

**USE OF AN ALTERNATE TESTING FRAMEWORK FOR  
CLASSIFICATION OF EYE IRRITATION POTENTIAL OF EPA  
PESTICIDE PRODUCTS**

5-31-2013

Office of Pesticide Programs  
U.S. Environmental Protection Agency  
Washington DC, 20460



## Table of Contents

LIST OF ABBREVIATIONS AND ACRONYMS .....	3
I. Summary and Scope of Policy.....	4
II. Scientific History Supporting Policy.....	5
III. Decision Tree Approach.....	6
A. Chemicals to which this policy applies.....	6
B. Selection of Appropriate Assays for Hazard Category Determinations.....	7
C. Assessing Results.....	7
IV. Submission Package Guidance.....	10
V. References .....	11
Appendix A: Results of ICCVAM Analysis and Antimicrobial Pilot Program.....	13
Appendix B: Alternative Eye Irritation Test Protocol Guidance.....	17

## LIST OF ABBREVIATIONS AND ACRONYMS

<b>AMCP</b>	Antimicrobial Cleaning Product
<b>ATWG</b>	Alternative Testing Working Group
<b>BCOP</b>	Bovine Corneal Opacity and Permeability
<b>BRD</b>	Background Review Document
<b>CM</b>	Cytosensor Microphysiometer
<b>EO</b>	EpiOcular Assay
<b>EPA</b>	Environmental Protection Agency
<b>GHS</b>	Globally Harmonized System
<b>ICCVAM</b>	Inter-Agency Coordinating Committee on the Validation of Alternative Methods
<b>IIVS</b>	Institute for <i>In Vitro</i> Sciences
<b>MRID</b>	Master Record Identification
<b>OPP</b>	Office of Pesticide Programs
<b>PPDC</b>	Pesticide Program Dialogue Committee

## I. SUMMARY AND SCOPE OF POLICY

This document establishes an alternative testing approach (using *in vitro/ex vivo* assays) for determination of eye irritation potential in the U.S. EPA's Office of Pesticide Programs (OPP) (U.S. EPA/OPP) under the U.S. EPA classification and labeling system. EPA/OPP has concluded from submission and review of alternative eye irritation tests conducted on antimicrobial pesticide products with cleaning claims (AMCPs) that the testing approach presented in this document is acceptable for determining the appropriate eye hazard classification and labeling for AMCPs (see section II of this document). Thus, the testing scheme presented in this document can be used to satisfy the *in vivo* data requirement for eye irritation in 40CFR Part 158W for AMCPs.

For other classes of pesticides and pesticide products, including conventional, biochemical, and other antimicrobial pesticides not in the scope of those with cleaning claims, the agency will consider alternative tests conducted and submitted on a case-by-case basis. In these situations, the applicant is encouraged to consult or submit the proposed test protocol to the EPA for review before conducting the study. The acceptability of test results for these other classes will be determined using a weight of evidence approach that considers, for example, any *in vivo* testing, structure-activity relationships, and bridging arguments from results on similar test materials to support a classification under this testing scheme.

As a science policy document, this testing approach is not binding on either EPA or any outside parties, and the EPA may depart from the approach where circumstances warrant and without prior notice. The methods contained in this approach are strongly recommended for generating the data that are the subject of the document, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the methods recommended in the document, with your supporting rationale. The Agency will assess such proposals on a case-by-case basis.

The alternative approach presented in this document utilizes a decision tree approach (presented in Section III) involving the use of three *in vitro/ex vivo* tests:

- Bovine Corneal Opacity and Permeability test (BCOP)
- EpiOcular assay (EO)
- Cytosensor Microphysiometer assay (CM)

It is acknowledged that other *in vitro* assays for eye irritation are available for assessment of eye irritation potential. (For example, see the [ICCVAM report on the validation status of in vitro eye injury test methods](#).) These include the Isolated Chicken Eye (ICE) test, the Hen's Egg Test – Chorioallantoic Membrane Test Method (HET-CAM) test, and the Isolated Rabbit Eye (IRE) test. Based on currently available

data, these other tests are currently limited in their ability to predict eye irritation across the EPA toxicity categories. The ICE method is acceptable to distinguish severe ocular irritants. However, because of the high false negative rate of the assay, submission of *in vivo* data is required if the ICE assay result is negative. Both the HET-CAM test and the IRE test require further development and optimization and are not recommended alternative tests at this time. As new methods are validated for use or as refinements are made to existing methods, the agency will consider incorporating these into the testing scheme as appropriate.

