymph node assay using radioactivity to detect sensitizers; NA = not available; NR = not reported; NT = not tested.

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[1] Vehicles apply to tests for the traditional LLNA and/or for the LLNA: BrdU-FC; ACE = acetone; AOO = acetone:olive oil (4:1); DaAE = dimethylacetamide/ acetone/ethanol; DMF = dimethylformamide; DMSO = dimethyl sulfoxide; L92 = 1% pluronic acid L92 surfactant in water; MEK = methyl ethyl ketone. Vehicle information reported for the LLNA: BrdU-FC is designated by "fc" after the corresponding vehicle; no vehicle information was available for the substances without SI values in the LLNA: BrdU-FC.

[2] + = sensitizer; - = nonsensitizer; +/- = equivocal (i.e., produced an equal number of divergent results when tested at least twice). The numbers below the "+" or "-" calls are the highest SI and the maximum concentration tested, unless the highest SI occurred at a lower concentration, in which case, that lower concentration is listed in addition to the maximum concentration.

[3] Calls are as noted above, and they are derived from ICCVAM (1999) based on studies using either the guinea pig maximization test or the Buehler test.

[4] Outcomes obtained by studies conducted with the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

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14   **APPENDIX D**

15   **MB Research Laboratories LLNA: BrdU-FC**

16   **Historical Data and Supplementary Studies Submitted in August 2008**

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53   **Appendix D1**
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55   **LLNA: BrdU-FC Hexyl Cinnamic Aldehyde Historical Data**
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71 **Appendix D1   MB Research Laboratories LLNA: BrdU-FC Hexyl Cinnamic Aldehyde (HCA) Historical Data**

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Vehicle: Dimethylacetamide: Acetone: Ethanol (DAE 433)

Approx. Date	Exp.	Conc.	#BrdU+ cells					Mean SI	Vehicle	#BrdU+ cells				
02/23/05	DAE1	25%	117760	83391	80372	46056	133028	8.0	DAE 433	17854	694	8547	10557	20171
03/09/05	DAE2	25%	118379	112905	66137	86912	78920	12.5	DAE 433	6070	4806	10336	6349	9540
04/13/05	DAE3	25%	143549	128742	75327	85217	114300	22.1	DAE 433	5921	2154	3028	7598	6046
02/21/07	DAE4	25%	69134	118012	142729	161192	197217	16.9	DAE 433	11958	11653	3193	8609	5434
04/24/07	DAE5	25%	153230	130402	108645	128360	166256	7.6	DAE 433	22439	16334	10622	24796	16524

Mean = 13.4
S.E.M.= 2.8
n = 5
SD = 6.159143609
CV = 45.9%

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Vehicle: Acetone

Approx. Date	Exp.	Conc.	#BrdU+ cells					Mean SI	Vehicle	#BrdU+ cells				
08/10/05	ACE1	25%	225451	188773	205942	174627	200441	25.9	Acetone	4692	12419	9429	4832	7066
01/15/06	ACE2	25%	169184	177223	357425	197383	---	16.8	Acetone	19075	2786	26691	9085	9444

Mean = 21.3
S.E.M.= 4.5
n = 2
SD = 6.433700706
CV = 30.1%

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Vehicle: Polyethylene Glycol (PEG)

Approx. Date	Exp.	Conc.	#BrdU+ cells					Mean SI	Vehicle	#BrdU+ cells				
11/02/06	PEG1	25%	59655	208656	170848	165583	163403	21.2	PEG 400	3466	2728	12807	7562	9706

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Vehicle: Acetone:Olive Oil (4:1) (AOO)

