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September 6, 2012

Dr. William Stokes
Director, NICEATM
Executive Director, ICCVAM
NIEHS
P.O. Box 12233, EC-17
Research Triangle Park, NC 27709

Dear Dr. Stokes:

I would like to nominate a new alternative dermal contact allergen identification assay to the Interagency Coordination Committee on the Validation of Alternative Methods (ICCVAM). This assay detects substances that cause dermal sensitization (delayed-type hypersensitivity) and discriminates them from irritants. The In Vitro Sensitivity Assay (IVSA) can predict allergic contact dermatitis (ACD) causing chemicals, or dermal sensitizers, using a completely *in vitro* system requiring no test animals. Thus, this assay nomination is within the mission of ICCVAM.

The IVSA is based on the secretion of IL-18 by human keratinocytes, cultured as a 3D differentiated skin model (EpiDerm™). Keratinocytes constitutively express IL-18 mRNA, expressed as a pre-IL-18, which is later converted and stored in the cytosol as IL-18. IL-18 acts as a master regulator during the sensitization signaling cascade, when activated by the inflammasome, which includes increased expression and release of IL-18. This cytokine release is dose-dependent upon increasing concentrations of sensitizer exposure. Quantification of IL-18 secretion is made by use of a commercially available ELISA kit, and the Stimulation Index (SI) test/control ratio is determined using the vehicle-only treatment as the control. An SI > 2.0 response is indicative of a dermal sensitizer, wheras an SI between 1.6 and 2.0 is a likely sensitizer (also a positive response) and an SI <1.6 is considered a negative response and therefore a non-sensitizer. The initial assessment of the IL-18 secretion for use in allergen screening was demonstrated by Deng et al. in abstract 2571, The Toxicologist, 120 (Sup.2), 2011. Where this EpiDerm™ tissue has been shown to be highly responsive to low concentrations of dermal sensitizers. We have since added the optimized cut-off SI = 1.6 (from the original 2.0 value).

Advantages of the IVSA include test concentrations typically covering a wide concentration range of one or two logarithmic decades. Another advantage to the IVSA is that direct application (neat) is possible, avoiding solubility issues when encountered, especially with formulated products and mixtures. Also, the IVSA can be implemented in any cell culture laboratory, needing only a spectrophotometer to measure the chromophore generated by IL-18 ELISA kit. The cost of the IVSA is lower than the existing LLNA and guinea pig tests, and other *in vitro* assay candidates such as the DPRA and Keratinosens. Moderate to high throughput is

possible due to 24-well and 96-well availability of the 3D tissue model. Total assay time is short and requires less than 2 days

I respectfully request that you accept this nomination to ICCVAM for consideration as an alternative assay for identification of contact allergens. Furthermore we propose that we work with ICCVAM and NICEATM to conduct validation studies to further optimize appropriate cut-off values and expand the applicability domain to maximize performance. Please find the details of the nomination in the attached documents.

Sincerely,

/s/

George DeGeorge, Ph.D., DABT
Chief Scientific Officer
In Vitro Test Labs
MB Research Laboratories

In Vitro Sensitization Assay (IVSA) — Summary of Data for ICCVAM Submission[1]

