

ANNEX A (Protocols)

A1 IIVS Cytosensor Protocol

CYTOSENSOR MICROPHYSIOMETER BIOASSAY USING L929 CELLS

1.0 PURPOSE

The purpose of this study is to evaluate the potential toxicity of a test article. The Cytosensor Microphysiometer method evaluates the potential ocular toxicity by measuring the test material induced reduction in the metabolic rate in treated cultures of L929 cells. Changes in metabolic rate are measured indirectly as a function of changes in the extracellular acidification rate. The dose which induces a 50% decrease in metabolic rate, the MRD₅₀ value (in units of mg/mL), is the endpoint of the assay.

2.0 SPONSOR

See Protocol Attachment 1

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article(s):

3.2 Controls: Positive: sodium lauryl sulfate (SLS)

Solvent: solvent (when other than Low-Buffered DMEM is used)

3.3 Determination of Strength, Purity, etc.

3.3.1 The sponsor will be responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions, as applicable.

3.3.2 Institute for In Vitro Sciences, Inc. (IIVS) will be responsible for the documentation of the analytical purity and composition of the SLS used for the stock or working dilution of the positive control. This may be accomplished by maintaining a certificate of analysis from the supplier.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Institute for In Vitro Sciences, Inc.

4.2 Address: 21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

4.3 Study Director: Greg Mun, B.A.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date:

5.2 Proposed Experimental Completion Date:

5.3 Proposed Report Date:

6.0 TEST SYSTEM

The L929 cells obtained from ATCC, Manassass, VA, will be used in the study. An isolated population of L929 cells will be exposed to increasingly concentrated doses of a test article starting at the lowest concentration. The concentration of test article that causes a 50% decrease in the acidification rate (MRD₅₀) will be determined.

7.0 EQUIPMENT : CYTOSENSOR MICROPHYSIOMETER

The Cytosensor Microphysiometer manufactured by Molecular Devices Corporation, Menlo Park, CA., measures the extracellular acidification rate of cell cultures. The Cytosensor Microphysiometer consists of a variety of components which include: 1) two Cytosensor Microphysiometer units which include eight built-in peristaltic pumps for each channel; 2) a computer which runs the Cytosensor Microphysiometer and collects the data; 3) a printer; and 4) sensor chambers. Various adherent cell types can be seeded in the capsule cup. Each cell culture-containing cell capsule (capsule cup and spacer assembly) is loaded into the sensor chamber. The capsule insert will not be included in the assembly. The bottom of the sensor chamber is made of the silicon sensor chip. This chip is capable of detecting very small changes in pH. Low-buffered medium is perfused across the cells in a stop/flow manner. When the flow is stopped, the change in pH due to acidic metabolites (e.g., lactate and CO₂) build-up is detected by the silicon sensor. The acidification of the medium occurs at a reproducible rate in the presence of a normal, undamaged cell population. Cells which have received a toxic insult will produce an altered acidification rate.

8.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The experimental design of this study consists of a solubility or miscibility test to confirm the solubility/workability of the test article in Low-Buffered DMEM (unless otherwise specified by the Sponsor or the Study Director), the determination of the pH of the neat test article if possible, the determination of the pH at the highest concentration of test article in the medium if possible, a dose range finding assay and at least two definitive assay trials. At the Study Director's discretion, additional definitive assay trials may be performed. Activity in the Cytosensor Microphysiometer assay is evaluated on the basis of reduction of the acidification rate of the individual cell population after the exposure to and subsequent washout of a series of test article concentrations. The concentration of test article which causes a 50% reduction in the acidification rate is calculated and termed the MRD₅₀ (Metabolic Rate Decrement 50%). The MRD₅₀ will be expressed in mg/mL.

The methods for conducting the Cytosensor Microphysiometer assay are modifications of procedures described in the Operator's Manual supplied by Molecular Devices Corporation. Additional background information is given by Parce et al. (1989).

8.1 Media and Reagents

8.1.1 Growth Medium: Dulbecco's Modified Eagle's Medium with 1.0 mM sodium pyruvate (DMEM) containing 10% Fetal Bovine Serum and 2.0 mM L-glutamine (Complete DMEM).

8.1.2 Seeding Medium: DMEM containing 1% Fetal Bovine Serum, 50 µg/mL gentamicin, 2.0 mM L-glutamine (Diluted DMEM).

8.1.3 Low-Buffered Medium: Serum-free, Sodium Bicarbonate-free, DMEM containing 50 µg/mL gentamicin, 2.0 mM L-glutamine, and additional NaCl for consistent osmolarity (Low-Buffered DMEM).

8.1.4 Ca⁺⁺Mg⁺⁺-Free Phosphate Buffered Saline (PBS)

8.1.5 0.05% Trypsin in Ca⁺⁺Mg⁺⁺-Free- Hanks' Balanced Salts Solution

8.1.6 Positive control - SLS 10% in water (stock)

8.2 Preparation and Delivery of Test Article

The test article will be dissolved in Low-Buffered DMEM. Other solvent systems will be used only after consultation with the Sponsor but should generally be avoided. If extraction of the test article is required, the extraction procedure will be determined in consultation with the Sponsor. It is essential that the test material be in a single phase solution/suspension in the highest dose used to prepare the subsequent dilutions (see section 8.7).

8.3 Route of Administration

The test article dosing solutions will be administered directly to the cells. Cells will be exposed to each concentration of test article for approximately 810 sec, after which time the test article is rinsed out of the sensor chamber with fresh medium. The acidification rate is immediately measured after washout of the sample. Dosing is generally conducted by testing lower concentrations first and gradually increasing the dose (the same cell chamber is used for each dose) until the MRD₅₀ point has been surpassed or until the highest concentration has been dosed.

8.4 pH Determination

The pH of the neat liquid test article (and/or dosing solution as appropriate) will be determined, if possible. The pH will be determined using pH paper (for example, with a pH range of 0 – 14 to estimate, and/or a pH range of 5 – 10 to

determine a more precise value). The typical pH increments on the pH paper used to report the pH are approximately 0.3 to 0.5 pH units. The maximum increment on the pH paper is 1.0 pH units.

8.5 Controls

The baseline acidification rate will serve as the internal control for each cell culture. For each sensor chamber used, baseline rates will fall between 50 and 200 microvolts/sec after a stabilization period of approximately 1 hour. The cell capsule in any chamber which fails to achieve these ranges will be replaced, or the channel will not be used in the assay, unless the Study Director determines the chamber to be acceptable.

Each assay will include a concurrent solvent control (when a solvent other than Low-Buffered DMEM is used) and a positive control. The positive control will be tested like a test article except that the dose range will be set based on historical data.

At the beginning of each assay, at least four to five stable rates are taken as the baseline rate. For each sensor chamber, these baseline data points should vary from their mean by no more than 10%, and will be determined just prior to introduction of the first sample dilutions. If the baseline data contain one out of five outlying points that can be explained (e.g., caused by a bubble), it is permissible to delete that data point and use only four for calculations.

8.6 Cell Maintenance and Preparation of the Capsule Cups

Stock cultures of L929 cells will be maintained and passaged in Growth Medium and incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO_2 in air. L929 cells will be seeded onto capsule cups at approximately 6.0×10^5 cells per capsule cup in Seeding Medium as described below.