Approx. Date	Exp.	Conc.	#BrdU+ cells					Mean SI	Vehicle	#BrdU+ cells				
02/23/05	AOO1	25%	50108	123592	76275	41412	33739	27.9	AOO	525	1829	1365	3805	4118
03/09/05	AOO2	25%	117369	148495	121476	136600	32292	6.5	AOO	30626	6815	10509	15880	21565
04/13/05	AOO3	25%	124002	150392	153822	176976	115336	12.8	AOO	5946	5197	9284	21832	14129
11/22/05	AOO4	25%	126189	163255	78671	200969	336779	10.1	AOO	3538	2489	29758	31287	22592
02/28/06	AOO5	25%	68308	85505	76858	31277	124326	9.4	AOO	16256	6124	7154	7450	3954
03/25/06	AOO6	25%	155430	65215	184582	141890	118440	3.9	AOO	32099	45208	39650	20933	---
04/10/06	AOO7	25%	266503	177602	122440	142171	179884	12.0	AOO	16610	21782	17291	12189	6193
04/25/06	AOO8	25%	137384	102485	140431	106428	---	8.7	AOO	15102	26657	7144	1744	19457
05/05/06	AOO9	25%	58859	163682	77451	138717	128234	5.7	AOO	29740	12262	8204	34350	14930
05/17/06	AOO10	25%	365495	254510	321232	332264	161854	16.0	AOO	20826	11739	18860	21130	17324
06/08/06	AOO11	25%	105487	215389	99657	68481	19448	15.3	AOO	2423	11427	14122	1672	3515
07/06/06	AOO12	25%	203918	100676	136413	88222	63197	7.7	AOO	6590	15167	21934	25128	8542
07/31/06	AOO13	25%	36131	103219	147375	96343	91964	10.9	AOO	9243	4981	1904	16774	10845
09/05/06	AOO14	25%	226175	56202	186005	132224	230580	14.1	AOO	14801	11833	13848	7452	11039
01/22/07	AOO15	25%	147252	302179	107616	194550	203769	9.1	AOO	26976	13237	5478	27951	30851
06/14/07	AOO16	25%	99081	89879	21027	74939	55015	6.4	AOO	13306	8842	10701	9743	---
07/05/07	AOO17	25%	79021	59237	133493	61678	122416	5.6	AOO	15241	6786	24130	23802	10941
07/10/07	AOO18	25%	48772	39221	43185	51414	---	7.4	AOO	4501	9760	1807	4159	10796
07/31/07	AOO19	25%	185052	214338	92611	84495	91560	13.9	AOO	11665	4185	5027	16462	10805

Mean =	10.7
S.E.M.=	1.3
n =	19
SD =	5.452191028
CV =	51.0%

(2001-2007: mean = 9.46, S.E.M = 0.89, n = 45)

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Vehicle: Dimethyl sulfoxide (DMSO)