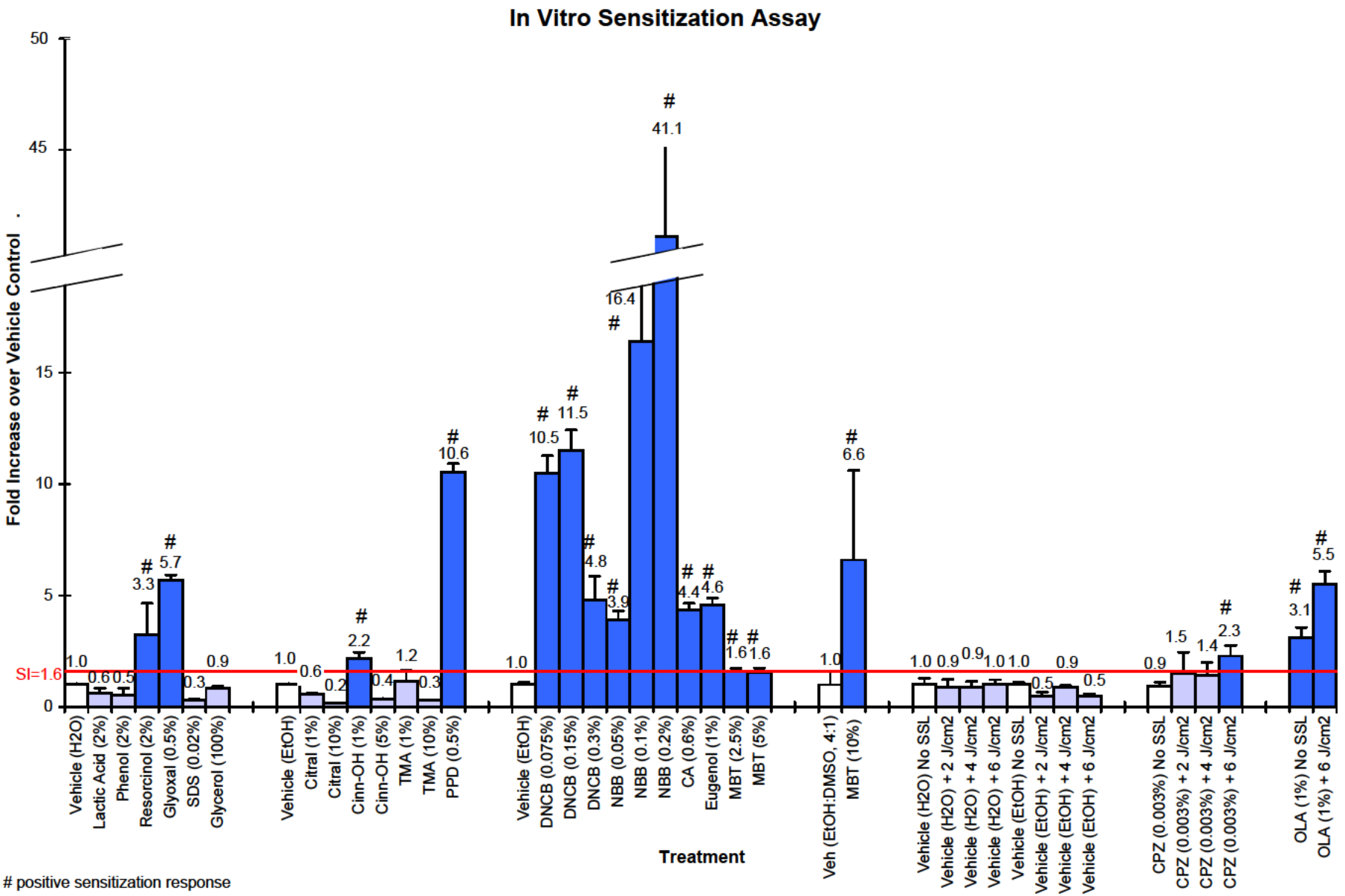
No.	Substance	Abv.	CASRN	Source	Vehicle	Conc. tested	SI	Result	Viability	Date	Solubility	Formula	(g/mol)	Phase	Class
1	Chlorpromazine	CPZ	69-09-0	Sigma	H_2O	0.003% no SSL	2.89	+	46.6%	03/23/12	H2O	$C_{17}H_{19}ClN_2S$*HCl	355.33	solid	Moderate* Photosensitizer
					H2O	0.003% 6J SSL	0.81	–	ND	03/23/12					
					H_2O	0.01% no SSL	8.48	+	32.4%	03/23/12					
					H2O	0.01% 6J SSL	1.24	–	ND	03/23/12					
					H_2O	0.003% no SSL	0.94	–	91.5%	05/18/12					
					H_2O	0.003% 2J SSL	1.49	–	91.6%	05/18/12					
					H_2O	0.003% 4J SSL	1.41	–	73.1%	05/18/12					
					H_2O	0.003% 6J SSL	2.29	+	81.9%	05/18/12					
2	Cinnamaldehyde	CA	104-55-2	SAFC	EtOH	0.6%	4.34	+	32.5%	03/08/12	EtOH, H2O	C_9H_8O	132.16	liquid	Moderate
3	Cinnamic alcohol	CAL	104-54-1	Aldrich	EtOH	1%	2.20	+	25.9%	04/19/12	EtOH, H_2O	$C_9H_{10}O$	134.18	solid	Weak
					EtOH	5%	0.40	–	14.0%	04/19/12					
4	Citral	CIT	5392-40-5	Aldrich	EtOH	1%	0.60	–	2.1%	04/19/12	EtOH, H_2O	$C_{10}H_{16}O$	152.23	liquid	Weak
					EtOH	10%	0.20	–	26.4%	04/19/12					
5	Dinitrochlorobenzene	DNCB	97-00-7	Aldrich	EtOH	0.075%	10.54	+	9.0%	03/08/12	EtOH, H_2O	$C_6H_3ClN_2O_4$	202.55	solid	Strong/Severe
					EtOH	0.15%	11.48	+	9.0%	03/08/12					
					EtOH	0.30%	4.78	+	8.2%	03/08/12					
6	Eugenol	EU	97-53-0	Aldrich	EtOH	1%	4.56	+	9.5%	03/08/12	EtOH	$C_{10}H_{12}O_2$	164.2	liquid	Weak
7	Glycerol	GLC	56-81-5	Sigma-Aldrich.	H_2O	100%	0.86	–	102.1%	03/23/12	H_2O	$C_3H_8O_3$	92.09	liquid	Non-toxin