The BCOP, EO, and CM tests are used under this policy to differentiate among the four eye irritation hazard categories currently used by the USEPA as shown in Table 1 below. The categories and the associated label statements are listed below.

**Table 1. USEPA Eye Irritation Hazard Categories**

<b>Toxicity Category</b>	<b>Signal Word</b>	<b>Eye Protection and Label Precautionary Language</b>
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.

For any pesticide product that is believed to be a severe ocular irritant, applicants can choose to accept EPA Category I labeling statements *in lieu* of testing for eye irritation.

## **II. SCIENTIFIC HISTORY SUPPORTING POLICY**

As a result of discussions on the use of alternative testing methods at the fall 2003 Pesticide Program Dialogue Committee (PPDC) meeting, the Alternative Testing Working Group (ATWG) was formed with the goal of developing a non-animal eye irritation testing approach for AMCPs. 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 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. Through an effort involving ICCVAM and The Institute for In Vitro Sciences (IIVS), companies that register AMCPs were encouraged to participate in the initiative and submit both *in vivo* data as well as *in vitro* data on eye irritation using these alternate tests. ICCVAM then conducted an analysis and technical review of the submitted data to determine the effectiveness of the methods to predict EPA labeling categories for eye irritation of AMCPs. These were published by ICCVAM and can be found at: <http://iccvam.niehs.nih.gov/methods/ocutox/AMCP-TMER.htm>

In addition to the analysis conducted through ICCVAM, in 2009 EPA's OPP initiated a pilot program through publication of a document entitled "Non-Animal Testing Approach to EPA Labeling for Eye Irritation." This pilot encouraged antimicrobial pesticide applicants to submit studies on eye irritation for AMCPs using the *in vitro* and *ex vivo* testing protocols mentioned above (BCOP, EO, and CM). The purpose of this pilot was to determine whether labeling classification decisions for eye irritation could be made using the approach.

The detailed results of the ICCVAM analysis as well as of the data submitted under the 2009 pilot are presented in Appendix A. Both of these data sets showed that for AMCPs, the *in vitro* BCOP and EO alternative tests did not under-predict the results of the traditional Draize rabbit eye test, supporting the use of these assays in the decision tree approach for classification of eye irritation potential of antimicrobial pesticides with cleaning claims. Although no assays using the CM test were submitted under the pilot program, an analysis of the predictions of the CM assay published in the 2009 OPP pilot document as well as a background review document by the European Union Reference Laboratory for Alternatives to Animal Testing (available at [http://ihcp.jrc.ec.europa.eu/our\\_labs/eurl-ecvam/validation-regulatory-acceptance/docs-eye-irritation/ECVAM-CMBRD-Aug08cleaned.pdf/view](http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/validation-regulatory-acceptance/docs-eye-irritation/ECVAM-CMBRD-Aug08cleaned.pdf/view)) supports the use of the CM assay for classification of mild and minimal irritants (EPA Category III and IV).

### **III. Decision Tree Approach**

#### **A. Test Materials for Which this Policy Applies**

In the development of the strategy using *in vitro/ex vivo* eye irritation tests, the AMCP formulations tested encompassed a range of chemistries associated with cleaning capacity. Products included AMCP formulations with alkaline and acidic chemistries, surfactant and solvent-based chemistries, and oxidizing (reactive) chemistries (such as hypochlorite, peroxide, percarbonate, oxygen, bleaches). Therefore, this testing strategy currently applies to AMCPs with these types of chemistries. Test materials assessed using these *in vitro/ex vivo* tests should be tested in the form and concentration that is intended to be marketed and/or sold unless otherwise noted in the protocol guidance (Appendix B). Exceptions to this should be supported by adequate scientific justification.

The decision tree approach presented in this document will be considered for other classes of pesticides and formulations (*e.g.*, biochemical pesticides, conventional pesticides, antimicrobial pesticides outside of those with cleaning claims) on a case by case basis using a weight of evidence approach that considers, for example, any *in vivo* testing, structure-activity relationships, and bridging arguments from results on similar test materials. This case by case approach for pesticides other than antimicrobial chemicals with cleaning claims is necessary due to the lack of comparative data on alternative non-animal and *in vivo* testing with these classes at this time.