Approx. Date	Exp.	Conc.	#BrdU+ cells					Mean SI	Vehicle	#BrdU+ cells				
02/23/05	DMSO1	25%	15809	8719	9832	8004	17100	2.7	DMSO	15809	8719	9832	8004	17100
04/13/05	DMSO2	25%	101903	125239	105577	138504	98554	6.5	DMSO	7046	17265	13294	21346	28184
07/01/05	DMSO3	25%	67259	181273	87790	117030	123779	14.2	DMSO	8063	3999	13009	7524	---
07/19/05	DMSO4	25%	70203	104623	99654	120584	77416	11.8	DMSO	8930	5643	7576	10798	7210
01/15/06	DMSO5	25%	112028	82753	178147	110841	---	3.0	DMSO	28566	52544	30805	51118	---
01/15/06	DMSO6	25%	227270	20466	128422	118931	152148	4.6	DMSO	21044	36669	15855	19022	49269
04/18/06	DMSO7	25%	132131	98561	119510	93410	178088	2.7	DMSO	28490	72858	51024	30434	49298
09/28/06	DMSO8	25%	211695	156574	151312	74446	114266	5.7	DMSO	21775	32297	27295	23873	18390
12/12/06	DMSO9	25%	97495	49978	54138	85401	74355	5.2	DMSO	8840	13048	16382	17891	13976
01/12/07	DMSO10	25%	172041	218448	116542	280886	46024	10.9	DMSO	29085	12604	10896	8673	---
01/26/07	DMSO11	25%	53150	77117	85523	53637	81475	5.2	DMSO	15749	21279	6544	10898	12460
02/02/07	DMSO12	25%	103862	93932	89628	188736	101616	4.6	DMSO	24400	31525	27998	8399	32456
03/16/07	DMSO13	25%	140651	96012	158106	197058	106934	7.2	DMSO	16739	18277	12192	32368	17100
04/10/07	DMSO14	25%	139703	198785	125939	172035	199951	4.0	DMSO	35130	39654	65667	37951	28430
02/13/07	DMSO15	25%	129539	106874	146418	127342	237263	5.6	DMSO	41034	19142	25764	14747	31912
02/06/07	DMSO16	25%	216671	307915	139560	340530	163616	4.4	DMSO	47918	38943	66799	34914	74646
03/01/07	DMSO17	25%	110908	176907	113883	53820	120371	5.3	DMSO	14668	35854	28421	14682	15499
03/16/07	DMSO18	25%	140651	96012	158106	197058	106934	7.2	DMSO	16739	18277	12192	32368	17100
04/10/07	DMSO19	25%	139703	198785	125939	172035	199951	4.0	DMSO	35130	39654	65667	37951	28430
05/18/07	DMSO20	25%	92412	65730	116261	101709	105241	4.4	DMSO	18989	7644	30958	23412	28324
05/29/07	DMSO21	25%	154771	136136	180802	111264	129183	4.8	DMSO	32820	27394	38248	29027	19367
07/13/07	DMSO22	25%	323337	265624	197125	205114	136441	10.0	DMSO	11940	36005	18375	19512	27045
07/31/07	DMSO23	25%	165766	208446	218559	162598	159683	15.3	DMSO	8086	12315	21440	5066	12739
07/31/07	DMSO24	25%	260973	282192	307322	91135	161727	9.9	DMSO	20948	17975	19557	15912	37521
08/13/07	DMSO25	25%	95302	72116	137090	218461	166605	5.2	DMSO	18621	44372	15750	22894	31689
09/07/07	DMSO26	25%	138814	125135	120691	234770	164258	9.0	DMSO	21375	19944	13594	20120	12398

Mean =	6.7
S.E.M.=	0.7
n =	26
SD =	3.436304432
CV =	51.6%

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Vehicle: N,N-Dimethylformamide (DMF)

Approx. Date	Exp.	Conc.	#BrdU+ cells					Mean SI	Vehicle	#BrdU+ cells				
02/23/05	DMF1	25%	48461	19562	100359	47334	13226	8.0	DMF	9263	690	5400	3563	9728
04/13/05	DMF2	25%	97677	182285	151496	136897	149246	9.0	DMF	15381	15852	21107	9102	18650
09/10/07	DMF3	25%	77779	225705	225930	194723	124102	14.6	DMF	2745	23103	20979	4757	6453
09/18/07	DMF4	25%	31201	237936	46442	172271	116864	3.4	DMF	12373	18538	38475	77203	33052

Mean =	8.7
S.E.M.=	2.3
n =	4
SD =	4.598956403
CV =	52.6%

Vehicle: Ethanol: Water (EtOH/dH2O) (50%/50%)

Approx. Date	Exp.	Conc.	#BrdU+ cells					Mean SI	Vehicle	#BrdU+ cells				
02/23/05	ETOH1	25%	25689	67158	48171	96889	94252	16.2	EtOH	3381	2644	4230	2175	8051
04/13/05	ETOH2	25%	145785	141734	83324	191720	109025	20.2	EtOH	6220	12361	1895	6070	
09/15/06	ETOH3	25%	148177	315068	423742	442438	395294	18.3	EtOH	24483	24563	14888	15210	14888
12/12/06	ETOH4	25%	54416	33223	40326	38914	45742	6.1	EtOH	1877	7936	8160	5878	10751

Mean =	15.2
S.E.M.=	3.1
n =	4
SD =	6.293414547
CV =	41.4%

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82 Abbreviations: BrdU = Bromodeoxyuridine; Conc. = Concentration; CV = Coefficient of variance; Exp. = Experiment identification; FC = Flow cytometry; LLNA = Local
83 lymph node assay; LLNA = Local lymph node assay; n = Number of values used in the calculation; SD = Standard deviation; S.E.M. = Standard error of mean; SI =
84 Stimulation index.