8	Glyoxal	GLO	107-22-2	Sigma	H_2O	0.5%	5.68	+	23.0%	03/23/12	H_2O	OHCCHO	58.04	liquid	Moderate
9	Lactic acid	LA	50-21-5	Fluka	H_2O	2%	0.63	–	85.6%	03/23/12	H_2O	$C_3H_6O_3$	90.08	liquid	Irritant
10	2-Mercaptobenzothiazole	MBT	149-30-4	Fluka	EtOH	2.5%	1.61	+	80.9%	03/08/12	EtOH, DMSO:EtOH 1:4, H_2O	$C_7H_5NS_2$	167.25	solid	Weak**
					EtOH	5%	1.56	–	84.4%	03/08/12					
					DMSO: EtOH 1:4	10%	6.60	+	59.1%	05/18/12					
11	4-Nitrobenzylbromide	NBB	100-11-8	Aldrich	EtOH	0.05%	3.89	+	62.4%	03/08/12	EtOH	$C_7H6BrNO_2$	216.03	solid	Strong/Severe
					EtOH	0.1%	16.40	+	50.4%	03/08/12					
					EtOH	0.2%	41.14	+	35.6%	03/08/12					
12	Olaquindox	OLA	23696-28-8	MP Biomedical	H_2O	1% no SSL	3.12	+	60.6%	05/18/12	H_2O	$C_{12}H_{13}N_3O_4$	263.25	solid	Moderate* Photosensitizer
					H_2O	1% 6J SSL	5.50	+	36.7%	05/18/12					
13	Phenol	PH	108-95-2	Sigma	H_2O	2%	0.52	–	12.7%	03/23/12	H_2O	C_6H_5OH	94.11	solid	Irritant
14	p-Phenyldiamine	PPD	106-50-3	Sigma	EtOH	1%	9.17	+	72.7%	03/08/12	EtOH, H_2O	$C_6H_8N_2$	108.14	solid	Strong/Severe
					EtOH	0.5%	10.60	+	97.7%	04/19/12					
					EtOH	1%	136.90	+	59.0%	04/19/12					
15	Resorcinol	RES	108-46-3	Sigma-Aldrich	H_2O	2%	3.25	+	22.7%	03/23/12	H_2O	$C_6H_6O_2$	110.11	solid	Moderate
16	Sodium dodecyl sulfate	SDS	151-21-3	Acros	H_2O	0.02%	0.30	–	102.2%	03/23/12	H_2O	$C_{12}H_{25}NaO_4S$	288.38	solid	Irritant
17	Trimellitic anhydride *1,2,4-Benzenetricarboxylic anhydride*	TMA	552-30-7	Sigma	EtOH	1%	1.20	–	76.1%	04/19/12	EtOH, H_2O	$C_9H_4O_5$	192.13	solid	Respiratory Sensitizer
					EtOH	10%	0.30	–	12.9%	04/19/12					