#### **B. Selection of Appropriate Assays for Hazard Category Determinations**

Generally, the BCOP assay is useful for distinguishing Toxicity Categories I or II while the CM and EO assays are useful for distinguishing those chemicals or formulations that may fall into Toxicity Categories III or IV.

As shown in the decision tree (Figure 1), the first step in determining the appropriate assay is whether the test material has oxidizing chemistry (e.g., hypochlorite, peroxide, percarbonate, oxygen bleaches, strong acids, etc.) or may be expected to be a severe or moderate eye irritant. If the answer is yes to either of these questions, the BCOP assay should be used. The BCOP assay can be used to test both liquids and solids (see Appendix B for the BCOP protocol guidance). Solids have been shown to produce high false negative rates in the assay, but since the purpose of the assay is to identify moderate or severe ocular irritants, confirmation that the solid test material is not a moderate or severe irritant could be demonstrated through conduct of a EO or CM assay.

Testing of ketones and alcohols in the BCOP has been shown to result in high false positive rates for the assay, but not all ketones or alcohols are over-predicted. It is up to the user of the BCOP whether over-prediction of eye irritation potential for these chemical classes can be accepted.

If the test material does not possess oxidizing chemistry or is not expected to be a severe or moderate eye irritant, the CM or EO assays can be conducted. Water-soluble test materials can be tested in either the EO or CM assays, while water-insoluble test materials can only be tested in the EO assay due to the physical constraints of the CM instrument (water-insoluble test materials would not be able to pass through the CM instrument to be tested). These assays are sensitive to small amounts of damage, and thus are not useful for more severe irritants, as virtually all of the tissue in the assay would be compromised.