Flasks of L929 cells to be passaged or seeded are selected at or near confluency. The size of flasks used will depend on the number of cells needed. The Growth Medium is decanted and the cell sheet washed twice with approximately 10 mL of PBS for each 75cm^2 of growth surface. The cells are trypsinized with approximately 3 mL of trypsin (for each 75cm^2 of growth surface) for 15 to 30 seconds. The trypsin solution is aspirated and the cells are incubated at room temperature for approximately 2 to 5 minutes, until the cells begin to round. The cells are dislodged by tapping the flask and approximately 5mL of Seeding Medium are for each 75cm^2 of growth surface. The cells are triturated using a pipet in order to break up clumps and are transferred by pipet to a conical centrifuge tube. If more than one flask is used, the contents of each are pooled. Cell counts are performed as required. The L929 cells will be seeded with approximately 6.0×10^5 cells per each capsule cup (0.5 mL of a 1.2×10^6 cell suspension) with 1.5 mL of Seeding Medium added to each outside well. The plate will be labeled with cell type, seeding density, and date. The plate will then be incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO_2 in air for 16 to 32 hours. Prior to the

start of the assay, the medium in capsule cups will be switched to Low-Buffered DMEM and a spacer will be added to each capsule cup and gently tapped down to the bottom. The cell capsules will be placed into the sensor chambers and exposed to Low-Buffered DMEM at $37 \pm 1^\circ\text{C}$.

For routine passaging, the stock cultures are trypsinized as described above, but are dislodged and resuspended using warm (approximately 37°C) Growth Medium, seeded into a culture flask(s), and returned to the humidified incubator maintained at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO_2 in air.

8.7 Dose Range Finding Assay

A dose range finding assay will be performed to establish an appropriate test article dose range for the definitive Cytosensor Microphysiometer assay. Dosing solutions will be prepared by serial three-fold dilutions (producing the same concentrations suggested in the following table) in sterile, Low-Buffered DMEM that has been allowed to equilibrate to room temperature.

IMPORTANT: Do not attempt to use preparations that separate into more than one phase in the Cytosensor. Similarly, do not attempt to use such preparations to make dilutions. At the discretion of the Study Director, a suspension that maintains a single phase may be assayed and used to prepare further dilutions.

If the sample does not go into a single phase with the medium at 10.0 mg/mL (maintaining a ratio of 100 mg/10 mL), prepare dilutions 2 or 3 as required. If a single phase test article/medium mixture is not achieved, the Study Director and Sponsor are to be consulted.

DILUTION #	CONCENTRATION
1	10 mg/mL
2	3.33 mg/mL
3	1.11 mg/mL
4	0.370 mg/mL
5	0.123 mg/mL
6	0.0412 mg/mL
7	0.0137 mg/mL

The test article will be evaluated by exposure to L929 cells contained in sensor chambers. The injection port for each sensor chamber will be labeled with the designated test article or positive control prior to exposure. After the baseline data points have been taken, the exposure cycle will begin with the lowest test article concentration. From these baseline data points, the spreadsheet will

compute the mean baseline value used in the MRD_{50} calculation. Each exposure cycle will take 20 minutes.

The maximum solvent concentration (other than Low-Buffered DMEM) will be 10% unless otherwise specified by the Sponsor or Study Director.

There will be three phases in the exposure cycle, with the following parameters selected within the Cytosensor Microphysiometer software (Cytosoft): First, a test article concentration will be introduced into the sensor chamber for 13 minutes and 30 seconds. The nominal rate of flow will be 100 μ L per minute for the first minute, and 20 μ L per minute for the next 12 minutes and 30 seconds. The second phase will be the wash-out phase which will be six minutes at a nominal rate of 100 μ L per minute. The test article will be washed out of the sensor chamber during this phase. Finally, the third phase will be the measurement of the acidification rate. For 25 seconds, there will be no flow and the rate of pH change will be measured.

The exposure cycle will repeat with increasing test article concentrations until either the highest test article concentration is reached or until the MRD_{50} value has been surpassed. Each test article concentration will be tested on a single set of cells. Positive control materials and solvent controls (for solvents other than Low-Buffered DMEM) will be tested in the same fashion. If possible, an MRD_{50} value will be calculated from the dose range finding assay.

The test article doses for the definitive assay will be chosen so that generally seven doses (spaced as three-fold dilutions) will be available for the determination of the MRD_{50} . Generally, three concentrations will be chosen to result in expected survivals lower than 50%, one concentration will be chosen to result in an expected survival of approximately 50%, and three or more concentrations will be chosen to result in expected survivals greater than 50%. If a test article fails to cause 50% toxicity in the dose range finding Cytosensor Microphysiometer assay, the maximum dose will generally be 270 mg/mL, or less based on its solubility/workability.

8.8 Definitive Assay

The definitive assay will be performed in the same manner as the dose range finding assay, with the exception that if the MRD_{50} value from the dose range finding assay is > 10 mg/mL, higher doses of test article will be prepared and tested in the definitive assay. At least seven doses, spaced at three-fold dilution intervals, up to a maximum of 270 mg/mL will be prepared. The determination of the final MRD_{50} will be based upon the results of at least two definitive assays and will generally also include the results of the dose range finding assay, if an MRD_{50} could be determined. At the Study Director's option, the results from additional definitive assays may also be incorporated into the calculation of the final MRD_{50} .

8.9 Data Analysis

The acidification rates which occur after exposure to each test article concentration are calculated by the Cytosoft program and compared to the mean acidification rate (basal acidification rate) of the same cells prior to exposure to a test material to determine the percent of control acidification rate for each dose. The dose response curve will be plotted with the percent of control acidification rates on the ordinate and the test article concentrations on the abscissa. The concentration of test material which results in a fifty percent reduction in acidification rate is interpolated from the curve and referred to as the MRD₅₀. MRD₅₀ data will be expressed in mg/mL.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The Cytosensor Microphysiometer assay will be accepted if the positive control MRD₅₀ falls within two standard deviations of the historical mean. The historical mean will be updated every three months.

Bruner, L.H., D.J. Kain, D.A. Roberts and Parker, R.D. (1991). Evaluation of seven in vitro alternatives for ocular safety testing. *Fundamental and Applied Toxicology* 17: 136-149.

Harbell, J.W., Osborne, R., Carr, G.J., and Peterson, A. (1997) Assessment of the Cytosensor microphysiometer assay in the COLIPA in vitro eye irritation validation study. Submitted, *Toxicology In Vitro*.

McConnel, H.M., Owicki, J.C., Parce, J.W., Miller, D.L., Baxter, G.T., Wada, H.G., and Pitchford, S. (1992) The Cytosensor microphysiometer: biological applications of silicon technology. *Science* 257:1906-1912.

Parce, J.W., Owicki, J.C., Kercso, K.M., Sigal, G.B., Wada, H.G., Muir, V.C., Bousse, L.J., Ross, K.L., Sikic, B.I, McConnell, H.M. (1989) Detection of cell-affecting agents with a silicon biosensor. *Science* 246: 243-247.

15.0 APPROVAL

SPONSOR REPRESENTATIVE

IIVS STUDY DIRECTOR

DATE

A2 COLIPA Cytosensor Protocol

Study Number:

**COLIPA VALIDATION STUDY OF THE
CYTOSENSOR MICROPHYSIOMETER BIOASSAY USING L929 CELLS**

1.0 PURPOSE

The purpose of this study is to compare the ocular toxicity of the test material as predicted using the Cytosensor Microphysiometer method with historical rabbit Draize eye test data. The Cytosensor Microphysiometer method evaluates the potential ocular toxicity by measuring the test material induced reduction in the metabolic rate in treated cultures of L929 cells. Change in metabolic rate are measured indirectly as a function of changes in extracellular acidification rate. The dose which induces a 50% decrease in metabolic rate is the end point of the assay.

2.0 SPONSOR

2.1 Name:

2.2 Address:

2.3 Representative:

2.4 Sponsor Project

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Articles: Test articles will be identified in an attachment(s)

3.2 Controls: Positive: sodium lauryl sulfate (SLS) (10% stock in water)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article. The testing laboratory will not assess the strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Microbiological Associates, Inc.