1 = Note: studies were not conducted according to the Good Laboratory Practices

* known positive, literature is inconclusive

** might be moderate

SSL = solar simulated light


In Vitro Sensitization Assay
Fold Increase over Vehicle Control
50
45
15
10
5
0
SI=1.6
Vehicle (H2O)
Lactic Acid (2%)
Phenol (2%)
Resorcinol (2%)
Glyoxal (0.5%)
SDS (0.02%)
Glycerol (100%)
Vehicle (EtOH)
Citral (1%)
Citral (10%)
Cinn-OH (1%)
Cinn-OH (5%)
TMA (1%)
TMA (10%)
PPD (0.5%)
Vehicle (EtOH)
DNCB (0.075%)
DNCB (0.15%)
DNCB (0.3%)
NBB (0.05%)
NBB (0.1%)
NBB (0.2%)
CA (0.6%)
Eugenol (1%)
MBT (2.5%)
MBT (5%)
Veh (EtOH:DMSO, 4:1)
MBT (10%)
Vehicle (H2O) No SSL
Vehicle (H2O) + 2 J/cm2
Vehicle (H2O) + 4 J/cm2
Vehicle (H2O) + 6 J/cm2
Vehicle (EtOH) No SSL
Vehicle (EtOH) + 2 J/cm2
Vehicle (EtOH) + 4 J/cm2
Vehicle (EtOH) + 6 J/cm2
CPZ (0.003%) No SSL
CPZ (0.003%) + 2 J/cm2
CPZ (0.003%) + 4 J/cm2
CPZ (0.003%) + 6 J/cm2
OLA (1%) No SSL
OLA (1%) + 6 J/cm2
Treatment
positive sensitization response

IVSA: Test Method and Results

This assay is based on the detection of the secretion of IL-18 into the culture medium of a 3D epidermal human skin model, EpiDerm™ produced by MatTek Corporation (Ashland, MA). We tested 17 chemicals consisting of 12 sensitizers and 5 irritating/non-irritating non-sensitizers. All test chemicals were dissolved and diluted in either distilled water, ethanol, or ethanol:dimethylsulfoxide 4:1 as solvent vehicles. All test concentration results were compared to vehicle by a simple ratio of the Stimulation Index (SI = test / vehicle) as per the protocol. SI values of >2.0 are considered positive sensitizer responses, whereas SIs between 1.6 – 2.0 are deemed "likely sensitizers" and a positive response, especially if a dose-dependent increase is observed. Those chemicals yielding an SI < 1.6 are considered non-sensitizers. IVSA correctly predicted sensitizers with an Accuracy of 94%, a Sensitivity of 92%, and Specificity of 100%. See contingency table with Cooper statistics below:

	Known +	**Known –**	
Tested +	11	0	11
Tested –	1	5	6
	12	5	17

Accuracy	94%	(16/17)
Sensitivity	92%	(11/12)
Specificity	100%	(5/5)
Positive Predictivity	100%	(11/121)
Negative Predictivity	83%	(5/6)