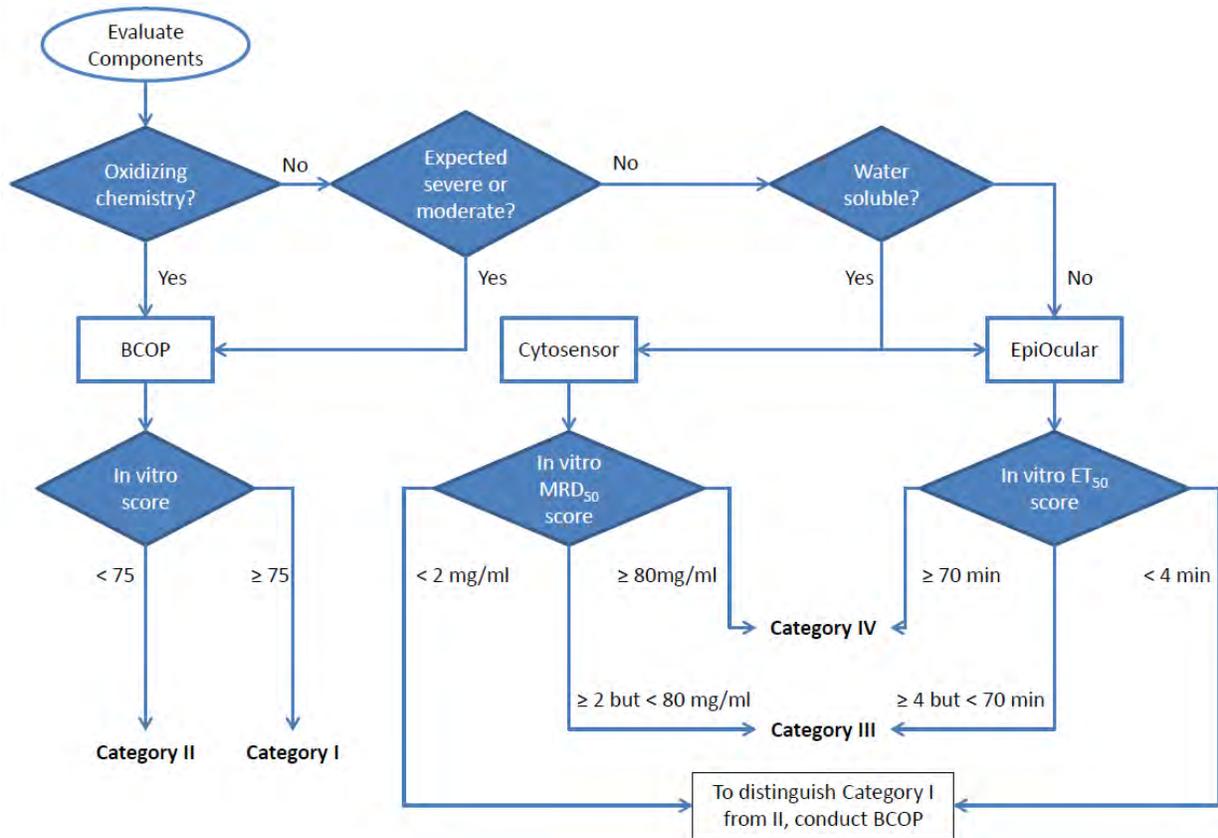
### **C. Assessing Results**

In the BCOP assay, irritancy is measured from calculation of the *in vitro* irritancy score (IVIS), a measure of corneal opacity induced by the test material. If the BCOP assay resulting IVIS score is  $\geq 75$ , the test material is considered a severe ocular irritant and would be labeled as Toxicity Category I. If the test material receives an IVIS score of  $< 75$  in the BCOP assay, the test material would be classified as Category II. The BCOP assay is not recommended for determination of test materials that may be mild irritants or non-irritants (Toxicity Category III or IV), as the assay tends to over-predict irritation potential for mild or non-irritants (ICCVAM, 2009). For determination of test materials that are believed to be mild or non-irritants, the EO or CM assay should be conducted.

In the EO assay, irritancy potential is measured by the exposure time required for the test substance to reduce tissue viability to 50% of controls ( $ET_{50}$ ). Viability is determined by the microsomal enzyme reduction of MTT in control and test material-treated cultures. The EO assay identifies toxicity category I, III and IV test materials based on the  $ET_{50}$ . If the  $ET_{50}$  score is  $< 4$  minutes, the test material is classified as an EPA Category I. The applicant may choose to accept this result or may conduct a BCOP to confirm whether the test material is a category I or II. If the  $ET_{50}$  score is  $\geq 4$  minutes, but  $< 70$  minutes, the test material is classified as an EPA Category III. If the  $ET_{50}$  score is  $\geq 70$  minutes, the test material is classified as an EPA Category IV.

In the CM assay, irritancy potential is measured as the concentration of test material which causes a 50% decrease in the acidification rate (MRD<sub>50</sub>) of the L929 cells used in the assay. If the MRD<sub>50</sub> score is <2 mg/mL, the test material is classified as an EPA Category I. The applicant may choose to accept this result or may conduct a BCOP to confirm whether the test material is a category I or II. If the MRD<sub>50</sub> score is ≥2 mg/mL, but <80 mg/mL, the test material is classified as an EPA Category III. If the MRD<sub>50</sub> score is ≥80 mg/mL, the test material is classified as an EPA Category IV.

**Figure1. Decision Tree: Selection and Evaluation of Assays for Hazard Labeling**



#### IV. SUBMISSION PACKAGE GUIDANCE

The following information is provided to applicants to assist with the materials that should be submitted with a data package in support of the alternative test method approach for eye irritation.

##### General Submission Package

- Raw data for the non-animal assay results (BCOP, EO, or CM).
- The following supplemental information is strongly encouraged if available;
  - Available Draize rabbit test results for similar or structurally related compounds.
  - Any other useful existing knowledge (e.g., chemical physical properties, other data about irritancy, Structural Activity Relationship (SAR) data on irritancy) for ocular hazard labeling.

##### Data and Reporting

The study report should include a description of the test material, the methods and the study results. At a minimum, the following should be 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 Practice (GLP) standards statement;
- rationale for selection of test (BCOP, EO, or CM);
- 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 of 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.

## V. REFERENCES

AMCP BRD: Background Review Document of an In Vitro Approach for EPA Toxicity Labeling Anti-Microbial Cleaning Products (AMCP); Rodger D. Curren, Ph.D.; Jennifer; R. Nash, M.S.; Angela Sizemore, B.S.; John Harbell, Ph.D.

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.

Botham, P.A., Osborne, R., Atkinson, K., Carr, G., Cottin, M., and Van Buskirk, R.G. (1997) IRAG working group 3: cell function-based assays. In: *Eye Irritation Testing: Practical Applications of Non-Whole Animal Alternatives*. Food and Chemical Toxicology 37:67-77.

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.

Buehler, E.V. and Newmann, E.A. A Comparison of Eye Irritation in Monkeys and Rabbits. *Toxicology and Applied Pharmacology* 6:701–710 (1964).

Curren R., Ph.D.; 8/26/08 presentation to ICCVAM's Ocular Toxicity Working Group. Co-author, Background Review Document of an In Vitro Approach for EPA Toxicity Labeling of Anti-Microbial Cleaning Products.