4.2 Address: 9900 Blackwell Road

Rockville, Maryland 20850

4.3 Study Director: Dr. John W. Harbell

5.0 TEST SCHEDULE (To be determined by the COLIPA Management Committee)

5.1 Proposed Experimental Initiation Date: 04/06/94

5.2 Proposed Experimental Completion Date: 05/13/94

5.3 Proposed Report Date: 05/27/94

6.0 TEST SYSTEM

The Cytosensor Microphysiometer ($\mu\phi$) manufactured by Molecular Devices Corporation, Menlo Park, CA., measures the acidification rate of cell populations. The $\mu\phi$ consists of a variety of components which include 1) two Cytosensor Microphysiometer units which include 8 built-in peristaltic pumps for each channel 2) a computer which runs the $\mu\phi$ and collects the data and a printer, and 3) cell chambers. Various adherent cell types can be seeded in the capsule cup. Each cell culture containing cell capsule (capsule cup, and spacer) is loaded into a sensor chamber. The capsule insert will not be included in the assembly. The bottom of the sensor chamber is made of the silicon sensor chip. This chip is capable of detecting very small changes in pH. Low-buffered medium (1-2 mM PO_4 without bicarbonate) is perfused across the cells in a stop/flow manner. When the flow is stopped, the change in pH due to acidic metabolites (e.g. lactate and CO_2) build up is detected by the silicon sensor. The acidification of the medium occurs at a reproducible rate in the presence of a normal, undamaged cell population. Cells which have received a toxic insult will produce an altered (generally decreased) acidification rate.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The experimental design of this study consists of several phases. A solubility or miscibility test is first performed to confirm the solubility of the test material in the assay medium at the highest dose tested. The pH of the neat test article (if possible) and the pH of the highest concentration of test article in the media are determined. A dose range finding assay and at least three definitive assays are performed. At the Study Director's discretion additional trials may be run. Activity in the $\mu\phi$ assay is evaluated on the basis of reduction of the acidification rate of the individual cell population after the exposure to and subsequent washout of a series of test article concentrations. The concentration of test article which causes a 50% reduction in the acidification rate is calculated and termed the MRD_{50} . The MRD_{50} is calculated in $\mu\text{g}/\text{ml}$. The \log_{10} of the MRD_{50} in $\mu\text{g}/\text{ml}$ will be reported to BIBRA.

The methods for conducting the $\mu\phi$ assay are modifications of procedures

described in the Operator's Manual for the $\mu\phi$ supplied by the Molecular Devices Corporation. Additional background information is given by Parce et al. (1989).

7.1 Media and Reagents

- 7.1.1 Growth Medium: Dulbecco's modified Eagle's medium (DMEM) (1 mg/ml glucose) complete with 10% Fetal Bovine Serum, 2.0 mM L-glutamine, and 1.0 mM sodium pyruvate.
- 7.1.2 Assay Medium: DMEM complete with 1% Fetal Bovine Serum, 5.0 $\mu\text{g/ml}$ gentamicin, 2.0 mM L-glutamine, and 1.0 mM sodium pyruvate.
- 7.1.3 Treatment Medium: Serum-free, Sodium Bicarbonate-free, DMEM with 5.0 $\mu\text{g/ml}$ gentamicin, 2.0 mM L-glutamine, and additional NaCl for consistent osmolarity (MDMEM). 11.1 ml of 4 M NaCl is required per liter.
- 7.1.4 Positive control - SLS 10% in water (stock)

7.2 Preparation and Delivery of Test Article

The test article will be dissolved in MDMEM. It is essential that the test material be in a single phase solution in the highest dose used to prepare the subsequent dilutions.

The stability of the test article under the actual experimental conditions will not be determined by the testing laboratory.

7.3 Route of Administration

The test article will be administered directly to the cells using the normal $\mu\phi$ procedures. Cells will be exposed to each concentration of test article for approximately 810 sec after which time the test article is rinsed out of the sensor chamber. The acidification rate is immediately measured after washout of the sample. Dosing is conducted by testing lower concentrations first and gradually increasing the dose (the same cell chamber is used for each dose) until the MRD_{50} point has been surpassed or until the highest concentration has been dosed.

7.4 Controls

The baseline acidification rate will serve as the internal negative control for each cell culture. Baseline rates will fall between 50 and 150 microvolts/sec after a stabilization period of at least 15 minutes. Replace the cell-containing insert in a chamber which fails to achieve these ranges.

When the 8 channel Cytosensor is used, a positive control assay will be performed with each definitive trial of the assay. When the 4 channel machine is used, a concurrent positive control trial will be performed with at least one of the definitive trials for each test material.

7.5 Growth of Cells

L929 cells will be grown on capsule cups using Assay Medium. L929 cells will be seeded at approximately 6.0×10^5 cells per each capsule cup and incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO_2 in air overnight. Prior to the start of the assay, the medium in capsule cups will be switched to Treatment Medium and cell capsules will be placed into the $\mu\phi$ sensor chambers and exposed to MDMEM at $37 \pm 1^\circ\text{C}$.

7.6 Dose-Range Finding Assay

A dose range finding $\mu\phi$ assay will be performed to establish an appropriate test article dose range for the definitive $\mu\phi$ assay. Prepare dilutions by serial three-fold dilution, as below, in sterile, low-buffered medium that has been left to equilibrate to room temperature.

IMPORTANT: Do not attempt to use in the Cytosensor, preparations that separate into more than one phase. Similarly, do not attempt to use such preparations to make dilutions.

If the sample does not go into solution (single phase) with the medium at 100.0 mg/ml (1.000 g/10 ml), leave the tube in a rack and prepare Dilution 8 by making up 0.333 g to 10 ml. If complete solubility is still not achieved, again leave the tube in a rack and prepare Dilution 7 by making up 0.111 g to 10 ml. If you still do not achieve complete solubility, declare the test sample as "Unsuitable for testing by the Cytosensor using standard techniques."

20 4

DILUTION #	CONCENTRATION	DILUTION
9	100 mg/ml	1.000g diluted to 10 ml (use weight not vol even if it is a liquid)
8	33.3 mg/ml	3 ml of Dilution 9 plus 6 ml medium
7	11.1 mg/ml	3 ml of Dilution 8 plus 6 ml medium
6	3.70 mg/ml	3 ml of Dilution 7 plus 6 ml medium
5	1.23 mg/ml	3 ml of Dilution 6 plus 6 ml medium
4	0.411 mg/ml	3 ml of Dilution 5 plus 6 ml medium
3	0.137 mg/ml	3 ml of Dilution 4 plus 6 ml medium
2	0.0457 mg/ml	3 ml of Dilution 3 plus 6 ml medium
1	0.0152 mg/ml	3 ml of Dilution 2 plus 6 ml medium

The test article will be tested by exposure to L929 cells contained in sensor chambers. After at least five stable rates are taken as the base rate, the exposure cycle will begin with the lowest test article concentration. Five baseline data points that vary from their mean by no more than about 10%, will be determined just prior to introduction of the first sample dilutions. If the baseline data contains one out of five points that can be explained by raw data as an outlier caused by a bubble, it is permissible to delete that data point and use only four for calculations. From these baseline data points, the Excel spreadsheet will compute the mean baseline value used in the MRD₅₀ calculation. Each exposure cycle will take 20 minutes.

There will be 3 phases in the exposure cycle. First, a test article concentration will be introduced into the sensor chamber for 13 minutes and 30 seconds. The rate of flow will be 100 μ l per minute for the first minute and 20 μ l per minute for next 12 minutes and 30 seconds. Second phase will be the wash-out phase which will be 6 minutes at 100 μ l per minute. The test article will be washed out of the sensor chamber during this phase. Finally, the third phase will be the measurement of the acidification rate. For 25 seconds, there will be no flow and the rate of pH change will be measured.