Classes of chemicals tested ranged from non-sensitizing to weak to strong/severe. In general, the concentrations needed to yield an SI ≥1.6 ($SI_{cut\text{-}off}$) and the magnitude of the SI response of each test chemical correlated well with its known human or animal test responses or classification (HRIPT, GP tests, or LLNA EC3). These seventeen test chemicals were selected from those tested in related assays by other researchers, especially Corsini et al. (*Toxicol In Vitro* 23 (5):789-796, 2009), Galbiati et al. (*Toxicol In Vitro* 25 (3):724-732, 2011), and Deng et al. (abstract 2571, The Toxicologist, 120 (Sup.2), 2011). In the case of photosensitizers, chemicals were chosen from the literature as moderate to strong sensitizers, and were irradiated by a Honle 500 solar simulator for the UVA light dose indicated in the summary data table.

MB RESEARCH LABORATORIES

In Vitro Sensitization Assay Protocol

1.0 TITLE OF STUDY: IN VITRO SENSITIZATION ASSAY (IVSA)

2.0 OBJECTIVE: To determine the possible sensitizing potential of a test article *in vitro* as an alternative assay for animal based test systems.

3.0 TEST ARTICLE:

3.1: Source: All test articles will be supplied by the Sponsor. Prior study termination, the Sponsor should provide the Study Director with test article characterization (refer to Section 13 herein).

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: Test Article Description: The observable physical properties of the test article will be recorded.

3.4: Storage: Refer to Section 13.

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

3.6: Vehicle: A suitable vehicle will be added to the test article to generate dilutions of the test article. The vehicle will be ethanol, unless the test article is not soluble or if otherwise chosen by the Study Director in consultation with the Sponsor based on their previous knowledge with the test article. When a vehicle or diluent other than ethanol will be used (see section 5.1), 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.

4.0 TEST SYSTEM AND JUSTIFICATION:

4.1: Test System: MatTek EpiDerm™ Skin Model (EPI-200) and Methyl thiazole tetrazolium (MTT) Kit (MTT-100) components (when applicable). Medical and Biological Laboratories (MBL) IL-18 ELISA kit.

4.2: Justification: The EpiDerm™ Skin Model closely parallels human skin, thus providing a useful *in vitro* means to assess dermal sensitization and cytotoxicity (cellular viability).

4.3: Storage: EpiDerm™ tissues and assay medium are stored at 2-8ºC. IL-18 ELISA kits are stored at 2-8ºC.

EXPERIMENTAL DESIGN:

5.1: Vehicle: Ethanol and water are the vehicles of preference for this assay. Solubility will be tested at no higher than 10%, first in ethanol. If an homogenous solution is not achieved, solubility may be tested in other vehicles listed below, in order of preference. The first vehicle that attains a solubility of 10% will be used for the study. If 10% is not achieved, the vehicle that provides the highest solubility will be used. Solubility testing will be documented in the raw data. The final dosing concentrations will be determined by the Study Director in consultation with the Sponsor.

5.1.2: Other Vehicles (Call MB Research):
4:1 Ethanol/Dimethyl sulfoxide (EtOH:DMSO)
4:1 Acetone:Olive Oil (AOO)

5.2: Selection of Concentrations:

5.2.1: Range Finder Screen (Week 1):

5.2.1.1: Test Article Concentrations: Eight concentrations of the test article will be tested in duplicate tissues. Test concentrations will be prepared based upon the maximum solubility of the test article in the vehicle. Concentrations will be prepared by diluting the stock test article by a constant factor (e.g. $\sqrt[2]{10} = 3.16$) in the selected vehicle, covering a large range starting at the highest achievable solubility (see Appendix A).

5.2.1.2: Stimulation Index: Secreted IL-18 will be measured and used to calculate the Stimulation Index (SI). The SI will be calculated as a fold change, derived from a ratio of the treated vs. control samples (see Section 7.1). The maximum SI achieved (SI_{max}) will be used to choose concentrations for the Definitive Test. In the event no SI (>1.6) is detected, the cellular viability will be evaluated for use in the Definitive Test.