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., Osborne, R., Carr, G.J., and Peterson, A. (1997) Assessment of the Cytosensor 11 microphysiometer assay in the COLIPA in vitro eye irritation validation study. Submitted, *Toxicology In Vitro*.

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.

ICCVAM (2010a): ICCVAM Test Method Evaluation Report: Current Validation Status of a Proposed *In Vitro* Testing Strategy for U.S. Environmental Protection Agency Ocular Hazard Classification and Labeling of Antimicrobial Cleaning Products. Available at: [http://iccvam.niehs.nih.gov/docs/ocutox\\_docs/AMCP/Body.pdf](http://iccvam.niehs.nih.gov/docs/ocutox_docs/AMCP/Body.pdf)

ICCVAM (2010b): ICCVAM Test Method Evaluation Report: Current Validation Status of *in vitro* Test Methods Proposed for Identifying Eye Injury Hazard Potential of Chemicals and Products. Available at: [http://iccvam.niehs.nih.gov/docs/ocutox\\_docs/InVitro-2010/TMER-Vol1.pdf](http://iccvam.niehs.nih.gov/docs/ocutox_docs/InVitro-2010/TMER-Vol1.pdf)

McConnel, H.M., Owicki, J.C., Parce, J.W., Miller, D.L., Baxter, G.T., Wada, H.G., and Pitchford, S. (1992) The Cytosensor 12microphysiometer: biological applications of silicon technology. *Science* 257:1906-1912.

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

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.

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.

U.S. EPA (2009): Non-Animal Testing Approach to EPA Labeling for Eye Irritation. Available at: <http://www.epa.gov/oppad001/eye-irritation.pdf>

## Appendix A: Results of ICCVAM Analysis and Pilot Program

In the Background Review Document published by ICCVAM (available at: [http://iccvam.niehs.nih.gov/docs/ocutox\\_docs/AMCP/AppC4-SRD-AnnexIV.pdf](http://iccvam.niehs.nih.gov/docs/ocutox_docs/AMCP/AppC4-SRD-AnnexIV.pdf), 28 antimicrobial products with cleaning claims that had been tested using the traditional Draize assay were also tested in the BCOP and EpiOcular assays. At the time the analysis was conducted, the antimicrobial products were identified using letter codes so that data confidentiality of the products would not be compromised. The results of testing of these products using the *in vitro* tests (shown in the Table 1A below) demonstrated that the *in vitro* alternative tests did not under-predict the results of the traditional Draize rabbit eye test, supporting the use of these assays for classification of eye irritation potential of antimicrobial pesticides with cleaning claims.

**Table 1A. Antimicrobial products (letter codes): Classification based on Non-Animal Tests and the Draize Rabbit Eye Test.**

Code	High Solvent <sup>1</sup>	<i>In Vivo</i> Classification		EpiOcular		BCOP <sup>3</sup>					
		GHS (Draize)	EPA (Draize)	ET <sub>50</sub> (min) <sup>2</sup>	EPA Classification	IVIS (10 min)	EPA Classification (Cat 1 ≥75; 10 min)	EPA Classification (Cat 1 ≥55.1; 10 min)	IVIS (3 min) <sup>4</sup>	EPA Classification (Cat 1 ≥75; 3 min) <sup>4</sup>	EPA Classification (Cat 1 ≥55.1; 3 min) <sup>4</sup>
H	No	Not Classified	II	9.4	III	14	III	III	2.85	-	-
I	No	Not Classified	III	12	III	0.6	III	III	-0.3	-	-
J	No	Not Classified	III	19.3	III	7.7	III	III	2.6	-	-
K	No	Not Classified	IV	>240	IV	0.3	III	III	0	-	-
P	No	Not Classified	IV	125.8	IV	1.1	III	III	-0.3	-	-
R	No	Not Classified	IV	>240	IV	0.2	III	III	-0.6	-	-
T	No	Not Classified	IV	31.6	III	1.8	III	III	0	-	-
W	No	Not Classified	IV	39.6	III	5.7	III	III	3.5	-	-
AG	No	1	I	<0.17	I	391.9	I	I	-	-	-
AH	No	1	I	0.4	I	255.7	I	I	-	-	-
AI	No	1	I	<0.17	I	354.7	I	I	-	-	-
AJ	No	1	I	<0.17	I	357.1	I	I	-	-	-
AK	Yes	1	I	<0.17	I	444.3	I	I	-	-	-
AL	Yes	2A	I	<0.17	I	353.6	I	I	-	-	-
AM	Yes	1	I	<0.17	I	135.8	I	I	-	-	-
AN	No	1	I	1.5	I	113.5	I	I	-	-	-
AO	Yes	1	I	<0.17	I	216.2	I	I	-	-	-