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98   **Appendix D2**
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100   **MB Research Laboratories LLNA: BrdU-FC Study No. 08-17098.26**
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119 **Appendix D2**   **MB Research Laboratories LLNA: BrdU-FC Study No. 08-17098.26**
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08-17098.26 MB Research

Treatment	Animal#	Total # Cells in Node x10³	%BrdU+	Lymphocyte Proliferation	SI	
AOO	1	1702	0.94	15994	0.5	
	2	2670	0.47	12547	0.4	
	3	4643	0.77	35749	1.0	
	4	4311	0.59	25436	0.7	
	5	6174	1.33	82118	2.4	
	Mean	3900	0.82	34369	**1.0**	
	StDev	1749	0.34	28176	0.8	
5% HCA lot/batch# 04072JE	6	346	20.98	72538	2.1	
	7	5283	0.74	39091	1.1	
	8	5639	1.13	63724	1.9	
	9	8992	0.74	66539	1.9	
	10	6808	1.38	93954	2.7	
	Mean	5414	4.99	67169	**2.0**	
	StDev	3182	8.94	19666	0.6	
10% HCA lot/batch# 04072JE	11	3530	1.27	44831	1.3	
	12	6967	1.05	73156	2.1	
	13	7057	1.54	108682	3.2	
	14	4813	1.07	51502	1.5	
	15	5116	1.02	52186	1.5	
	Mean	5497	1.19	66071	**1.9**	
	StDev	1506	0.22	26087	0.8	
25% HCA lot/batch# 04072JE	16	8328	1.61	134073	3.9	
	17	14855	1.73	256987	7.5	
	18	9217	1.29	118899	3.5	
	19	6490	2.18	141487	4.1	
	20	12697	1.65	209492	6.1	
	Mean	10317	1.69	172188	**5.0**	a
	StDev	3394	0.32	58774	1.7	
0.025% DNCB lot/batch# 10505DD	21	2954	1.36	40178	1.2	
	22	3872	0.67	25942	0.8	
	23	6971	0.90	62737	1.8	
	24	450	1.87	8410	0.2	
	25	1952	0.70	13662	0.4	
	Mean	3240	1.10	30186	**0.9**	
	StDev	2442	0.51	21935	0.6	
0.05% DNCB lot/batch# 10505DD	26	2285	1.33	30384	0.9	
	27	7659	0.96	73529	2.1	
	28	10723	2.28	244484	7.1	
	29	3644	1.81	65956	1.9	
	30	5343	1.04	55570	1.6	
	Mean	5931	1.48	93985	**2.7**	
	StDev	3347	0.56	85696	2.5	

0.1% DNCB lot/batch# 10505DD	31	4140	0.82	33948	1.0	
	32	10752	2.52	270938	7.9	
	33	6755	1.70	114827	3.3	
	34	10595	2.05	217187	6.3	
	35	7509	1.15	86354	2.5	
	Mean	7950	1.65	144651	**4.2**	a
	StDev	2783	0.68	97151	2.8	
DMSO (vehicle)	36	4046	0.88	35605	2.0	
	37	3061	0.58	17751	1.0	
	38	1111	0.78	8666	0.5	
	39	2772	0.44	12198	0.7	
	40	2968	0.54	16027	0.9	
	Mean	2792	0.64	18049	**1.0**	
	StDev	1061	0.18	10424	0.6	
25% SDS lot/batch# 046K0085	41	665	1.69	11243	0.6	
	42	7899	0.90	71091	3.9	
	43	9321	1.03	96006	5.3	
	44	5741	1.26	72333	4.0	
	45	4253	0.47	19987	1.1	
	Mean	5576	1.07	54132	**3.0**	
	StDev	3366	0.45	36667	2.0	
25% MBT lot/batch# 11020DE	46	7694	1.58	121557	6.7	
	47	10210	1.57	160289	8.9	
	48	5323	1.22	64938	3.6	
	49	748	0.89	6659	0.5	
	50	9050	1.34	121263	6.7	
	Mean	6605	1.32	94941	**5.3**	a
	StDev	3745	0.29	59928	3.3	