The exposure cycle will repeat with increasing test article concentrations until either the highest test article concentration is reached or until the MRD₅₀ value has been surpassed. Each test article concentration will be tested on a single set of cells.

7.7 Definitive Assays

As with the range-finding experiment, prepare nine test sample dilutions. However, for the definitive experiments, these should be made as a quarter log series that brackets the MRD₅₀. The range-finding assay MRD₅₀ is used to help set the range midpoint (Dilution 5) as described below. Four dilutions above and four dilutions below the midpoint are also prepared.

Refer to both columns shown below and select the concentration that is closest to the range-found MRD₅₀. It will be given in terms of -Log g/ml by the Excel spreadsheet but you may find it easier to interpret in terms of mg/ml. Once you find this value, it becomes the new dilution 5. Count up 4 places in the left hand column. If the concentration is 100, 10, 1, or 0.1 mg/ml use this to start the dilution series. If not, go up to the next such concentration and prepare a stock solution in medium at that concentration for your dilution series.

CONCENTRATION (mg/ml)	-LOG g/ml
316.2	0.50
177.8	0.75
100.0	1.00
56.2	1.25
31.6	1.50
17.8	1.75
10.0	2.00
5.62	2.25
3.16	2.50
1.78	2.75
1.00	3.00
0.562	3.25
0.316	3.50
0.178	3.75
0.100	4.00
0.056	4.25
0.032	4.50

To make a quarter log dilutions, prepare dilution tubes containing 5.46 ml of MDMEM medium and use transfer volumes of 7.00 ml. If the range-finding assay MRD_{50} is greater than about 14 mg/ml, it may be necessary to include concentrations higher than those used in the range-finding study. The maximum dose will be 316.2 mg/ml.

The determination of the MRD_{50} will be based upon the results of three definitive assays.

For the positive control, make dilutions of the 10% SLS stock solution using MDMEM medium and consider that the 10% solution is the "neat" test material. Prepare the initial dilution on a weight to volume basis. A dose range finding assay should be performed once on the positive control to set the appropriate ranges for the subsequent definitive trials.

7.8 Data Analysis

The acidification rates which occurred after exposure to each test article concentration are calculated by the $\mu\phi$ software (Cytosoft) and compared to the mean acidification rate (base acidification rate) of the same cells prior to exposure to a test material. The percent of control acidification rate will be determined by comparing the dose response acidification rate to the base acidification rate. The dose response curve will be plotted with the percent of control acidification rates on the ordinate and the test article concentration on the abscissa. The concentration of test material which results in a fifty percent reduction in acidification rate is interpolated from the curve and referred to as the MRD_{50} . These calculations can be performed using the Excel spreadsheet program provided for this study. Note that this program may give MRD_{50} values in mg/ml. **MRD_{50} values will be determined in ug/ml and converted to $\log_{10}(MRD_{50})$ for reporting (see Attachment 2).** Expressing the MRD_{50} in ug/ml will ensure that almost all $\log(MRD_{50})$ values will be greater than zero. In cases where the test article is found to be insoluble (see section 7.6), the result "Unsuitable for testing" will be entered into the $\text{Log}(MRD_{50})$ to indicate that the sample was "Unsuitable for testing by the Cytosensor using standard techniques".

7.9 Prediction of the Draize MMAS from the mean Log(MRD₅₀)

A predicted MMAS score can be generated for each test article for which a mean log(MRD₅₀) can be obtained. The predicted MMAS is calculated using the formula developed by Osborne et al (The Procter & Gamble Company):

$$MMAS = \frac{A}{1 + e^{(B \cdot \log_{10} MRD_{50} - G)}}$$

Where A = 148.0, B = 1.813, and G = -2.329. This three parameter model was prepared from the combined historical data and is an unrestricted model as to the highest possible in vivo MMAS value.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

Assay acceptance criteria are normally based on the performance of the positive control. The $\mu\phi$ assay would be accepted if the positive control MRD₅₀ fell within 2 standard deviations of the historical range. The acceptable range for SLS will be provided by the lead laboratory. The positive control assay will not be performed with each trial on the 4 channel machine. Therefore, acceptance of those trials, lacking a positive control, will be based on the judgement of the study director.

9.0 REPORT

The report form for the data is included as Attachment 2 of this protocol. Additional copies should be made to submit data on all of the test materials.

10.0 RECORDS AND ARCHIVES

A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of each laboratory.

11.0 REFERENCES

Parce, J.W. et al., Detection of Cell-Affecting Agents with a Silicon Biosensor, Science 246:243-247, 1990.

Cytosensor Microphysiometer System User's Manual

12.0 APPROVAL

STUDY DIRECTOR

DATE

PROTOCOL AMENDMENT

DATE: 3 July 1995

SPONSOR: The Procter & Gamble Company

SPONSOR'S TEST ARTICLE

DESIGNATION: COLIPA Test Articles (A94BR08 to A94BR62)

MA STUDY NO: A94BR08-62.200014

PROTOCOL NO: SPAT200014

PROTOCOL TITLE: COLIPA VALIDATION STUDY OF THE CYTOSENSOR
MICROPHYSIOMETER BIOASSAY USING I.929 CELLS

AMENDMENT(S):

1) Location: Page 8, Section 7.9, Prediction of the Draize MMAS from the mean
Log(MRD₅₀)

Amendment: Replace the section with:

"A predicted MMAS score can be generated for each test article for which a mean log(MRD₅₀) can be obtained. The predicted MMAS is calculated using the formula developed by Osborne et al (The Procter & Gamble Company):

$$MMAS = \frac{A}{1 + e^{B(\log(MRD_{50}) - G)}}$$

Where A = 148.0, B = 1.813, and G = 2.329. This three parameter model was prepared from the combined historical data and is an unrestricted model as to the highest possible in vivo MMAS value."

Reason: Correct two typographical errors. The first typographical error is in the formula where the "(" should be placed to the left of "log" rather than to the left of "B". The second typographical error is in the constant G. Constant G should be positive.

APPROVAL:


AUTHORIZED REPRESENTATIVE

4 July 1995
DATE


STUDY DIRECTOR

3 July 1995
DATE

 MICROBIOLOGICAL
ASSOCIATES, INC.

A3 IIVS EpiOcular Protocol

**TOPICAL APPLICATION OCULAR IRRITATION SCREENING ASSAY
USING THE EPIOCULAR™ HUMAN CELL CONSTRUCT**

1.0 PURPOSE

The purpose of this study is to evaluate the potential ocular irritation of the test article by measuring 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye conversion by the EpiOcular™ tissue construct after topical exposure to the test article.

2.0 SPONSOR

2.1 Name:

2.2 Address:

2.3 Representative:

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article(s):

3.2 Controls: Positive: 0.3% Triton®-X-100

Negative: negative (Sterile deionized water or other solvent as appropriate)
blank control (MTT reading only)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Institute for In Vitro Sciences, Inc.