5.2.1.3: Cellular Viability: The percentage of viability will be calculated using MS Excel. The concentration at which the percent viability has dropped to 50% is considered the CV_{50} value. If no increases in the SI are detected, then the CV_{50} will be used as starting concentration in the Definitive Test. If no SI or CV_{50} can be calculated, the screen may need to be repeated.

5.2.2: Definitive Test (Week 2): Based on the results of the range finder screen, the dilution factor in the concentration series for the Definitive Test should be smaller than that in the range finder screen. Eight concentrations of the test article will be tested in duplicate tissues. The final dosing concentrations will be determined by the Study Director in consultation with the Sponsor.

5.3: Vehicle Control: Vehicle alone will serve as a baseline used for comparison. In consultation with the Sponsor, especially when a new vehicle is being used, duplicate undosed tissues will be concurrently evaluated under identical conditions.

5.4: Negative Control: The non-sensitizing irritating material, Lactic Acid (2.0%) will serve as a negative control.

5.5: Positive Control: Positive controls (supplied by MB) will be either 0.15% 1-Chloro-2,4-Dinitrobenzene (DNCB, in ethanol) or 0.5 - 2.0% Glyoxal (in water).

5.6 Additional Controls: Additional positive, negative, or irritant controls may be added by the Study Director in consultation with the Sponsor (see Section 13).

5.7: Reduction of MTT: When possible, MTT reduction by the test article will be measured prior to starting the study. If the test article results in a reduction of MTT or is likely to stain the tissue, frozen (dead) tissues will be run concurrently for each time point tested (or at minimum, the highest concentration tested).

6.0 TEST PROCEDURE:

6.1: Pre-Incubate: EpiDerm™ samples will be placed in 6-well plates containing pre-warmed assay media and will be equilibrated in a humidified 37ºC ± 1ºC, 5% ± 1% CO_2 incubator overnight.

6.2 Replace Media: The next day, assay media below the Millicell will be replaced prior to dosing.

6.3: Dose application: Following replacement of the assay media below the tissue, the appropriate volume and concentration of test material (administered using a positive displacement pipette) will be added into the Millicell atop the EpiDerm™ tissue on the stratum corneum at the apical side. Vehicle controls will be treated in an identical manner to the dosed inserts. Application volume and concentrations will be documented in the raw data.

6.4: Incubate: The 6-well plates containing the dosed EpiDerm™ samples will be returned to the incubator for 24 hours.

6.5: Media for IL-18 Analysis: Following 24-hour exposure to test materials, the assay media from the 6-well plates will be sampled for IL-18 cytokine analysis. Whenever possible, samples will be analyzed immediately. Otherwise, samples to be assayed for IL-18 will be stored at -70ºC ± 10ºC until all samples can be run concurrently for the ELISA.

6.6: MTT Conversion: Following 24-hour exposure to test materials and controls, any liquid remaining atop the EpiDerm™ tissues will be discarded. Each insert will be individually rinsed extensively with Phosphate Buffered Saline (PBS) to remove residual test material and the tissue will be incubated with 300 µL of 1 mg/mL MTT, diluted in Dulbecco's Modified Eagle Medium (DMEM), for 3 hours at 37ºC ± 1°C, 5% ± 1% CO_2.

6.7: MTT Extraction: Following the MTT incubation period, each insert will be removed individually and gently rinsed with PBS to remove any residual MTT solution. The inserts will be immersed using 2.0 mL of extractant solution per well, completely covering the EpiDerm™ sample. The extraction plate will be sealed and covered to reduce evaporation of extractant.

6.7.1: If the test article is colored or does not rinse off, only 1.0 mL of extractant solution will be added to each well, allowing extraction to occur through the bottom of the insert.

6.8: Extraction Conditions: The extraction will be allowed to precede overnight at room temperature in the dark. Alternatively, the extraction can be allowed to proceed for at least 2 hours, with shaking, at room temperature.