X = Outlier   a = SI ≥ 3
ND = No Data; animal did not survive

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136   **Appendix D3**
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138   **MB Research Laboratories LLNA: BrdU-FC Study No. 08-17150.26**
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155 **Appendix D3**   **MB Research Laboratories LLNA: BrdU-FC Study No. 08-17150.26**
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MBR 08-17150.26

Treatment	Animal#	Total # Cells in Node x10³	%BrdU+	Lymphocyte Proliferation	SI	
AOO (Vehicle 1)	1	3027	0.76	23003	0.6	
	2	5820	0.70	40737	1.1	
	3	3117	0.55	17142	0.5	
	4	6386	0.97	61944	1.7	
	5	3867	1.11	42924	1.2	
	Mean	4443	0.82	37150	**1.0**	
	StDev	1563	0.22	17758	0.5	
5% HCA	6	2404	0.80	19234	0.5	
	7	5046	0.41	20689	0.6	
	8	5819	0.58	33752	0.9	
	9	7018	0.67	47017	1.3	
	10	7085	0.78	55265	1.5	
	Mean	5474	0.65	35191	**0.9**	
	StDev	1917	0.16	15889	0.4	
10% HCA	11	4373	1.00	43728	1.2	
	12	8529	1.09	92962	2.5	
	13	9308	1.09	101457	2.7	
	14	2459	1.32	32452	0.9	
	15	3834	0.79	30287	0.8	
	Mean	5700	1.06	60177	**1.6**	
	StDev	3032	0.19	34321	0.9	
25% HCA	16	11948	1.69	201921	5.4	
	17	11354	1.91	216861	5.8	
	18	15249	1.63	248555	6.7	
	19	10212	1.59	162375	4.4	
	20	7273	1.45	105455	2.8	
	Mean	11207	1.65	187033	**5.0**	a
	StDev	2889	0.17	55135	1.5	
0.05% DNCB	21	1899	3.38	64178	1.7	
	22	4846	1.30	62992	1.7	
	23	10236	2.55	261018	7.0	
	24	5662	1.56	88319	2.4	
	25	8965	1.72	154189	4.2	
	Mean	6321	2.10	126139	**3.4**	a
	StDev	3335	0.85	84018	2.3	
0.1% DNCB	26	7445	2.42	180169	4.8	
	27	6145	2.24	137648	3.7	
	28	10100	2.17	219165	5.9	
	29	3407	2.55	86885	2.3	
	30	4604	3.53	162530	4.4	
	Mean	6340	2.58	157279	**4.2**	a
	StDev	2598	0.55	49300	1.3	