4.2 Address: 21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

4.3 Study Director:

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date:

5.2 Proposed Experimental Completion Date:

5.3 Proposed Report Date:

6.0 TEST SYSTEM

The EpiOcular™ human cell construct, provided by the MatTek Corporation, will be used in this study. The use of EpiOcular™ cultures offers features appropriate for a model for ocular irritation. First, the model is composed of stratified human keratinocytes in a three-dimensional structure. Secondly, test materials can be applied topically to the model so that water insoluble materials may be tested. Prior to use, each plate (6, 12, and 24-well) will be uniquely identified with a number written in permanent marker, on the plate and its cover, the test article number, and the exposure time.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The experimental design of this study consists of the determination of the pH of the neat liquid test article if possible (and/or dosing solution as appropriate) and a single definitive assay. The toxicity of the test article will be evaluated by the exposure time required to reduce tissue viability to 50% of controls (ET₅₀). Viability will be determined by the NAD(P)H-dependent microsomal enzyme reduction of MTT (and to a lesser extent, by the succinate dehydrogenase reduction of MTT) in control and test article-treated cultures (Berridge, et al., 1996). Data will be presented in the form of relative survival (relative MTT conversion) versus test article exposure time.

One of two exposure time ranges may be used. The standard exposure time range extends up to four hours and is used for most materials to be tested. For extremely mild materials, such as those that might be applied around or in the eyes, a long exposure assay might be used. For the long exposure study, exposure times of up to 24 hours could be used. In general, the standard exposure range will be used, unless the Sponsor specifies an alternative exposure time range or if the Study Director determines that the class of test articles warrants the use of an alternative exposure time range.

7.1 Media and Reagents

- 7.1.1 Assay Medium: supplied by MatTek Corporation
- 7.1.2 EpiOcular™ Tissue: OCL-200 supplied by MatTek Corporation
- 7.1.3 Dulbecco's Modified Eagle's Medium (DMEM) containing 2mM L-glutamine by Quality Biological (or equivalent) (MTT Addition Medium)
- 7.1.4 Sterile deionized water by Quality Biological (or equivalent)
- 7.1.5 3-[4,5 - dimethylthiazol-2-yl] - 2,5 - diphenyltetrazolium bromide (MTT) Solution: 1 mg/mL MTT in MTT Addition Medium
- 7.1.6 Ca⁺⁺ and Mg⁺⁺ Free Dulbecco's Phosphate Buffered Saline (Ca⁺⁺Mg⁺⁺Free-DPBS)
- 7.1.7 Extraction Medium: Isopropanol

7.2 Preparation and Delivery of Test Article

Test articles will generally be tested neat. End use concentrations or other forms may be used as directed by the Sponsor. One hundred μL of pipettable substances, such as liquids, gels, creams, and foams, will be applied directly on the tissue so as to cover the upper surface. To aid in filling the pipet for pipettable materials that are viscous, the test article may first be transferred to a syringe. The pipet tip of the positive displacement pipet will be inserted into the dispensing tip of the syringe so that the material can be loaded into the displacement tip under pressure. Simultaneously, the syringe plunger is depressed as the pipet piston is drawn upwards. If air bubbles appear in the pipet tip, the test article should be removed (expelled) and the process repeated until the tip is filled without air bubbles. This method should be used for any materials that cannot be easily drawn into the pipet such as gels, (e.g., toothpastes, mascaras, and face creams) and solid test articles that are creamed like lipsticks and antiperspirants/deodorant sticks. A dosing device (a flat headed cylinder of slightly less diameter than the inner diameter of the tissue insert) may be placed over the test article to assure even spreading, if required. Dry powders will be ground with a mortar and pestle and passed through a #40 copper sieve, if needed. Powders will be placed directly onto the culture at approximately 30 mg/culture. Materials that are too viscous to spread over the tissue will first be spread onto the flat end of a dosing device. The dosing device will then be placed into the Millicell[®] to bring the test article in contact with the tissue. When the test article must first be applied to a dosing device, approximately 30 μL or 30 mg of material will be applied to the dosing device so as to cover the dosing surface. The sample should be spread to form a relatively smooth even layer on the surface of the dosing device to maximize uniform tissue contact. Solids such as lipsticks or antiperspirant/deodorant sticks can be pre-softened by creaming a portion in a weigh boat. The softened portion can be transferred to a syringe affixed with a three way stopcock attached to a second syringe. The sample is pushed from syringe to syringe until it is of a consistency which can be pipetted. The exact exposure conditions used for other test article forms will be determined after consultation with the Sponsor and/or the Study Director. All exposure conditions will be documented in the study workbook.

The stability of the test article under the actual experimental conditions will not be determined by the Institute for In Vitro Sciences, Inc. (IIVS).

7.3 Route of Administration

The test article(s) will be administered by topical application to the construct.

7.4 pH Determination

The pH of the neat liquid test article (and/or dosing solution as appropriate) will be determined, if possible. The pH will be determined using pH paper (for example, with a pH range of 0 – 14 to estimate, and/or a pH range of 5 – 10 to determine a more precise value). The typical increments on the pH paper used to

report the pH are approximately 0.3 to 0.5 pH units. The maximum increment on the pH paper is 1.0 pH units.

7.5 Controls

Generally, at least two negative control exposure times will be used. One negative control exposure time will be selected to fit the range of the shortest test article or positive control exposure times (the minimum negative control exposure time will be 15 minutes). The second negative control exposure time will be selected to match the longest test article or positive control exposure time (whichever is longer, up to 240 minutes). On occasion, the second negative control exposure time may be selected to fit the longest test article exposure time of a test article run concurrently, but from an independent study. For the long exposure assay (exposures of greater than 240 minutes), multiple negative control exposure times may be selected to fit the range of test article exposure times. If all exposure times are one hour and less, a single negative control exposure time may be used. Additional negative control exposure times may be selected at the discretion of the Study Director. Positive control cultures are treated with 0.3% (3 mg/mL) Triton[®]-X-100 prepared in sterile deionized water and are exposed for 15 and 45 minutes. At least two cultures will be used for each negative and positive control exposure time.

7.6 Assessment of Direct Test Article Reduction of MTT

It is necessary to assess the ability of each test article to directly reduce MTT. A 1.0 mg/mL MTT solution will be prepared in warm MTT Addition Medium as described in §7.8. Approximately 100 µL (liquid test articles) or 30 mg (solid test articles) will be added to 1 mL of the MTT solution and the mixture incubated in the dark at 37±1°C in a humidified atmosphere of 5±1% CO₂ in air (standard culture conditions) for approximately one hour. The negative control (100 µL) will be run concurrently. If the MTT solution color turns blue/purple, the test article is presumed to have reduced the MTT. Water insoluble test materials may show direct reduction (darkening) only at the interface between the test article and the medium.

7.7 Receipt of the EpiOcular[™] model

Upon receipt of the EpiOcular[™] assay materials, the solutions will be stored as indicated by the manufacturer. The tissue will be stored at 2-8°C until used.

On the day of dosing, EpiOcular[™] Assay Medium will be warmed to approximately 37°C. Nine tenths (0.9) mL of Assay Medium will be aliquoted into the appropriate wells of pre-labeled 6-well plates. The 6-well plates will be labeled with the test article(s) and exposure time(s). Each tissue will be inspected for air bubbles between the agarose gel and Millicell[®] insert prior to opening the sealed package. Cultures with air bubbles under greater than 50% of the Millicell[®] area will not be used. Each 24-well shipping container will be removed from its plastic bag and its surface disinfected by wiping with 70% ethanol-soaked tissue paper. An appropriate

number of tissues will be transferred aseptically from the 24-well shipping containers into the 6-well plates. The EpiOcular™ tissues will be incubated at standard culture conditions for at least one hour. The medium will be aspirated and 0.9 mL of fresh Assay Medium will be aliquoted into each assay well below the tissue. Upon opening the bag, any unused tissues remaining on the shipping agar at the time of tissue transfer will be briefly gassed with an atmosphere of 5% CO₂/95% air, and the bag will be sealed and stored at 2-8°C for subsequent use.

7.8 Definitive MTT Assay

The Sponsor and/or Study Director have requested the following assay:

- Short term exposure assay.
- Long term exposure assay.
- See Protocol Attachment 1.

