US Environmental Protection Agency
Office of Pesticide Programs

NON-ANIMAL TESTING APPROACH TO EPA LABELING FOR EYE IRRITATION

May 11, 2009

VOLUNTARY PILOT PROGRAM TO EVALUATE USE OF A NON-ANIMAL TESTING APPROACH TO EPA LABELING FOR EYE IRRITATION FOR CERTAIN AN TIMICROBIAL PRODUCTS WITH CLEANING CLAIMS; 5/11/2009; John Redden, MS; Mark J. Perry, MPH; Timothy Leighton, Jonathan Chen, Ph.D.; Tim McMahon, Ph.D.

I. INTRODUCTION

The EPA Office of Pesticide Programs (OPP) currently uses the Draize rabbit eye test to determine ocular hazards, and the required hazard labeling for pesticide products. This voluntary pilot project is designed to evaluate the effectiveness of a specific alternative testing approach, as a potential replacement for the Draize rabbit eye test, for labeling antimicrobial products with cleaning claims. The proposed testing strategy uses three assays; the Bovine Corneal Opacity and Permeability test (BCOP), the EpiOcular™ model (EO), and the Cytosensor Microphysiometer assay (CM).

This approach is intended to allow OPP to differentiate among the four eye irritation hazard categories used by the Agency. These categories and the associated label statements are listed below. Along with the three alternative assays, OPP is asking participating registrants to submit available consumer incident data and any existing Draize test results on similar or structurally-related chemicals or products as further support for the testing approach.

Table 1. Eye Irritation-Triggered Label Statements and Eye Protection.

Toxicity Category	Signal Word	Eye Protection and Label Precautionary Language
I	DANGER	Goggles, face shield, or safety glasses. Corrosive. Causes irreversible eye damage.
II	WARNING	Goggles, face shield, or safety glasses. Causes substantial but temporary eye injury.
III	CAUTION	Protective eyewear if appropriate. Causes moderate irritation.
IV	CAUTION	No statements are required.

II. OBJECTIVE

The objective of this voluntary pilot project is to evaluate and gain experience with certain non-animal testing methods (*i.e., ex vivo* and *in vitro*) to assess eye irritation and generate labeling for certain antimicrobial products with cleaning claims. The Agency, which has formed a workgroup to manage this pilot, also intends to use the resulting data set to further evaluate the individual non-animal assays. In addition, this pilot incorporates the goals of the 3 R's of animal testing:

- **Refinement alternative**: New or modified test method/s that refine/s procedures to lessen or eliminate pain or distress in animals or enhances animal well-being.

- **Reduction alternative**: New or modified test method/s that reduce/s the number of animals required for a test method, while remaining consistent with sound scientific practices necessary to obtain valid results.

- **Replacement alternative**: New or modified test method/s that replace/s animals with non-animal systems or replace/s an animal species with a phylogenetically lower species.

The pilot test strategy and supporting science have been reviewed and approved by the EPA's Office of Pesticide Programs Science Policy Council (SciPoC). The Science Policy Council consists of senior staff from all of the science and regulatory divisions and serves as a central forum that assists OPP in reviewing and transitioning new science policies and methods into the pesticide program. As mentioned above, the assays that will serve as the basis of the pilot approach for eye irritation labeling include the Bovine Corneal Opacity and Permeability test, the EpiOcular model and the Cytosensor Microphysiometer assay. These non-animal assays, **when used in a tiered approach,** are being considered as a potential replacement to the Draize rabbit test. A brief description of these assays follows:

- **Bovine Corneal Opacity and Permeability Assay (BCOP) -** is an assay that uses bovine (cow) eyes which are received shortly after the slaughter of the animal so the cells are still viable. The corneas are excised and treated with a chemical to determine its potential to damage the eye. The BCOP model is a model with endpoints similar to many human corneal responses.

- **EpiOcular ™ (EO) Model** - this test is an *in vitro* model of the human corneal epithelium composed of normal human-derived epidermal keratinocytes and is used to evaluate the eye irritation potential of chemicals, particularly surfactants.

- **Cytosensor Microphysiometer (CM) Assay** - this assay evaluates the potential eye toxicity of a chemical by measuring the dose required to reduce the metabolic rate in treated cells in vitro. A very sensitive instrument called a microphysiometer is used to electronically measure the metabolic rate of cell populations through small changes in acidic metabolites in the medium. The rate is constant in an undamaged cell population and if the cells are injured, an altered metabolic rate is found.

It is intended during the pilot phase that labeling decisions will be made using data derived from these non-animal tests if the testing methods and testing results are deemed by the Agency to be adequate and appropriate to support such regulatory decisions. Antimicrobial products with cleaning claims may include formulations of different composition (water or surfactant-based; oxidizing chemistry; solvent-containing; or non-aqueous soluble formulations). Proposed testing strategies for these various types of formulations in this pilot may differ.

III. PILOT DETAILS

A. Scope and Duration

As mentioned previously, this pilot project is limited to antimicrobial products with cleaning claims. Additionally, because this subset of pesticide products typically accounts for over 100 registrations per year, for practical purposes the pilot will be limited initially to a timeframe of 18 months. After 18 months a decision will be made to determine if: 1) the non-animal testing strategy is valid and adequate, or 2) the pilot needs to be extended to gather more data. Also, since one of the goals of the pilot is to collect non-animal testing data for further

evaluation, OPP does not expect to generally allow data submitted under this pilot to be cited or bridged from one product to another.

B. Review Process

The review process for the pilot project will involve determining the effectiveness of the decision tree (see Section IV), as well as whether submitted assays were conducted according to the published guidance and are acceptable. Initially, packages submitted to the Agency under the pilot will be reviewed by the workgroup. One member of the workgroup will perform the primary review, while secondary review will be performed by one or more of the remaining workgroup members. Any issues which arise during this initial phase of the pilot will be discussed by the workgroup as a whole. Toxicity reviewers from the Antimicrobials Division (AD), who will ultimately be responsible for assessing these packages, will participate in these discussions. The assays will be reviewed for compliance with the following protocols (see Appendix I, Annex I, II and III):

- Ocular Irritation Assay for Antimicrobial products with cleaning claims using the Bovine Corneal Opacity and Permeability Assay and Histology

- Ocular Irritation Assay for Antimicrobial products with cleaning claims using the EpiOcular™ Human Cell Construct