DMSO (Vehicle 2)	31	5184	0.59	30587	0.8
	32	9141	0.53	48446	1.3
	33	7382	0.66	48718	1.4
	34	4897	0.60	29384	0.8
	35	2377	0.97	23054	0.6
	Mean	5796	0.67	36038	**1.0**
	StDev	2578	0.17	11804	0.3
25% MBT	36	10640	1.33	141515	3.9
	37	7276	1.61	117144	3.3
	38	7586	1.22	92543	2.6
	39	9444	1.53	144486	4.0
	40	10093	1.41	142308	3.9
	Mean	9008	1.42	127599	**3.5** a
	StDev	1504	0.16	22547	0.6
DMF (Vehicle 3)	41	7987	0.78	62301	0.9
	42	2762	0.59	16294	0.2
	43	1804	0.69	12448	0.2
	44	3335	0.72	24008	0.3
	45	12234	1.87	228780	3.3
	Mean	5624	0.93	68766	**1.0**
	StDev	4396	0.53	91617	1.3
25% MBT	46	6113	1.16	70909	1.0
	47	8182	1.50	122726	1.8
	48	6351	1.02	64783	0.9
	49	2094	1.07	22400	0.3
	50	11420	0.92	105062	1.5
	Mean	6832	1.13	77176	**1.1**
	StDev	3394	0.22	38877	0.6
DMA (Vehicle 4)	51	3701	0.76	28130	0.6
	52	7703	0.87	67018	1.5
	53	6398	0.83	53103	1.2
	54	4342	0.92	39946	0.9
	55	4615	0.90	41533	0.9
	Mean	5352	0.86	45946	**1.0**
	StDev	1652	0.06	14732	0.3
25% MBT	56	8884	1.20	106611	2.3
	57	7137	1.39	99208	2.2
	58	3524	0.97	34185	0.7
	59	3382	1.05	35511	0.8
	60	7608	1.97	149868	3.3
	Mean	6107	1.32	85077	**1.9**
	StDev	2506	0.40	49769	1.1

X = Outlier   a = SI ≥ 3
ND = No Data; animal did not survive

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171 **Appendix D4**
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173 **MB Research Laboratories LLNA: BrdU-FC Study No. 08-17158.26**
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189   **Appendix D4**   **MB Research Laboratories LLNA: BrdU-FC Study No. 08-17158.26**
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MBR 08-17158.26

Treatment	Animal#	Total # Cells in Node x10^3	%BrdU+	Lymphocyte Proliferation	SI	
AOO	1	3228	0.84	27117	1.7	
(Vehicle 1)	2	4385	0.64	28061	1.8	
	3	1495	0.48	7177	0.5	
	4	1527	0.95	14509	0.9	
	5	267	0.53	1414	0.1	
	Mean	2180	0.69	15656	**1.0**	
	StDev	1621	0.20	11846	0.8	
0.025%	6	3308	1.62	53582	3.4	
DNCB	7	3174	0.87	27614	1.8	
	8	4285	1.34	57416	3.7	
	9	251	13.98	35090	2.2	
	10	3031	0.89	26971	1.7	
	Mean	2810	3.74	40134	**2.6**	
	StDev	1513	5.73	14448	0.9	
0.05%	11	5210	1.82	94819	6.1	
DNCB	12	2969	2.10	62354	4.0	
	13	2502	0.68	17015	1.1	
	14	1818	1.50	27270	1.7	
	15	924	2.27	20963	1.3	
	Mean	2685	1.67	44484	**2.8**	
	StDev	1608	0.63	33380	2.1	
0.1%	16	4635	2.48	114944	7.3	
DNCB	17	4893	1.81	88563	5.7	
	18	6219	1.76	109446	7.0	
	19	4066	1.92	78067	5.0	
	20	6313	2.13	134467	8.6	
	Mean	5225	2.02	105097	**6.7**	a
	StDev	997	0.29	22262	1.4	
5%	21	1434	0.72	10321	0.7	
HCA	22	2103	0.59	12405	0.8	
	23	5175	1.21	62621	4.0	
	24	2918	1.08	31512	2.0	
	25	908	0.81	7353	0.5	
	Mean	2507	0.88	24842	**1.6**	
	StDev	1670	0.26	23147	1.5	
10%	26	1353	1.02	13796	0.9	
HCA	27	3082	0.86	26505	1.7	
	28	2892	0.75	21692	1.4	
	29	1283	0.76	9747	0.6	
	30	1717	0.73	12530	0.8	
	Mean	2065	0.82	16854	**1.1**	
	StDev	860	0.12	6984	0.4	

