APPENDIX A

Flow Cytometery – Local Lymph Node Assay Protocol

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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:

4.1: Animal Requirements:		IRRITATION PRESCREEN	QUANTITATIVE IRRITATION TEST (if needed)	MAIN TEST
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)		

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.

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.

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.

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^{o}C will not be used unless the substance is known to be heat stable.

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.

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^k+ 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°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.

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

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.

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^k+ 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: _____ (signature) _____ (date)

_____ (typed name)

_____ (title)

_____ (email)

FOR: _____ (company Name)

_____ (address)

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

_____ (phone) _____ (fax)

Additional Sponsor Representative: _____

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]

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