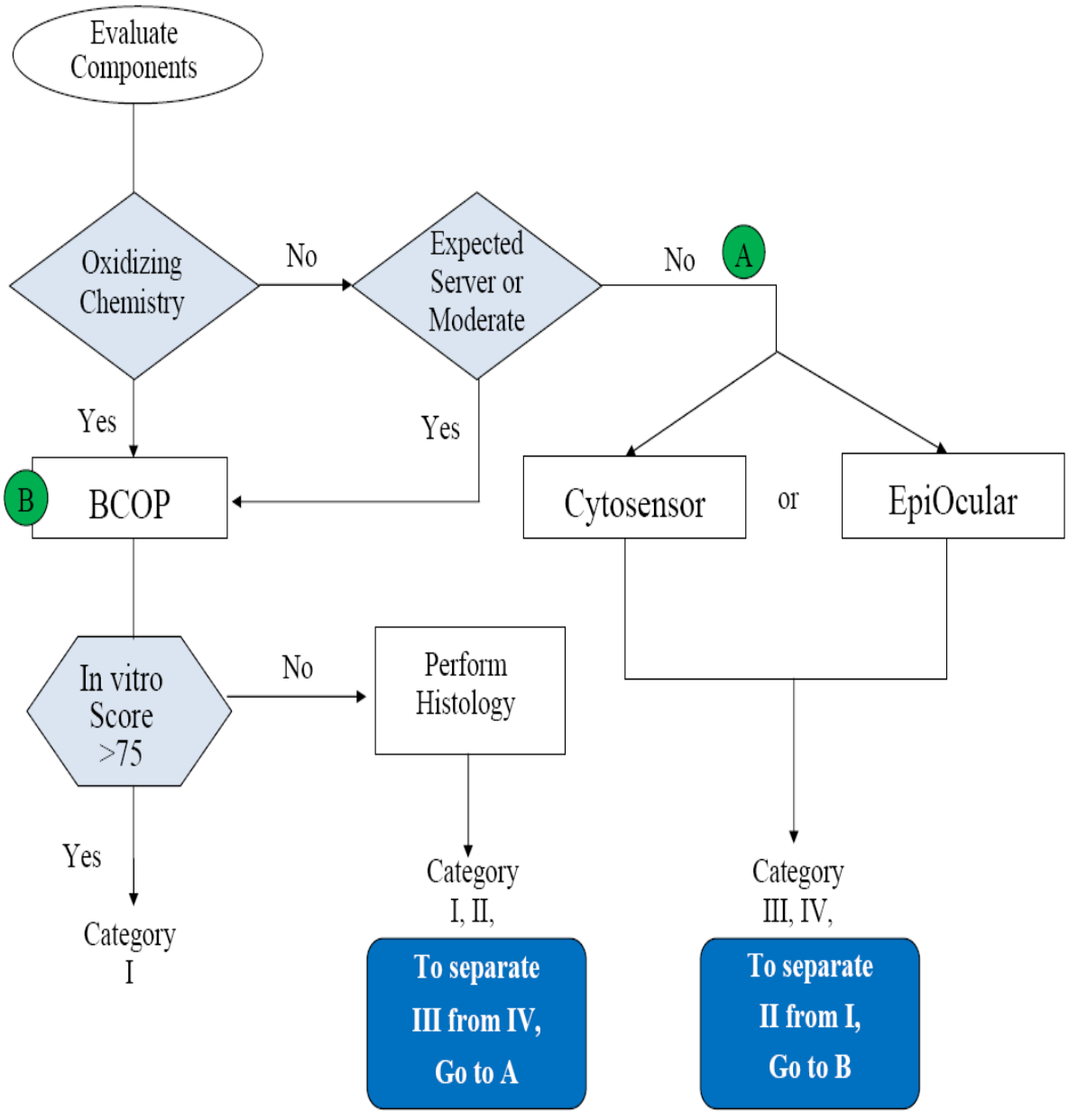
- Ocular Irritation Assay for Antimicrobial products with cleaning claims using the Cytosensor Microphysiometer Bioassay

Once the Workgroup has gained experience reviewing the studies, the primary and secondary review responsibility will be turned over to the Antimicrobials Division. The workgroup will meet regularly with the AD reviewers to discuss any issues related to the assessment of these packages. If needed, the workgroup will be available at any point in the process to perform secondary reviews of submitted data packages, or to discuss study-related issues with registrants and/or performing laboratories.

C. Pilot Project Assessment:

Eighteen months after the start of the pilot project, the workgroup will compile and analyze the study data. This process will involve an evaluation of the resulting eye irritation categories, the types of products received for review, and a comparison of the study findings with submitted incident data and/or existing Draize data. Consumer incident data and Draize data will be considered from the subject product undergoing registration, as well as from other pesticidal and non-pesticidal products deemed similar to the subject product. Product similarity will be verified by the workgroup prior to inclusion of incident or Draize data in the assessment document. The final assessment document will be presented to OPP's Science Policy Council upon completion for their recommendations on the way forward with this non-animal approach.

IV. DECISION TREE



The first step in the decision tree is to evaluate the existing information (including the chemical and physical properties) on the active ingredient/formulation, and existing Draize results or *in vitro* data on related compounds. If the formulation is based on oxidizing (reactive) chemistries or the components fall in a class of chemicals that are severe irritants, it should be tested in the BCOP assay. If the formulation is not based on oxidizing (reactive) chemistries, a scientific judgment should be made (based on the type or concentration of formulation ingredients, past registration of similar products, in-use information from similar, non anti-microbial products, etc.) as to the expected ocular hazard category of the formulation. **This decision does not affect the outcome of the evaluation. If the initial judgment is incorrect, that fact will be revealed in the testing strategy and a second test may have to be used. For example, if EpiOcular indicates Category I, then BCOP will need to be performed. The initial judgment is only made for efficiency; if correct, only one assay will have to be conducted.**

If the material is a "high solvent" formulation, it should be tested with a 3 minute exposure. All other materials are tested with a ten-minute exposure. A high solvent formulation is considered to have equal to or greater than 5% concentrations of organic solvents (e.g. alcohols, glycol ethers). Because of earlier indications that some solvent-containing materials might be over predicted, IIVS – for the last several years – has tested such materials in the BCOP assay using two different exposure times: 3 minutes and 10 minutes. Based on this experience it has been generally noticed that the three minute exposure gives a better prediction of the actual irritancy potential than does the 10 minute exposure (extracted from: **Background Review Document of an *In Vitro* Approach for EPA Toxicity Labeling of Anti-Microbial Cleaning Products see: http://iccvam.niehs.nih.gov/methods/ocutox/PeerPanel09.htm).**

If the formulation receives a score of >75 in the BCOP assay it is labeled as a Category I. Although such formulations are placed in Category I, performance of histopathology is strongly encouraged. If it receives a score of <75, histopathology should be performed and the depth of injury should be used to determine if the final Category should be I, II, or a lower. The data presented by the AMCP supports the selection of >75 for Category I as well as <75 (with histopathology) for differentiating Category I and II (see BRD: Background Review Document of an In Vitro Approach for EPA Toxicity Labeling Anti-Microbial Cleaning Products; Rodger D. Curren, Ph.D.; Jennifer; R. Nash, M.S.; Angela Sizemore, B.S.; John Harbell, Ph.D). Although it should be noted that a standard scoring scheme for judging histopathology results has not been established.