25% **HCA**	31	7852	1.24	97359	6.2
	32	12647	1.72	217528	13.9
	33	11764	1.71	201164	12.8
	34	8966	1.80	161388	10.3
	35	12993	2.00	259865	16.6
	Mean	10844	1.69	187461	**12.0** a
	StDev	2302	0.28	61507	3.9
DMF(Vehicle 2)	36	804	0.44	3535	0.2
	37	5252	0.73	38336	2.1
	38	4903	0.65	31868	1.7
	39	403	1.26	5078	0.3
	40	1913	0.65	12435	0.8
	Mean	2655	0.75	18250	**1.0**
	StDev	2283	0.31	15912	0.9
2.5% **MBT**	41	3219	0.60	19316	1.1
	42	2824	0.59	16660	0.9
	43	2867	0.56	16055	0.9
	44	104	6.98	7242	0.4
	45	2672	0.54	14429	0.8
	Mean	2337	1.85	14740	**0.8**
	StDev	1264	2.87	4546	0.2
5% **MBT**	46	2204	0.73	16087	0.9
	47	2240	0.94	21054	1.2
	48	3679	0.48	17660	1.0
	49	1374	1.18	16210	0.9
	50	2012	0.59	11871	0.7
	Mean	2302	0.78	16576	**0.9**
	StDev	845	0.28	3307	0.2
10% **MBT**	51	3674	0.85	31228	1.7
	52	1737	0.64	11117	0.6
	53	3027	0.80	24216	1.3
	54	3617	1.32	47748	2.6
	55	5021	1.07	53727	2.9
	Mean	3415	0.94	33607	**1.8**
	StDev	1189	0.26	17353	1.0
25% **MBT**	56	6857	1.52	104230	5.7
	57	5298	1.30	68874	3.8
	58	3823	1.31	50081	2.7
	59	538	2.53	13618	0.7
	60	4456	1.43	63721	3.5
	Mean	4195	1.62	60105	**3.3** a
	StDev	2339	0.52	32782	1.8
DMSO **(Vehicle 3)**	61	1739	0.46	7998	0.5
	62	3634	0.43	15626	1.1
	63	2299	0.56	12872	0.9
	64	1560	1.47	22925	1.5
	Mean	2308	0.73	14855	**1.0**

	StDev	939		0.50		6236	0.4
25%	65	ND		ND		ND	ND
SLS	66	3810		2.24		85344	5.7
	67	4003		1.39		55638	3.7
	68	ND		ND		ND	ND
	Mean	3906		1.82		70491	**4.7** a
	StDev	136		0.60		21005	1.4

X = Outlier   a = SI ≥ 3

ND = No Data; animal did not survive

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220   **Appendix D5**
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238 **Appendix D5**   **MB Research Laboratories LLNA: BrdU-FC Study No. 08-17195.26**
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MBR 08-17195.26

Treatment	Animal#	Total # Cells in Node x10^3	%BrdU+	Lymphocyte Proliferation	SI	
AOO	1	2112	0.71	14993	0.9	
(Vehicle 1)	2	4799	0.37	17754	1.1	
	3	2242	0.51	11432	0.7	
	4	4810	0.53	25492	1.6	
	5	749	1.26	9441	0.6	
	Mean	2942	0.68	15822	**1.0**	
	StDev	1797	0.35	6283	0.4	
5%	6	5491	0.59	32394	2.0	
HCA	7	6313	0.91	57444	3.6	
	8	2599	0.83	21570	1.4	
	9	675	2.41	16255	1.0	
	10	3018	0.94	28372	1.8	
	Mean	3619	1.14	31207	**2.0**	
	StDev	2282	0.73	15924	1.0	
10%	11	7334	0.99	72604	4.6	
HCA	12	6449	0.69	44498	2.8	
	13	3096	0.83	25693	1.6	
	14	6409	1.15	73701	4.7	
	15	2051	2.96	60710	3.8	
	Mean	5068	1.32	55441	**3.5**	a
	StDev	2336	0.93	20374	1.3	
25%	16	8679	1.46	126717	8.0	
HCA	17	10482	1.23	128932	8.1	
	18	18066	1.18	213173	13.5	
	19	7424	1.33	98736	6.2	
	20	11618	1.74	202153	12.8	
	Mean	11254	1.39	153942	**9.7**	a
	StDev	4136	0.22	50614	3.2	
0.025%	21	5456	0.84	45826	2.9	
DNCB	22	6522	0.75	48917	3.1	
	23	6367	1.21	77041	4.9	
	24	2272	1.23	27946	1.8	
	25	226	1.57	3548	0.2	
	Mean	4169	1.12	40656	**2.6**	
	StDev	2792	0.33	27191	1.7	
0.05%	26	7532	1.50	112976	7.1	
DNCB	27	7425	1.34	99495	6.3	
	28	6132	1.29	79106	5.0	
	29	8813	0.86	75788	4.8	
	30	641	1.95	12504	0.8	
	Mean	6109	1.39	75974	**4.8**	a
	StDev	3200	0.39	38603	2.4	