Four to five exposure times will be tested for each test article. The exposure times may be suggested by the Sponsor, or selected by the Study Director. In the short term exposure assay, if the expected range of toxic response is unknown, a 20 minute exposure time may be performed first to determine the remaining exposure durations. The maximum exposure time will be 240 minutes unless otherwise directed by the Sponsor.

Each test article and control exposure time will be tested by treating two tissues. The dosing procedure will be determined as indicated in §7.2. Generally, exposure times of ten minutes or greater will be incubated at standard culture conditions.

The positive control will be exposed for 15 and 45 minutes. A second negative control will be exposed for the longest exposure time used for the test or control articles up to 240 minutes.

At the end of the treatment time, the test article will be removed by extensively rinsing both sides of the culture with room temperature Ca⁺⁺ and Mg⁺⁺-Free Dulbecco's Phosphate Buffered Saline (Ca⁺⁺Mg⁺⁺Free-DPBS). The process will be performed until the culture appears free from test article. If it is not possible to remove all of the visible test material, this will be noted in the workbook.

After rinsing, the tissue will be transferred to 5 mL of Assay Medium for a 10 to 20 minute incubation at room temperature. This rinse is intended to remove any test article absorbed into the tissue.

A 10X stock of MTT prepared in PBS (filtered at time of batch preparation) will be thawed and diluted in warm MTT Addition Medium to produce the 1.0 mg/mL solution no more than two hours before use. Alternatively, a 1.0 mg/mL MTT solution will be prepared in warm MTT Addition Medium and filtered through a 0.45 µm filter to remove undissolved crystals. Three hundred µL of the MTT solution will be added to each designated well of a pre-labeled 24-well plate. The tissue will be transferred to the appropriate wells after rinsing, and the plates incubated for 3 ± 0.1 hours at standard culture conditions.

After 3 ± 0.1 hours, the bottom of the EpiOcular™ tissue constructs will be blotted on absorbent paper, cleared of excess liquid, and transferred to a prelabeled 24-well plate containing 2.0 mL of isopropanol in each designated well. The plates will be sealed with parafilm and stored in the refrigerator (2-8°C) until the last exposure time is harvested. The plates, then, will be shaken for at least 2 hours at room temperature. At the end of the extraction period, the liquid within each Millicell® insert will be decanted into the well from which it was taken. The extract solution will be mixed and 200 µL transferred to the appropriate wells of a prelabeled 96-well plate(s). Two hundred µL of isopropanol will be added to the wells designated as blanks. The absorbance at 550 nm (OD₅₅₀) of each well will be measured with a Molecular Devices Vmax plate reader.

7.9 Killed Controls for Assessment of Residual Test Article Reduction of MTT

In cases where the test article is shown to reduce MTT, only test articles that remain bound to the tissue after rinsing, resulting in a false MTT reduction signal, present a problem. To demonstrate that residual test article is not acting to directly reduce the MTT, a functional check is performed in the definitive assay to show that the test material is not binding to the tissue and leading to a false MTT reduction signal.

To determine whether residual test article is acting to directly reduce the MTT, a freeze-killed control tissue is used. Freeze killed tissue is prepared by placing untreated EpiOcular™ constructs in the -20°C freezer at least overnight, thawing to room temperature, and then refreezing. Once refrozen, the tissue may be stored indefinitely in the freezer. To test for residual test article reduction, killed tissues are treated with the test article in the normal fashion. Generally, each test article will be evaluated for at least the shortest and longest exposure times (or longest exposure time if all exposures are 1 hour or less) in single replicate killed tissues. All assay procedures will be performed as for the viable tissue. A killed control treated with sterile deionized water (negative killed control) will be tested in parallel since a small amount of MTT reduction is expected from the residual NADH and associated enzymes within the killed tissue.

If little or no MTT reduction is observed in the test article-treated killed control, the MTT reduction observed in the test article-treated viable tissue may be ascribed to the viable cells. If there is appreciable MTT reduction in the treated killed control (relative to the amount in the treated viable tissue), additional steps must be taken to account for the chemical reduction or the test article may be considered untestable in this system. The OD₅₅₀ values from the killed controls will be analyzed as described in §7.10.

7.10 Presentation of Data

The raw absorbance values will be captured, and the following calculations made:

The mean OD₅₅₀ of the blank control wells will be calculated. The corrected mean OD₅₅₀ of the exposure time control(s) will be determined by subtracting the mean OD₅₅₀ of the blank control from their mean OD₅₅₀s. The corrected OD₅₅₀ of the individual test article exposure times and the positive control exposure times will be determined by subtracting the mean OD₅₅₀ of the blank control from their respective OD₅₅₀s. When applicable, corrected OD₅₅₀ values will be calculated for the control and test article-treated killed controls, as well. Generally, all calculations will be performed using Microsoft Excel.

$$\text{Corr. test article exposure time OD}_{550} = \text{Test article exp. time OD}_{550} - \text{Blank mean OD}_{550}$$

If killed controls (KC) are used, the following additional calculations will be performed to correct for the amount of MTT reduced directly by test article residues. The OD₅₅₀ value for the negative control killed control will be subtracted from the OD₅₅₀ values for each of the test article-treated killed controls (at each exposure time), to determine the net OD₅₅₀ values of the test article-treated killed controls.

$$\text{Net OD}_{550} \text{ for each test article KC} = \text{Raw OD}_{550} \text{ test article KC} - \text{Raw OD}_{550} \text{ negative control KC}$$

The net OD₅₅₀ values represent the amount of reduced MTT due to direct reduction by test article residues at specific exposure times. In general, if the net OD₅₅₀ value is greater than 0.150, the net amount of MTT reduction will be subtracted from the corrected OD₅₅₀ values of the viable treated tissues, at each corresponding exposure time, to obtain a final corrected OD₅₅₀ value. These final corrected OD₅₅₀ values will be used to determine the % of Control viabilities at each exposure time.

$$\text{Final Corrected OD}_{550} = \text{Corrected test article OD}_{550} \text{ (viable)} - \text{Net OD}_{550} \text{ test article (KC)}$$

Finally, the following % of Control calculations will be made:

$$\% \text{ of Control} = \frac{\text{corrected OD}_{550} \text{ of each Test Article or Positive Control exposure time}}{\text{corrected mean OD}_{550} \text{ of Negative Control}} \times 100$$

The individual % of Control values are then averaged to calculate the mean % of Control per exposure time. Viability calculations for test articles treated in the long exposure time assay may be performed by comparing the corrected OD₅₅₀s of each test article exposure time to the appropriate exposure time control(s).

Exposure time response curves may be plotted with the % of control on the ordinate and the test article exposure time on the abscissa. Other plot forms may be used as requested by the Sponsor. The ET₅₀ will be interpolated from each plot. To determine the ET₅₀, two adjacent points will be selected, one that shows greater than 50% survival and one that shows less than 50% survival. The two selected points will be used to determine the slope and the y-intercept for the equation $y = m(x) + b$. Finally, to determine the ET₅₀, the equation will be solved for

$y = 50$. If all of the exposure time points show greater than 50% survival, the ET_{50} will be listed as greater than the longest exposure time. If all of the exposure times show less than 50% survival, the ET_{50} will be presented as less than the shortest exposure time. At the Study Director's option, additional assays may be performed to produce the final ET_{50} value.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The assay will be accepted if the positive control, 0.3% Triton[®]-X-100, causes an ET_{50} within two standard deviations of the historical mean. The historical mean is updated every three months. The corrected mean OD_{550} value for the minimum negative control exposure time must be within 20% of the corrected mean OD_{550} value for the maximum negative control exposure time (up to 240 minutes).