If it is thought that the product might be a Category III or IV material, companies may chose to use either the CM or EO test which can confirm the moderate Category III or Category IV materials. If at the beginning of the testing scheme it is thought that the product is likely a mild/moderate product, either the CM or EO assays should be chosen. The CM, however, could only be used with completely water soluble materials (limitation noted in data submitted to ICCVAM for CM). The results from either of these tests would determine whether the material was a Category I, III, or IV. If the company thought the material might actually be a Category II, then an additional BCOP assay with histopathology should be conducted to determine if this is the case and to support a different classification.

It should be noted that the above strategy is self-correcting if the initial estimate of irritation potential of a test substance is incorrect. If a highly irritating material is tested in the Cytosensor or EpiOcular assays, it will receive a score indicating that it is a highly irritating (Category I) material. If further resolution is desired (to determine if it is actually only a

Category II material rather than a Category I material), the formulation can then be further tested in the BCOP assay. Similarly a mild material will be identified as a Category III material by the BCOP assay. If it is important to the company to distinguish between a Category III and IV for labeling and marketing purposes, then an additional Cytosensor or EpiOcular assay may be helpful in making that determination.

The proposed *in vitro* strategy is considered conservative and generally results in over labeling of some products, especially many EPA category IV materials which could be over-predicted to be EPA category III. The participating companies are aware of the potential for over-predictions and have accepted it as a small consequence of adopting non-animal testing strategy.

V. SUBMISSION PACKAGE GUIDANCE

A. General Package

- Raw data on the non-animal assays (BCOP, EO, and/or CM).

- Available Draize rabbit test results for similar or structurally related compounds.

- Available consumer incident data on the tested product or similar products/formulations. Incident data should be provided on similar EPA-registered pesticide products, as well as similar unregistered consumer products, if available. The following should also be reported if applicable:

    - EPA File Symbol/Registration Number
    - Active ingredient(s)/PC Code(s) associated with each incident
    - Symptoms/clinical effects
    - Specific effects should be specified whenever possible/available
    - Duration of symptoms
    - Symptom reversibility
    - Medical outcome/severity of incident
    - Level of health care required (another indicator of severity/burden)
    - Exposure route
    - Exposure site
    - Exposure reason
    - Age
    - Sex
    - Year of incident

- Any other useful existing knowledge (e.g., chemical physical properties, other data on irritancy, Structural Activity Relationship (SAR) data on irritancy) for ocular hazard labeling

B. Data and Reporting

The study report should include a description of the test material, the methods and the study results. At a minimum, all data should be detailed in tabular form for each individual protocol and the following reported:

- physical nature, and, where appropriate, concentration and pH value for the test substance;
- description of any pre-test conditioning;
- manufacturer, source, purity, and lot number of test substance;
- Good Laboratory Standard (GLP) standards employed;
- rationale for selection of test (BCOP, EpiOcular or Cytosensor);
- identification, composition, and characteristics of any vehicles (e.g., diluents, suspending agents, emulsifiers, and anesthetics) or other materials used in administering the test substance;
- a list of references cited in the body of the report, i.e., references to any published literature used in developing the test protocol; performing the testing, making and interpreting observations, and compiling and evaluating the results;
- description of the method used to score the irritation;
- description of any lesions observed (BCOP);
- any effects other than ocular which were observed;
- narrative description of the degree and nature of irritation or corrosion observed, and;
- a tabular description irritant/corrosive* response data for each individual test.

* *Eye corrosion* is the production of irreversible tissue damage in the eye following application of a test substance to the anterior surface of the eye. *Eye irritation* is the production of reversible changes in the eye following application of a test substance to the anterior surface of the eye. (Note: Reversible changes can not be measured *ex vivo*. However it is proposed that a score less than 75 in the BCOP with histopathology and the weight of evidence from other sources will allow this determination.)

VI. SCIENTIFIC BASIS

A. Regulatory Background

As a result of discussions on the use of non-animal testing methods at the fall 2003 Pesticide Programs Dialogue Committee (PPDC), the Alternative Testing Working Group (ATWG) was formed with the goal of developing a non-animal eye irritation testing approach for antimicrobial products with cleaning claims. In 2004 the ATWG, which is comprised of industry representatives from the PPDC, developed an approach which uses three tests (BCOP, EO and CM) to determine an ocular irritation category. Subsequently, EPA's Office of Pesticide Programs (OPP) requested the assistance of the National Toxicology Program's Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) with the evaluation of this approach. The ICCVAM formed the Ocular Toxicity Working Group (OTWG) which has proposed an alternate strategy employing only the BCOP and EO assays to assign materials to categories I and IV, respectively. A peer review of the OTWG approach is scheduled for May 2009 at the National Institutes of Health.

The OTWG chose not to include the Cytosensor Microphysiometer in their proposed strategy due to its reliance on Low Volume Eye Test (LVET) data for validation. The LVET, which was developed as an alternative to Draize, involves application of 0.1 ml of test material directly on the cornea of the test animal. Based on studies which indicate that LVET data under-predicts severe irritants when compared to the Draize, the OTWG concluded that the LVET is not an acceptable *in vivo* reference test method against which to compare *in vitro* test method results. However, a recent European Centre for the Validation of Alternative Testing (ECVAM) evaluation of Cytosensor Microphysiometer supports its use to classify category I and category IV ocular irritants based on non-LVET reference data. Following the ECVAM evaluation, the OTWG concurred with this decision.