6.9: Decant Extractant in 24-well Plate: After the extraction period is complete, the liquid within each insert will be decanted back into the well from which it was taken, i.e. the solution will be mixed with the extractant in the well. The inserts will be discarded.

6.9.1: If the test article necessitated that only 1.0 mL of extractant was added to each well as per Section 6.7.1, an additional 1.0 mL of extractant will be added to each well to bring the volume to 2.0 mL.

6.10: Transferring to 96-Well Plate: An aliquot of 200 µL of the mixed extraction solution will be pipetted into a 96-well microliter plate.

6.11: Measuring Optical Density: The optical density of the extracted samples will be determined at a wavelength of 540 nm, subtracting out a background reading for all samples at the reference wavelength of 690 nm and using 200 µL of the extractant as a blank.

6.12: IL-18 measurement: Secreted IL-18 will be analyzed by ELISA as per manufacturer's instructions using an automated microplate reader. The stopped reaction will be measured at a wavelength of 450 nm, subtracting out a background reading for all samples at 620 nm.

7.0 DATA ANALYSIS:

7.1: Stimulation Index:

For analysis of each test article concentration, the **Stimulation Index (SI)**, i.e. the ratio of secreted IL-18 in response of each test article divided by that of the mean vehicle control tissue, will be calculated according to Equation 1 below:

Equation 1:

$$\text{SI per concentration} = \frac{\text{Mean OD of each test article concentration}}{\text{Mean OD of Vehicle}}$$

7.2: Calculating % Viability: The percent viability will be determined at each of the dosed concentrations using the following formula:

$$\% \text{ viability} = 100 \times [\text{OD(sample)}/\text{OD(vehicle control)}]$$

7.3: Data Interpretation: A test article will be regarded as a sensitizer if at least one concentration of the test article results in a 2-fold increase relative to that of Vehicle Control, as indicated by an SI ≥ 2.0 (Positive response). SI values of < 1.6 will indicate a negative response.

7.4: Indeterminate or Inconclusive Results: In cases where secretion of IL-18 results in dose-related SI values between 1.6 and 2.0, will be deemed a result of "Probable Sensitizer". Additional tests may be required using higher concentrations of the test article (or in another vehicle) if possible. In such cases, the effect of the vehicle on the outcome should also be examined.

7.5: Statistical Analysis: For each concentration, the Mean and Standard Deviation for each pair of duplicate values of tissue viability, IL-18 and SI will be calculated.

7.6: Criteria For Determination of a Valid Test: The negative control should yield an SI of < 1.6. The positive control should yield an SI of ≥ 2.0.

8.0 TEST DURATION: The duration of this assay is at least 2 weeks.

9.0 REFERENCE:

There are no references applicable to this protocol.

10.0 AMENDMENT TO THE PROTOCOL:

Any amendment to or deviation from this protocol will be fully documented in the study file, including the reason for the change, authority for change and the date.

11.0 RECORDS TO BE MAINTAINED:

11.1: Collection of Data: All data generated during the conduct of this study is recorded in ink on worksheets. All entries are dated and initialed.

11.2: Final Report: The final report may include, but is not limited to, a description of the methods and experimental design, results, discussion, conclusion, data table and the Quality Assurance statement. The content of the final report will meet the requirements of the applicable Good Laboratory Practice Regulations.

11.3: Retention of Data: All data generated during the conduct of this study will be archived at MB Research for at least 10 years from the date of the final report. The Sponsor will be contacted in writing to determine final disposition of the records. If the Sponsor fails to respond within 90 days, the archived items will be properly discarded.

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: Refer to section 13 for test article disposition. If this study exceeds 28 days, it is recommended that the Sponsor archive a sample of the test article to meet the applicable Good Laboratory Practices Regulations.

11.3.4: Test Article Mixtures: These are not routinely retained. However, upon written request from the Sponsor, and at additional cost, an aliquot of the test article mixture will be forwarded to the Sponsor.

12.0 GOOD LABORATORY PRACTICES:

This study will not routinely be conducted in full accordance with the Good Laboratory Practices.