9.0 EVALUATION OF TEST RESULTS

Significance of the ET_{50} is dependent on the class of materials tested. The Sponsor should refer to existing information in the literature to determine the significance of the ET_{50} of this test article.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. A summary will be prepared reporting the ET_{50} values for each test article as well as the positive control data. A copy of the protocol used for the study and any significant deviation(s) from the protocol will appear as a part of the final report.

11.0 RECORDS AND ARCHIVES

A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data, reports and specimens will be retained in the archives for a period of either a) 5 years, b) the length of time specified in the contract terms and conditions, or c) as long as the quality of the preparation affords evaluation, whichever is applicable.

12.0 REFERENCES

MTT Effective Time 50 (ET-50) Protocol, MatTek Corporation

Berridge, M.V., Tan, A.S., McCoy, K.D., Wang, R. (1996) The Biochemical and Cellular Basis of Cell Proliferation Assays That Use Tetrazolium Salts. **Biochemica** 4:14-19.

13.0 APPROVAL

SPONSOR REPRESENTATIVE

DATE

(Print or Type Name)

IIVS STUDY DIRECTOR

DATE

A4 IIVS BCOP Protocol

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH OPTIONAL HISTOLOGY

1.0 PURPOSE

The purpose of this study is to evaluate the potential ocular irritancy/toxicity of a test article as measured by the test article's ability to induce opacity and permeability to fluorescein in an isolated bovine cornea.

2.0 SPONSOR

2.1 Name:

2.2 Address:

2.3 Representative:

2.4 Sponsor Project Number:

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article(s): See Protocol Attachment 1

3.2 Controls: Positive:
Ethanol (CAS #64-17-5) Neat (liquid test articles)
Imidazole (CAS #288-32-4) 20% (w/v) in Complete MEM (solid test articles)
Negative:
Sterile deionized water or appropriate solvent

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions, if required.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Institute for In Vitro Sciences, Inc.

4.2 Address: 21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

4.3 Study Director:

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date:

5.2 Proposed Experimental Completion Date:

5.3 Proposed Report Date:

6.0 TEST SYSTEM

The test system (target tissue) is the isolated bovine cornea obtained as a by-product from freshly slaughtered animals. The procedures for preparing and handling the test system were developed by Gautheron et al. (1992). The assay measures two important components which are predictive of eye irritation; corneal opacity and permeability. Each cornea holder will be uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The treatment of each cornea will be identified with the test article number (or control) written in permanent marker on colored tape, affixed to each holder. Furthermore, when requested by the Sponsor, the depth and degree of injury may be assessed by histological evaluation.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

Liquid test articles will be tested neat unless otherwise directed by the Sponsor. If the liquid test article is to be diluted, the study may begin with a solubility or miscibility test in sterile, deionized water or appropriate solvent designated by the Sponsor. Solid test articles will be diluted in sterile deionized water unless otherwise directed by the Sponsor. The pH of each neat (liquid) test article or diluted test article will be determined, if possible, and recorded. Two or three corneas treated with sterile, deionized water will serve as the negative control. Two or three corneas will be exposed to the positive control. Four or five corneas will be treated with each neat test article or test article solution/suspension. One of two treatment methods will be used depending on the physical state and chemical characteristics (liquid or surfactant versus non-surfactant solid) of the test article. Changes in opacity, permeability to fluorescein and tissue architecture (depth of injury) will be measured and used to assess the relative potential for ocular irritancy of the test articles.

7.1 Reagents

7.1.1 Hanks' Balanced Salt Solution with Ca^{++} and Mg^{++} (containing Penicillin/Streptomycin) (HBSS)

7.1.2 Fetal Bovine Serum (FBS)

7.1.3 Minimum Essential Medium (EMEM) without phenol red

7.1.4 Minimum Essential Medium (EMEM) with phenol red
(used for rinsing of test substances only)

7.1.5 Sodium Fluorescein – diluted in DPBS

7.1.6 Sterile Deionized Water

7.1.7 10% Buffered formalin solution

7.2 Bovine Eyes

Bovine eyes will be obtained from the abattoir of J.W. TREUTH & SONS, Inc., Baltimore, MD. The eyes will be excised by an abattoir employee (as soon after slaughter as possible) and held in HBSS on ice. Once the required number of eyes has been obtained, the eyes will be transported to IIVS. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas will be initiated.

7.3 Preparation of Corneas

All eyes will be carefully examined for defects (opacity, scratches, pigmentation, etc.) and those exhibiting defects discarded. The tissue surrounding the eyeball will be carefully pulled away and the cornea will be excised leaving a 2 to 3 mm rim of sclera. The isolated corneas will be stored in a petri dish containing HBSS prior to mounting. Corneas will then be mounted in the corneal holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber will then be positioned on top of the cornea and tightened with screws. The chambers of the corneal holder will then be filled with EMEM (without phenol red) containing 1% FBS and 2mM L-glutamine (Complete MEM). The posterior chamber will always be filled first. The corneas will be incubated for the minimum of one hour at $32\pm 1^{\circ}\text{C}$.

7.4 Sample Preparations

When appropriate, test articles will be diluted or suspended in either sterile deionized water or other Sponsor-directed solvent. Samples will be diluted on a w/v basis, unless otherwise specified by the Sponsor.

7.5 Treatment of Corneas

At the end of the one-hour incubation period, the medium will be removed from both chambers and replaced with fresh Complete MEM without phenol red. An initial opacity measurement will be performed on each of the corneas. Two or three corneas with opacity readings approximately equivalent to the median opacity of all corneas will be selected as the negative control corneas. The opacity of each cornea (including the negative control corneas) will be read against an air-filled chamber and recorded. Corneas that have an initial opacity reading that is 10 or more units greater or lower than the average opacity of all used corneas will not be dosed. The medium will be removed from the anterior chamber and replaced with the test article, negative control, or positive control.

Protocol Attachment 1 will provide the test article designation(s), any preparation (including dilution and handling of the test material), the method to be used (if

applicable), the length of the treatment, the post-treatment incubation time(s) and the applicable regulations to be followed.

7.5.1 Method A:

Liquids will generally be tested neat (undiluted), unless the Sponsor requests a specific dilution. Surfactants (either solids or liquids) will generally be tested at a 10% concentration in sterile deionized water unless otherwise directed by the Sponsor. Formulations will be tested after consultation with the Sponsor.

Seven hundred and fifty μL of test substance (test article, negative control or positive control) will be introduced into the anterior chamber. The holder will be slightly rotated (with the corneas maintained in a horizontal position) to ensure uniform distribution of the test substance over the cornea. Alternatively, the test material may be applied as a spray to cover the corneal surface. Spray application will be used only when directed by the Sponsor and will follow the specific procedure indicated. The test article and negative control treated corneas will be incubated at $32\pm 1^\circ\text{C}$ for 10 minutes or as directed by the Sponsor. The positive control will be incubated at $32\pm 1^\circ\text{C}$ for 10 minutes. On occasion, the negative control exposure time may be selected to fit the longest test article exposure time of a test article run concurrently, but from an independent study. The test substance will then be removed and the epithelium will be washed at least 3 times (or until no visual evidence of test substance can be observed) with Complete MEM (containing phenol red). Once the media is free of test substance, the corneas will be given a final rinse with Complete MEM (without phenol red). If the test article cannot be removed from the cornea a note will be documented in the raw data record. The anterior chamber will then be refilled with fresh Complete MEM without phenol red, and an opacity measurement will be performed. The corneas will then be incubated for approximately 2 hours at $32\pm 1^\circ\text{C}$. At the completion of the incubation period, a second measure of opacity will be performed (final opacity). The values obtained at this second measurement will be used in calculating the corneal opacity.