In addition, the OTWG concluded that the histopathology data for the BCOP did not give the degree of sensitivity expected, and as a result, recommended that the BCOP only be used for severe irritants (Toxicity Category I). It was noted that based on a limited data set (n=29) using BCOP to identify corrosives followed by EpiOcular to identify non-irritants appears to be an effective strategy for these two categories. **The OTWG further concluded that there are inadequate data to determine the usefulness of this strategy for identifying mild and moderated irritants.**

Additional data using BCOP, EpiOcular and Cytosensor Microphysiometer should be submitted by the registrant community to the Agency. As noted above, there will be a May 2009 ICCVAM peer review to analyze the proposed strategy using BCOP, EpiOcular and Cytosensor. Additional data from this pilot would strengthen the case for finalizing this pilot testing strategy for certain antimicrobial products with cleaning claims. Before initiating this pilot, the Agency has reviewed the data and concluded that the evidence available supports that this approach will identify Toxicity Category I, II, III and IV labeling for eye irritation.

B. Agency Conclusions on LVET Data

The LVET is not a guideline study, and the Agency does not consider it sufficient, by itself, to satisfy the eye irritation data requirement for pesticides. The Agency concurs with the OTWG that the LVET is not an acceptable *in vivo* reference test method against which to compare *in vitro* Test Method results.

C. Cytosensor

Because Cytosensor is considered, as reported at ICCVAM/OTWG, useful for identifying ocular corrosives/severe irritants (top-down approach) and nonirritants (bottom-up approach) specifically for surfactants and surfactant-based formulations, and because many antimicrobial products with cleaning claims are surfactant containing formulations, SciPoc believes that Cytosensor may be useful in a testing strategy for identifying ocular irritation.

As part of the testing scheme, chemical physical properties of the chemical of interest, registrant gathered consumer incident data, and existing Draize test results on structurally related compounds would be assessed to evaluate labeling decisions regarding product safety. This weight of evidence approach should allow greater confidence in the results reported in the *in vitro* and *ex vivo* studies.

D. BCOP Decision Criteria

BCOP is not intended to characterize EPA Toxicity Category IV. The AMCP BRD (see reference, Section VII) gives the following guidance for when to do histopathology for BCOP. The histopathology endpoint has often been added to the BCOP test to allow the actual extent of damage to the cornea to be visualized and assessed. The initial area and depth of injury to the cornea has been hypothesized to be directly related to the reversibility of the injury. The AMCP BRD concluded that the greater the depth of injury, the less likely that the lesion is reversible.

Table 2: AMCP BRD recommendations

In Vitro Score	EPA Category	GHS Category	Histopathology
≥ 75	I	1	No histopathology needs to be conducted
< 75 and ≥ 25	II	2A	They should be further assessed with a histopathological evaluation and given the final categorization of whichever determination (in vitro score or histological evaluation) is more severe.
< 25	III	2B	They should be assessed with a histological evaluation and given the final categorization of whichever determination (in vitro score or histological evaluation) is more severe.

Table 3: AMCP BRD Histopathology Decision Criteria

Extent of Damage	Suggested EPA Category	Suggested GHS Category
Cell loss or damage extending no further than midway through the epithelium	IV	NL
Cellular damage or collagen matrix damage extending no further than the upper third of the stroma	III	2B
Cellular damage or collagen matrix damage extending no further than two-thirds of the way through the stroma	II	2A
Cellular damage or collagen matrix damage extending into the lower third of the stroma and/or causing damage o the endothelial cells	I	1

According to the BCOP BRD "The *in vitro* irritation classification schemes used for this evaluation were based on two different predetermined ranges of *in vitro* scores. The differences between the two ranges are attributed to two different criteria used to identify ocular corrosives and severe irritants (i.e., EPA Category I, EU R41, and GHS Category 1)." One approach (**Table 4**) included the ICCVAM recommended decision criteria for identifying an ocular corrosive/severe irritant (i.e., IVIS ≥ 55.1, ICCVAM 2006).

Table 4. *In Vitro* Ocular Irritancy Classification Scheme for the BCOP Test Method (ICCVAM 2006)

In Vitro Score Range	*In Vitro* Classification
0-3.0	**Not Labeled**
3.1 - 25	**Mild irritant**
25.1 - 55	**Moderate irritant**
≥ 55.1	**Severe irritant**

The second approach (**Table 5**) included an alternative decision criteria used for identifying an ocular corrosive/severe irritant in the AMCP BRD submission (i.e., IVIS ≥ 75).

Table 5. *In Vitro* Ocular Irritancy Classification Scheme for the BCOP Test Method (AMCP BRD Submission)

In Vitro Score Range	*In Vitro* Classification
0-3.0	**Not Labeled**
3.1 - 25	**Mild irritant**
25.1 – 74.9	**Moderate irritant**
≥ 75	**Severe irritant**

E. OTWG/ICCVAM Conclusions Regarding BCOP Histopathology

For 10 out of 17 of the AMCP materials, the EPA category classification using only BCOP was inconsistent with the EPA Category determined by Draize. For 11 out of 17 of the AMCP materials, the final EPA Category (using BCOP with histopathology) was inconsistent with the EPA Category by Draize. It appears that histopathology does not improve BCOP accuracy, but this observation is based on very limited data (N=17). An important aspect of this pilot project is to evaluate additional data to determine if modifying the histopathology decision criteria could improve accuracy.

F. ICCVAM-Proposed Alternative AMCP Strategy Using BCOP and EpiOcular

Two strategies have been evaluated by NICEATM/ICCVAM:

1. Test in BCOP first ("top-down" strategy); Any substance classified as EPA Category I or II based on BCOP would be classified as such and no further testing required. All other substances would be subsequently tested in EpiOcular to classify as either EPA Category III or IV.

2. Test in EpiOcular first ("bottom-up" strategy); Any substance classified as EPA Category III or IV based on EpiOcular would be classified as such and no further testing required. All other substances would be subsequently tested in BCOP to classify as either EPA Category I or II.

NOTE: Further Draft recommendation from OTWG: 1) overall database for BCOP and EpiOcular indicate that BCOP is reliable in identifying Category I, while EpiOcular is reliable in identifying Category IV; and 2) there are insufficient data with which to adequately demonstrate that the ICCVAM-proposed alternative AMCP testing strategy can identify all four required EPA hazard categories for ocular irritation/corrosion.

G. Details on Determining Hazard Categories.

The proposed strategy is intended to identify Categories I-IV materials using a combination of assays and a weight of the evidence approach, which is strengthened by available consumer incident data and/or existing Draize test results on similar or structurally-related chemicals. Using this approach, the EO and CM assays are intended to identify Category III and IV materials, while the BCOP assay is intended to identify Category I and II materials. If the Agency concludes that the data submitted is not convincing then the Agency will make the more conservative decision.