12.1: 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.

12.2: Quality Assurance: The Quality Assurance Unit will review the raw data and the report in accordance with the Standard Operating Procedures of MB and applicable governmental regulations.

13.0 SPONSOR REQUEST:

13.1: The Sponsor requests that this protocol be implemented:
☐ As written *(or)* ☐ Modifications (per attached)

13.2: Range Finder: ☐ Conduct as written ☐ Other:_____

13.3: Vehicle (see Section 5.1): ☐ Ethanol ☐ Water ☐ Other:_____

13.4 Positive Controls (see Section 5.3.3): ☐ 0.15% DNCB ☐ 0.5-2.0 Glyoxal) ☐ Other_____

13.5 Additional Controls (see 5.3):_____

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

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

Lot/Batch #:_____

Special Handling Precautions:_____

13.7.1: Storage Requirements: ☐ Room Temperature ☐ Refrigerated (2-8ºC) ☐ Other: _____

13.7.2: 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 termination 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

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

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

13.7.5: Disposition of Test Article at Study Termination: (Call for costs)
☐ UPS / Ambient temperature *(no charge)* ☐ Express carrier / Ambient temperature
☐ Overnight carrier / ☐ Dry Ice or ☐ Ice Packs ☐ Discard (proper disposal):_____

13.8: Authorization Statement: This protocol is authorized for implementation at MB Research.

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

_____ _____
(type/print name) (address)

_____ _____
(title) (city) (st) (zip)

_____ _____
(email) (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: ☐ Not supplied by Sponsor, or

☐ Received and Reviewed by Study Director

14.2: MB Project Number assigned to this study: _____

14.3: Supplier: The tissue equivalent 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 3 weeks following Experimental Term Date.

14.5: Approval: This protocol is approved for implementation at MB Research by the below named MB Study Director.

BY: _____

Study Director (date)
Testing Facility MB Research Laboratories
1765 Wentz Road, P. O. Box 178
Spinnerstown, PA 18968

APPENDIX A: DECIMAL GEOMETRIC CONCENTRATION SERIES

In general, dose-response relationships are nonlinear, but can be linearized to some extent by logarithmic transformation of the x-axis. Usually this has to be done when EC_{50} values are calculated either by regression analysis or by graphical estimation. If dose series (in cell culture: concentration series!) are done with arithmetic steps, transformation of the x-axis will result in an unequal distribution of measuring points. Therefore, the use of geometric concentration series (= constant dilution factor) is recommended. The simplest geometric series are dual geometric series, e.g. factor 2. These series have the disadvantage of permanently changing chains within the series (2, 4, 8, 16, 32, 64, 128, 256, etc.). The decimal geometric series, first described by *Hackenberg & Bartling (1959)*[1] for the use in toxicological and pharmacological studies, has the advantage that independent experiments with wide and narrow dose factors can be easily compared, and under certain circumstances can even be merged together.

The dose factor of 3.16 (= $\sqrt[2]{10}$) divides a decade into 2 equal chains, the dose factor of 2.15 (= $\sqrt[3]{10}$) divides a decade into 3 equal chains, the dose factor of 1.47 (= $\sqrt[6]{10}$) divides a decade into 6 equal chains, and the dose factor of 1.21 (= $\sqrt[12]{10}$) divides the decade into 12 equal chains. Therefore, for reasons of an easier biometrical evaluation of the data it is recommended to use decimal geometric concentration series rather than dual geometric series. The technical production of decimal geometric concentration series is very easy. An example is given for 1.47:

Dilute one volume of highest dose by adding 0.47 volumes of diluent. After equilibration, dilute one volume of this solution by adding 0.47 volumes of diluent...(and so on). Thus, assuming an initial concentration of 10%, and using the dose factor of 1.47 (= $\sqrt[6]{10}$), the resulting concentrations would be 10.0%, 6.8%, 4.6%, 3.2%, 2.2%, 1.5%, 1.0% and 0.68%.