7.5.2 Method B:

Solid materials will generally be tested as a 20% dilution (w/v) in sterile deionized water (or Sponsor directed solvent). Different concentrations may be evaluated at the Sponsor's request.

Seven hundred and fifty μL of test substance (test article, negative control or positive control) will be introduced into the anterior chamber. The holder will be slightly rotated (with the corneas maintained in a horizontal position) to ensure uniform distribution of the test substance over the cornea. The corneas will be incubated in a horizontal position at $32\pm 1^\circ\text{C}$ for approximately 4 hours or as specified by the Sponsor. The test substance will then be removed and the epithelium washed at least 3 times

(or until no visual evidence of test substance can be observed) with Complete MEM (containing phenol red). Once the media is free of test substance, the corneas will be given a final rinse with Complete MEM (without phenol red). If the test article cannot be removed from the cornea a note will be recorded in the raw data record. The anterior and the posterior chambers will then be refilled with fresh Complete MEM without phenol red, and an opacity measurement performed immediately (without any further incubation)(final opacity).

7.5.3 Method C:

In some cases, a test compound may require a different exposure and post-treatment incubation time and/or test concentration. The specific concentration and treatment times will be determined after consultation with the Sponsor. The treatment will then be performed in the same manner as Method A. Depending on the nature of the test articles exposure times of 10 minutes and less may be performed at room temperature. The exposure time will be subtracted from the 2-hour post-exposure incubation period for test articles with exposure times greater than 10 minutes.

7.6 Opacity Measurement

The opacitometer will determine the difference in the light transmission between each treated or control cornea and an air-filled chamber, and a numerical opacity value (arbitrary unit) will be displayed and recorded.

7.7 Permeability Determinations

Methods A & C:

After the second opacity measurement is performed, the medium will be removed from both chambers of the holder. The posterior chamber will be refilled with fresh Complete MEM without phenol red. One mL of a 4 mg/mL sodium fluorescein solution will be added to the anterior chamber.

Method B:

After the opacity measurement is performed, the medium will be removed from the anterior chamber only and replaced with 1 mL of a 5 mg/mL sodium fluorescein solution.

After the addition of the fluorescein solution to the anterior chamber, the corneas will be incubated in a horizontal position for approximately 90 minutes at $32\pm 1^{\circ}\text{C}$. The medium from the posterior chamber will be removed at the completion of the incubation period, and its optical density at 490 nm (OD_{490}) determined using a spectrophotometer. Samples reading 1.500 and above (OD_{490}) will be diluted to bring the reading within the linear range of the platereader and the plate read again.

7.8 Fixation of the Corneas

After the medium is removed for the fluorescein determination, each cornea will be carefully removed from its holder and transferred to a prelabelled tissue cassette. The endothelial surface will be placed on sponge to protect it. The cassettes will be placed in 10% neutral buffered formalin and fixed for a minimum of 24 hours. If the Sponsor elects to have histological evaluation performed, the corneas will be sent to the histology laboratory for processing. If the Sponsor elects not to have histology performed, the corneas will be discarded.

7.9 Histological Evaluation (if elected by the Sponsor)

7.9.1 Dr. Stewart B. Jacobson

Charles River Laboratories, Pathology Associates (PAI)
15 Worman's Mill Court, Suite I
Frederick, MD 21701
301-624-2027

7.9.2 The fixed tissues will be transferred to Charles River Laboratories-Pathology Associates (PAI) in Frederick, MD, for embedding, sectioning, staining and histological evaluation. Dr. Stewart B. Jacobson at PAI will serve as the Principle Investigator for the histological portion of the study. Each cornea will be bisected and a section from each half will be cut, placed in a cassette and embedded in paraffin to produce a single slide. Each slide will then be stained with hematoxylin and eosin. The resulting slides will be evaluated by either the Institute for In Vitro Sciences, Inc. or by Dr. Jacobson. Cornea sections will be examined for the presence of changes in the epithelium, stromal, and endothelial areas of the tissue. Particular emphasis will be placed on assessment of depth of injury into the stromal elements (Harbell et al, 1999, and Curren et al, 1999). Treated tissues will be compared to the negative and positive control tissues. Representative fields will be photographed for illustration of the changes.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The test will be accepted if the positive control causes an *in vitro* score that falls within two standard deviations of the historical mean.

8.1 EVALUATION OF TEST RESULTS

The change in opacity for each cornea (including the negative control corneas) will be calculated by subtracting the initial opacity reading from the final opacity reading. These values will then be corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value for each treatment will be calculated by averaging the corrected opacity values of each cornea for a given treatment.

The mean OD₄₉₀ for the blank wells will be calculated. The mean blank OD₄₉₀ will be subtracted from the OD₄₉₀ of each well (corrected OD₄₉₀). Any dilutions that are made to bring the OD₄₉₀ values into the linear range of the platereader (OD₄₉₀ should be less than 1.500), will have each diluted OD₄₉₀ value multiplied by the dilution factor. The final corrected OD₄₉₀ of the test article(s) and the positive control will be calculated by subtracting the average corrected OD₄₉₀ of the negative control corneas from the corrected OD₄₉₀ value of each treated cornea:

$$\text{Final Corrected OD}_{490} = (\text{OD}_{490} - \text{mean blank OD}_{490}) - \text{average corrected negative control OD}_{490}$$

The mean OD₄₉₀ value of each treatment group will be calculated by averaging the final corrected OD₄₉₀ values of the treated corneas for that treatment condition.

9.1 *In Vitro* Score Calculation

The following formula will be used to determine the *in vitro* score:

$$\text{In Vitro Score} = \text{Mean Opacity Value} + (15 \times \text{Mean OD}_{490} \text{ Value})$$

9.2 Data Interpretation

The following classification system was established by Sina et al. based on studies with a wide range of test materials. While this classification system provides a good initial guide to interpretation of these *in vitro* data, these specific ranges may not be applicable to all classes of materials or other exposure times. Whenever possible, results should be compared to “benchmark” materials tested under similar exposure conditions.

In Vitro Score:

≤ 25	= mild irritant
from 25.1 to 55	= moderate irritant
from 55.1 and above	= severe irritant

10.0 REPORT

A report of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. A summary will be presented for each treatment group. The report will also include a discussion of results. A copy of the protocol used for the study and any significant deviation(s) from the protocol will appear as a part of the final report.

11.0 RECORDS AND ARCHIVES

A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data, reports and specimens will be retained in the archives for a period of either a) 5 years, b) the length of time specified in the contract terms and conditions, or c) as long as the quality of the preparation affords evaluation, whichever is applicable.

All data and materials generated by PAI will be shipped or delivered to the study director at the Testing Facility upon finalization of the pathology report, or within three months of the issuance of the draft pathology report, whichever occurs first

REFERENCES

Curren, R.D., Evans, M.G., Raabe, H.A., Ruppalt, R.R., Harbell, J.W. (2000) An histological analysis of damage to bovine corneas in vitro by selected ocular toxicants. **The Toxicologist** 54(1):188.

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Harbell, J.W., Raabe, H.A., Evans, M.G., and Curren, R.D. (1999) Histopathology associated with opacity and permeability changes in bovine corneas in vitro. **The Toxicologist** 48:336-337.

Sina, J.F., Galer, D.M., Sussman, R.G., Gautheron, P.D., Sargent, E.V., Leong, B., Shah, P.V., Curren, R.D., and Miller, K. (1995) A collaborative evaluation of seven alternatives to the Draize eye irritation test using pharmaceutical intermediates. **Fundamental and Applied Toxicology** 26:20-31.

APPROVAL

SPONSOR REPRESENTATIVE

DATE

(Print or Type Name)

IIVS STUDY DIRECTOR

DATE