Cytosensor

OPP believes that the CM can identify Category III materials. As can be seen in the following graph of 105 materials, materials which scored between 2 mg/ml and 80 mg/ml are designated as Category III materials (Figure 1). They are clearly less toxic than the Category I and II materials which all scored below 2 mg/ml.

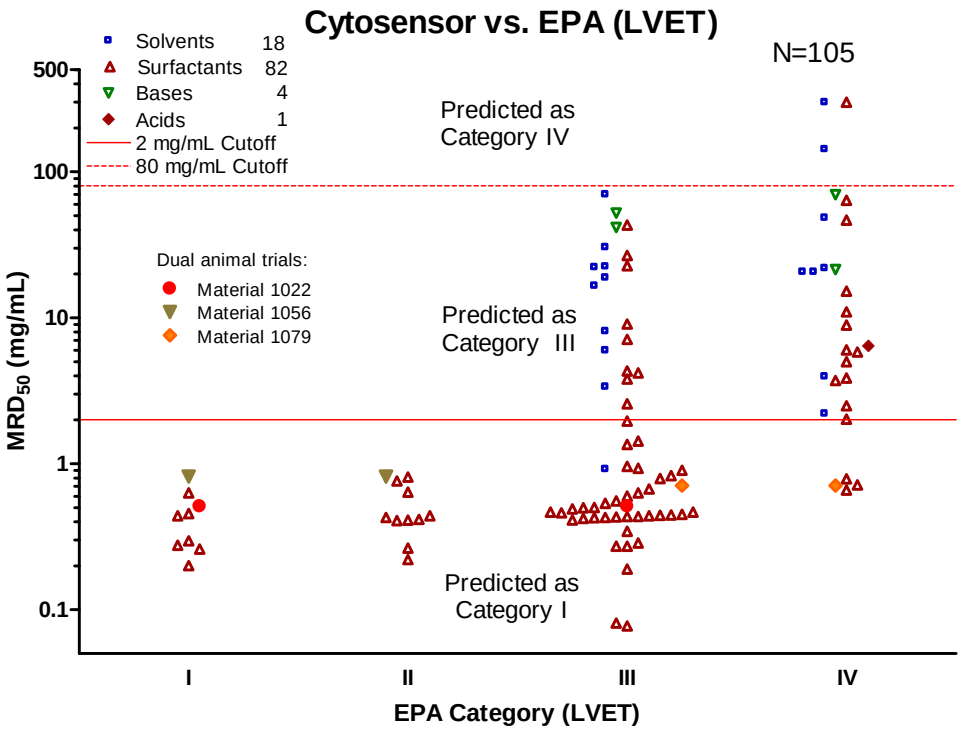


Figure 1. Cytosensor MRD_{50} values plotted against EPA toxicity categories determined by the LVET. Suggested cut-off values with their predicted EPA categories are included. There are 105 unique materials; however, 3 materials are graphed with 2 different EPA categories since they were tested twice in the animal trials with different results each time (BRD).

EpiOcular

OPP believes that the EO assay can identify Category III materials. As can be seen in Figure 2, materials having a score between 4 and 70 minutes would be considered Category III. Admittedly there were not an overwhelming number of materials tested, but an additional 25 materials which had LVET data showed the same pattern.

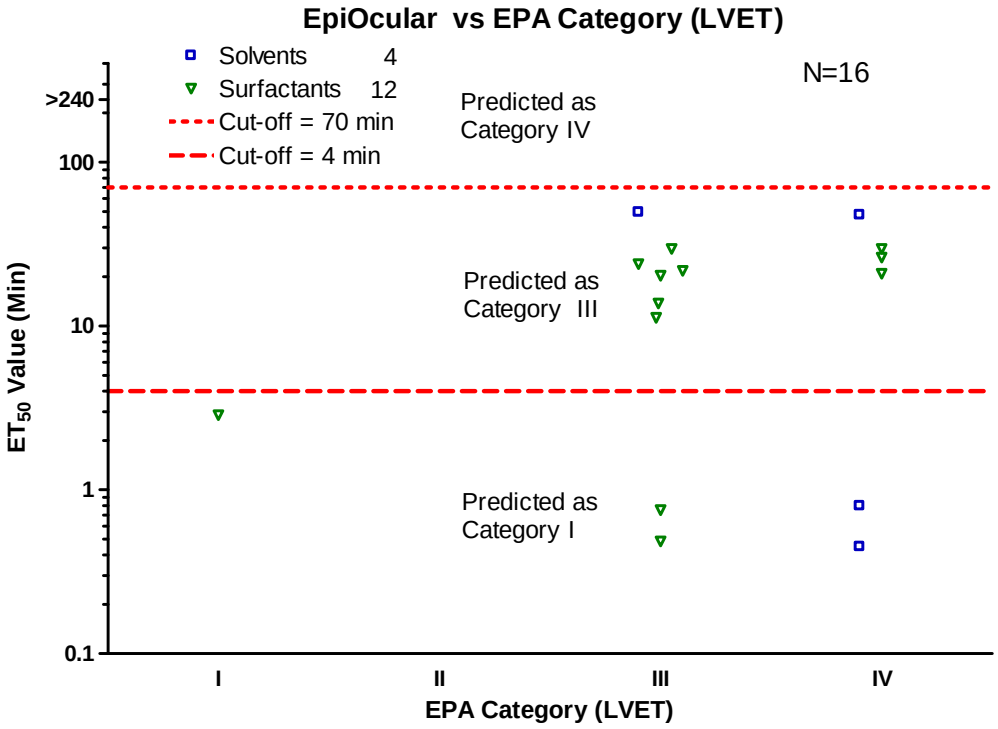


Figure 2. EpiOcular ET_{50} values plotted against EPA categories determined by the LVET. Oxidizers have been removed since they will be tested only in the BCOP assay. Suggested cut-off values with their predicted EPA categories are included (BRD).

BCOP

OPP believes that the BCOP assay can identify Category II materials. As can be seen in the Figure 3, all materials scoring between 25 and 75 are considered Category II materials. These are clearly different than the Category I's (>75) or Category III's (<25). In order to assure accurate classification for Category II and Category III products, EPA is also asking participating registrants to submit the consumer incident data and existing Draize test results on related compounds for EPA to further evaluate the reliability of this interim pilot project.