0.1% **DNCB**	31	8476	1.82	154268	9.7	
	32	2122	2.49	52825	3.3	
	33	9311	1.84	171327	10.8	
	34	9196	2.28	209657	13.3	
	35	1697	2.74	46498	2.9	
	Mean	6160	2.23	126915	**8.0**	a
	StDev	3897	0.40	73354	4.6	
DMSO(Vehicle 2)	36	794	1.10	8731	0.6	
	37	2435	0.71	17287	1.1	
	38	3416	0.69	23569	1.6	
	39	567	1.53	8667	0.6	
	40	3594	0.48	17250	1.1	
	Mean	2161	0.90	15101	**1.0**	
	StDev	1424	0.42	6385	0.4	
5% **MBT**	41	7469	0.91	67970	4.5	
	42	4815	1.33	64043	4.2	
	43	7762	0.95	73741	4.9	
	44	2299	0.78	17932	1.2	
	45	2923	1.51	44134	2.9	
	Mean	5054	1.10	53564	**3.5**	a
	StDev	2518	0.31	22820	1.5	
10% **MBT**	46	11088	1.31	145253	9.6	
	47	6690	1.29	86301	5.7	
	48	9184	1.45	133168	8.8	
	49	6881	0.95	65365	4.3	
	50	4024	1.43	57547	3.8	
	Mean	7573	1.29	97527	**6.5**	a
	StDev	2683	0.20	39708	2.6	
25% **MBT**	51	9343	1.27	118660	7.9	
	52	11058	1.24	137119	9.1	
	53	4847	1.00	48468	3.2	
	54	2353	1.00	23533	1.6	
	55	13702	2.04	279516	18.5	
	Mean	8261	1.31	121459	**8.0**	a
	StDev	4614	0.43	100190	6.6	
DaAE 433 **Vehicle 3**	56	2479	0.78	19332	0.8	
	57	4315	0.52	22435	1.0	
	58	2354	0.48	11297	0.5	
	59	2990	0.56	16745	0.7	
	60	2580	1.76	45404	2.0	
	Mean	2943	0.82	23043	**1.0**	
	StDev	803	0.54	13151	0.6	
5% **MBT**	61	7187	0.58	41682	1.8	
	62	4595	0.39	17919	0.8	
	63	947	0.82	7767	0.3	
	64	3023	0.49	14813	0.6	
	65	1552	1.38	21411	0.9	

	Mean	3461	0.73	20718	**0.9**
	StDev	2516	0.40	12751	0.6
10%	66	4122	0.37	15252	0.7
MBT	67	4657	0.57	26542	1.2
	68	6459	0.49	31650	1.4
	69	5619	0.73	41021	1.8
	70	6794	0.59	40083	1.7
	Mean	5530	0.55	30910	**1.3**
	StDev	1142	0.13	10620	0.5
25%	71	4419	0.96	42425	1.8
MBT	72	4558	0.66	30080	1.3
	73	1402	0.74	10371	0.5
	74	4822	0.93	44847	1.9
	75	462	2.54	11735	0.5
	Mean	3133	1.17	27891	**1.2**
	StDev	2041	0.78	16367	0.7

X = Outlier   a = SI ≥ 3
ND = No Data; animal did not survive

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