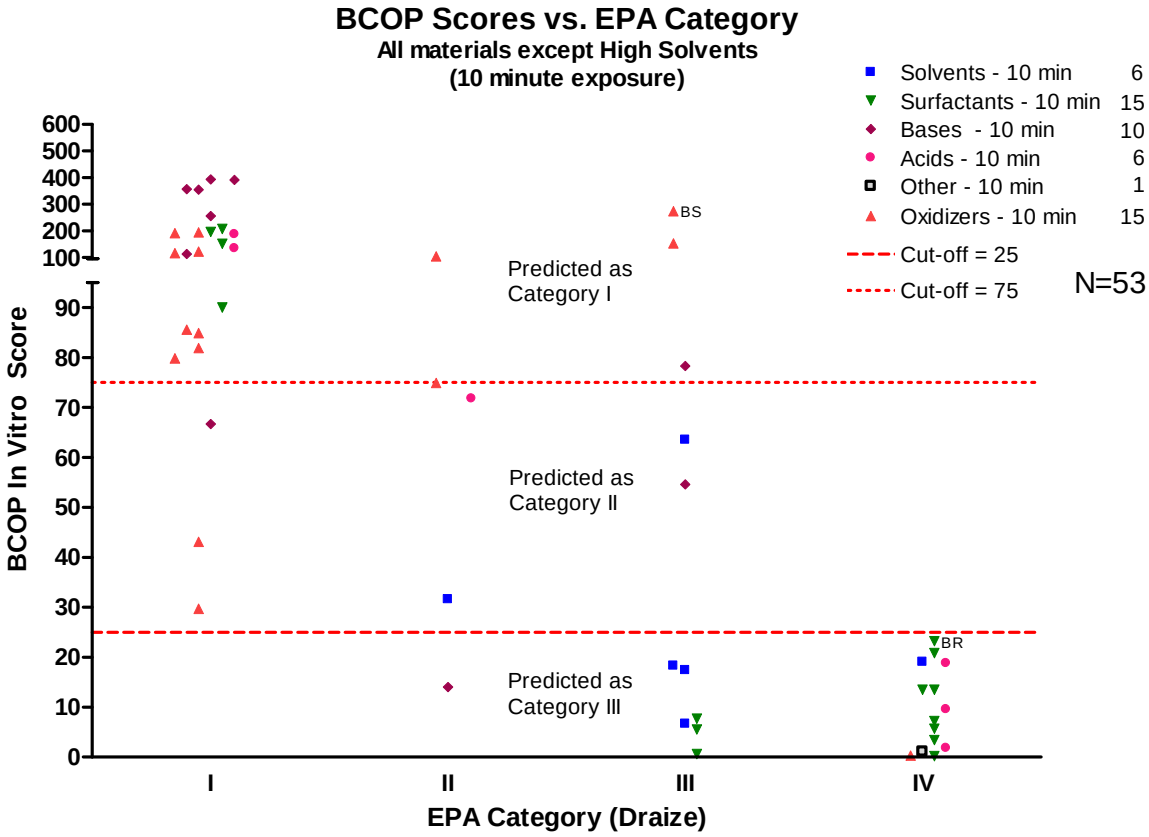


Figure 3. BCOP *in vitro* scores for non-High Solvent materials plotted against EPA categories determined by the Draize test. Proposed cut-off values with their predicted EPA categories are included. The EPA categories for test materials BR and BS were determined by using the results of an LVET assay (BRD).

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APPENDIX I

Each of the three *in vitro* test methods has a slightly different area of strength. The CM and EO assays are very sensitive to small amounts of damage, and can be useful in the less irritancy side of the toxicity range. For example, they can separate EPA Category IV materials from EPA Category III and higher materials. In contrast, the BCOP assay uses a much more robust tissue and can be useful to correctly distinguish between EPA Category I and II materials. The CM assay especially is not useful to differentiate between Category I and Category II materials since virtually all of the tissue would be destroyed by just a Category II product; more toxicity caused by a Category I material could not be measured.

As stated above, generally only one test should be necessary to determine the appropriate hazard classification, but in some cases the registrant may fine tune the final hazard classification by using a second test that is either more robust or more sensitive. For example, if BCOP assay indicated that the AMCP should have only a Category III or less classification, the EO or CM assay could then be employed to help make the final decision of whether the product was actually a Category III material or a Category IV material.

Antimicrobial products with cleaning claims can be formulated in different ways depending on the desired cleaning capacity for the product. For example, the formulation can rely on its alkaline or acidic properties for cleaning, or on surfactants and solvents, or on oxidizing (reactive) chemistries. Most of these classes react similarly in the *in vitro* assays, i.e. the hazard categories of the various types of formulations are similarly predicted. However, formulations with oxidizing (reactive) chemistries and those with a high solvent concentration (>5%) should be treated somewhat differently from the others (see below). It is also useful to determine the water solubility of the formulation since only fully water soluble materials can be tested in the Cytosensor Microphysiometer (limitation noted in data submitted to ICCVAM for CM)..

In summary:

- Three *in vitro* assays are included in the pilot program.
- The *in vitro* assays were selected to address the commonly recognized modes of action for eye irritants.
- The assays complement each other to cover the range of irritancy potential.
- The testing strategy is consistent with current practice (using an up/down approach) and with the proposed ECVAM strategy developed with input from ICCVAM representatives.

AREA OF APPLICATION

The testing strategy described in this document is proposed for use only with certain antimicrobial products with cleaning claims. Antimicrobial products with cleaning claims are defined as either water or surfactant-based; excluding solvents.

There are some differences in how some antimicrobial products with cleaning claims are tested, depending on certain characteristics of their formulation. These are:

1) Oxidizing materials

   Formulations containing specific reactive chemicals, *e.g.,* hypochlorite, peroxide, percarbonate, oxygen bleaches, etc.

   These materials are best tested in the BCOP assay (i.e. usually severe ocular irritants). Because of the very reactive nature of these formulations, they are often over predicted by the EO and CM assays, where there is less substrate available to bind the reactive materials than in the human or animal cornea. In addition there are some delayed effects which can only be visualized in the longer-term BCOP assay.

2) "High Solvent" formulations

   Formulations having >5% concentrations of organic solvents, e.g. alcohols, glycol ethers, etc.

   These materials often can be over predicted in the BCOP assay when using the conventional exposure time of 10 minutes (see data presented in Appendix B – Background Review Document of an *In Vitro* Approach for EPA Toxicity Labeling of Antimicrobial products with cleaning claims, and ICCVAM recommendations for the use of the BCOP assay). When such materials are tested in the BCOP assay a shorter exposure time of 3 minutes should be used (addressed earlier in document).

3) Non-aqueous soluble formulations

   These materials should only be tested in the EO or BCOP assays (i.e. usually a slight or nonirritant). Because of physical constraints imposed by the pumps and tubing used to circulate the test material, only fully water-soluble materials can be tested in the CM.

ANNEX I: OCULAR IRRITATION ASSAY FOR CERTAIN ANTIMICROBIAL PRODUCTS MAKING CLEANING CLAIMS USING THE BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY AND HISTOLOGY (Courtesy of Institute for In Vitro Sciences, Inc.)

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 cause opacity and/or permeability in an isolated bovine cornea.

2.0 SPONSOR

2.1 Name:

2.2 Address:

2.3 Representative:

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Articles: 1

3.2 Controls: Positive: Ethanol(CAS #64-17-5) Neat
Negative: Sterile deionized water

3.3 Determination of Strength, Purity, etc.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name:

4.2 Address:

4.3 Study Director:

4.4 GLP: 40 CFR Part 160 Good Laboratory Practice Standards (GLP) apply to this assay

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 three components which are predictive of eye irritation: corneal opacity, permeability, and tissue architecture. 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, the depth and degree of injury is assessed by histological evaluation.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

Liquid test articles will be tested neat unless otherwise directed by the Sponsor. The pH of each 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. Three corneas will be treated with each test article at each exposure time.

7.1 Reagents:

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

7.1.2 Fetal Bovine Serum (FBS)

7.1.3 Minimum Essential Medium (EMEM) without phenol red

7.1.4 Complete MEM: EMEM without phenol red containing 1%FBS and 2mM L-glutamine

7.1.5 Minimum Essential Medium (EMEM) with phenol red (used for rinsing test substances from corneas only)

7.1.6 Complete MEM: EMEM with phenol red containing 1% FBS and 2mM L-glutamine

7.1.7 Sodium Fluorescein – diluted in DPBS

7.1.8 Sterile Deionized Water

7.1.9 10% Buffered formalin solution

7.2 Bovine Eyes

Bovine eyes will be obtained from a local abattoir or other commercial supplier. 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 the testing facility. 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 (Complete MEM). The posterior chamber will always be filled first. The corneas will be incubated for the minimum of one hour at 32±1ºC.

7.4 Sample Preparations

Liquid test articles will be tested neat whenever possible. 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 Initial Opacity Reading

At the end of the one-hour incubation period, the medium will be removed from both chambers and replaced with fresh Complete MEM. 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.

7.6 Treatment of Corneas

Antimicrobial products making cleaning claims described by the Sponsor as being a High Solvent (defined as having a solvent concentration of ≥5%) will be tested as in 7.6.1 below, <u>with the exception that the exposure time will be 3 minutes</u>.

7.6.1 Method A: Liquids

Liquids will be tested undiluted, unless otherwise directed by the Sponsor. At least three corneas will be dosed per material. Approximately seven hundred and fifty µL of test substance (test article, negative control or positive control) will be introduced into the anterior chamber. Highly viscous materials will be applied directly to the corneal surface. The holder will be slightly rotated (with the corneas maintained in a horizontal position) to ensure uniform distribution over the cornea. The test article

treated corneas will be exposed for 10 minutes at 32±1ºC. The negative and positive controls will be tested for 10 minutes also. At the end of the exposure time, the test substance will 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 a total of approximately 2 hours at 32±1ºC. 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.

7.6.2 Method B: Solids

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±1ºC 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).

7.7 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.

7.8 Permeability Determinations

Method A: Liquids

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 fluorescein solution will be added to the anterior chamber.

Method B: Solids

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 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±1ºC. The medium from the posterior chamber will be removed at the completion of the incubation period, and 360 µL will be transferred to the appropriate wells of a prelabeled 96-well plate. Three hundred and sixty µL of fresh Complete MEM without phenol red will be added to the wells designated as blanks. The optical density at 490 nm (OD_{490}) will be 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.

7.9 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 a sponge to protect it. The cassettes will be placed in 10% neutral buffered formalin and fixed for a minimum of 24 hours.

7.10 Histological Evaluation

7.10.1 Name of Evaluator

7.10.2 The fixed tissues will be transferred to the pathology laboratory for embedding, sectioning, staining and histological evaluation. If the histological evaluation is conducted off-site, a Principal Investigator will be assigned by the sub-contractor. 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. 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.

9.0 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 OD_{490} value of each treatment group will be calculated by averaging the OD_{490} values of the treated corneas (less the average negative control values) for each treatment condition.

9.1 *In Vitro* Score Calculation

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

In Vitro Score = Mean Opacity Value + (15 x Mean OD_{490} Value)

9.2 Data Interpretation

All antimicrobial products making cleaning claims having an I*n Vitro* Score of ≥75 will be classified as an EPA Category I. Antimicrobial products making cleaning claims having an *In Vitro* Score <75 and ≥25 are given a preliminary classification of EPA Category II, but will be further assessed with a histopathological examination (as part of the weight of evidence) and given the final categorization of whatever determination (*In Vitro* Score or histopathology) is more severe. Antimicrobial products making cleaning claims having an *In Vitro* Score <25 are given a preliminary classification of EPA Category III then either an EpiOcualr of CM assay must be performed.

Histological changes will be reported for each treatment group of three corneas. Injury to each tissue layer will be scored and representative photographs taken to illustrate the degree of damage.

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 allows 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.

12.0 REFERENCES

Curren, R., Evans, M., Raabe, H., Dobson, T., and Harbell, J.(1999) Optimization of the bovine corneal opacity and permeability assay: histopathology aids understanding of the EC/HO false negative materials. **ATLA** 27:344.

Gautheron, P.D., Dukic, M., Alix, D., and Sina, J.F. (1992) Bovine Corneal Opacity and Permeability Test: An *in Vitro* Assay of Ocular Irritancy. **Fundamental and Applied Toxicology** 18:442-449.

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.

13.0 APPROVAL

_____ _____

SPONSOR REPRESENTATIVE DATE

(Print or Type Name)

_____ _____

STUDY

ANNEX II: OCULAR IRRITATION ASSAY FOR CERTAIN ANTIMICROBIAL PRODUCTS MAKING CLEANING CLAIMS USING THE EpiOcular™ HUMAN CELL CONSTRUCT (Courtesy of Institute for In Vitro Sciences, Inc.)

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.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name:

4.2 Address:

4.3 Study Director:

4.4 GLP: 40 CFR Part 160 Good Laboratory Practice Standards (GLP) apply to this assay

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 EpiOcular™ cultures offers features appropriate for a model for ocular irritation. First, the model is composed of stratified human keratinocytes (neonatal foreskins) 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 (and/or dosing solution as appropriate), if possible, 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 (ET_{50}). 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.

The standard exposure time range extends up to 90 minutes and is used for most materials to be tested. In general, a standard exposure range of 2, 15, 45 and 90 minutes 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 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, and solid test articles that are creamed. 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. 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 testing facility.

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 90 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. 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±1ºC in a humidified atmosphere of 5±1% CO_2 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 2-8ºC until used.

On the day of dosing, EpiOcular™ Assay Medium will be warmed to approximately 37ºC. Nine tenths (0.9) mL of Assay Medium will be aliquoted into the appropriate wells of prelabeled 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% CO_2/95% air, and the bag will be sealed and stored at 2-8ºC for subsequent use.

7.8 Definitive MTT Assay

Four to five exposure times will be tested for each test article. The exposure times will generally be 2, 15, 45 and 90 minutes, although other 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.

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 prelabeled 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 (2-8ºC) 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 (OD_{550}) of each well will be measured with a Molecular Devices Vmax plate reader (or equivalent).

7.9 Freeze 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 –20ºC 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 OD_{550} 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 OD_{550} of the blank control wells will be calculated. The corrected mean OD_{550} of the exposure time control(s) will be determined by subtracting the mean OD_{550} of the blank control from their mean OD_{550}s. The corrected OD_{550} of the individual test article exposure times and the positive control exposure times will be determined by subtracting the mean OD_{550} of the blank control from their respective OD_{550}s. When applicable, corrected OD_{550} values will be calculated for the control and test article-treated killed controls, as well. Generally, all calculations will be performed using Microsoft Excel.

Corr. test article exposure time OD_{550} = Test article exp. time OD_{550} – 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 OD_{550} value for the negative control killed control will be subtracted from the OD_{550} values for each of the test article-treated killed controls (at each exposure time), to determine the net OD_{550} values of the test article-treated killed controls.

Net OD_{550} for each test article KC = Raw OD_{550} test article KC – Raw OD_{550} negative control KC

The net OD_{550} values represent the amount of reduced MTT due to direct reduction by test article residues at specific exposure times. In general, if the net OD_{550} value is greater than 0.150, the net amount of MTT reduction will be subtracted from the corrected OD_{550} values of the viable treated tissues, at each corresponding exposure time, to obtain a final corrected OD_{550} value. These final corrected OD_{550} values will be used to determine the % of Control viabilities at each exposure time.

Final Corrected OD_{550} = Corrected test article OD_{550} (viable) – Net OD_{550} test article (KC)

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

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

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 OD_{550}s 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 ET_{50} will be interpolated from each plot. To determine the ET_{50}, 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 ET_{50}, 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

If the antimicrobial product making a cleaning claim has an ET_{50} score of <4 minutes, it is classified as an EPA Category I. However, a BCOP must be done to confirm this result. If the antimicrobial product making a cleaning claim has an ET_{50} score of ≥4 minutes, but <70 minutes, it is classified as an EPA Category III. If the antimicrobial product making a cleaning claim has an ET_{50} score of ≥70 minutes, it is classified as an EPA Category IV.

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 allows 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

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SPONSOR REPRESENTATIVE DATE
(Print or Type Name)

_____ _____

STUDY DIRECTOR DATE

ANNEX III: OCULAR IRRITATION ASSAY FOR CERTAIN ANTIMICROBIAL PRODUCTS MAKING CLEANING CLAIMS USING THE CYTOSENSOR MICROPHYSIOMETER BIOASSAY (Courtesy of Institute for In Vitro Sciences, Inc.)

1.0 PURPOSE

The purpose of this study is to evaluate the potential ocular toxicity of a test article by measuring the test material-induced reduction in the metabolic rate of treated 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 MRD_{50} value [in units of mg/mL]) is the endpoint of the assay.

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: sodium lauryl sulfate (SLS)

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

3.3 Determination of Strength, Purity, etc.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name:

4.2 Address:

4.3 Study Director:

4.4 GLP: 40 CFR Part 160 Good Laboratory Practice Standards (GLP) apply to this assay

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

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 (MRD_{50}) 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 may 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 perused 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 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 MRD_{50} (Metabolic Rate Decrement 50%). The MRD_{50} 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 MRD_{50} 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 ± 1°C and 5 ± 1% CO_2 in air. L929 cells will be seeded onto capsule cups at approximately 6.0 x 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 X 10^5 cells per each capsule cup (0.5 mL of a 1.2 X 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 ± 1°C and 5 ± 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 ± 1°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 ± 1°C and 5 ± 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 µL per minute for the first minute, and 20 µL 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 µL 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 MRD_{50}. MRD_{50} 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 MRD_{50} falls within two standard deviations of the historical mean. The historical mean will be updated every three months.

10.0 EVALUATION OF TEST RESULTS

If the antimicrobial product making a cleaning claim has an MRD_{50} score of <2 mg/mL, it is classified as an EPA Category I. However, a BCOP must be conducted to confirm this result. If the antimicrobial product making a cleaning claim has an MRD_{50} score of ≥2 mg/mL, but <80 mg/mL, it is classified as an EPA Category III. If the antimicrobial product making a cleaning claim has an MRD_{50} score of ≥80 mg/mL, it is classified as an EPA Category IV.

11.0 REPORT

A report of the results of this study will be prepared by the Testing Facility and will accurately describe all methods used for the generation and analysis of the data. For each test article, the individual MRD_{50} values from each assay trial, and the average MRD_{50} value from at least two valid definitive trials will be presented. The MRD_{50} value from the dose range finding assay will be included in the calculation of the average MRD_{50}, if one can be determined. A separate summary will be prepared reporting the MRD_{50} values for each assay with 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 and the SOPs of the Testing Facility will appear as a part of the final report.

12.0 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 allows evaluation, whichever is applicable.

13.0 REFERENCES

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14.0 APPROVAL

SPONSOR REPRESENTATIVE DATE

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