Code	High Solvent <sup>1</sup>	In Vivo Classification		EpiOcular		BCOP <sup>3</sup>					
		GHS (Draize)	EPA (Draize)	ET <sub>50</sub> (min) <sup>2</sup>	EPA Classification	IVIS (10 min)	EPA Classification (Cat 1 ≥75; 10 min)	EPA Classification (Cat 1 ≥55.1; 10 min)	IVIS (3 min) <sup>4</sup>	EPA Classification (Cat 1 ≥75; 3 min) <sup>4</sup>	EPA Classification (Cat 1 ≥55.1; 3 min) <sup>4</sup>
AP	No	1	I	<0.17	I	393.3	I	I	-	-	-
AT	No	1	I	<1	I	85.6	I	I	49.8	-	-
AU	No	1	I	<1	I	122.2	I	I	64.5	-	-
AV	No	1	I	<1	I	191.8	I	I	68.8	-	-
AX	Yes	1	I	<0.17	I	157.3	I	I	-	-	-
BB	Yes	SCNM	IV	>240	IV	2	III	III	0	III	III
BE	No	Not Classified	III	4	III	15	III	III	-	-	-
BK	No	Not Classified	III	9.4	III	6.7	III	III	2.6	-	-
BM	Yes	Not Classified	IV	4.9	III	25.4	II	II	11.6	III	III
BL	Yes	Not Classified	IV	6.7	III	6	III	III	7.7	III	III
BN	No	Not Classified	IV	1.8	I	13.5	III	III	-	-	-

Abbreviations: BCOP = bovine corneal opacity and permeability, EPA = U.S. Environmental Protection Agency, GHS = Globally Harmonized System, IVIS = *in vitro* irritancy score, SCNM = study criteria not met.

<sup>1</sup> High solvents are substances with solvent or glycol ether concentrations >5% (as defined in the AMCP BRD).

<sup>2</sup> ET<sub>50</sub> values represent the time needed to reduce cell viability by 50%.

<sup>3</sup> The BCOP test method data were evaluated using either the decision criteria in the AMCP BRD (IVIS ≥75 for EPA Category I) or in the 2006 ICCVAM BRD (IVIS ≥55 for EPA Category I).

<sup>4</sup> Three-minute exposure data were only considered for substances identified as high solvents.

In addition to the results of the tests presented in the ICCVAM document, the Antimicrobials Division evaluated the results of submitted *in vitro/ex vivo* eye irritation studies under the 2009 pilot program. Four products with existing *in vivo* data were submitted and reviewed. The results of these reviews are summarized in the table below. As shown in Table 2A, the results of *in vitro* eye testing were comparable or more restrictive compared to the results of *in vivo* testing, adding further support to the use of the *in vitro* approach to assessment of eye irritation for antimicrobial products with cleaning claims.

**Table 2A. Results of 2009 Pilot For Antimicrobial Products with Cleaning Claims.**

Product (EPA Reg #)	In vivo MRID	Tox Category	In vitro MRID	Tox Category
4582-72 (Ultimate Clean)	47565407	III	48108401 (BCOP)	III <sup>1</sup>
4822-LUT (DeeDee 1) (Dexter 1)	46677706	IV	46679503 46679504 (BCOP)	II
5813-RNR (Tuck)	48312805	III	48312808 (BCOP)	II
4822-576 (Petrie 1)	48171106	IV	48171107	III

<sup>1</sup>The classification for this product was assessed under the 2009 pilot using weight of evidence, and suggested that the product was less severe than a Category II; however, criteria for classifying a test material as EPA Category III using the BCOP assay alone have not been formally developed at this time.

Based on the results of these data, in conjunction with the previously analyzed data by ICCVAM, the Agency concludes that the use of the *in vitro/ex vivo* studies described in this paper in a decision tree approach provide a valid alternative to the Draize eye test for classification of antimicrobial pesticides with cleaning claims. Tests with other classes of pesticides and pesticide formulations will be considered on a case by case basis as noted in this document.

## **Appendix B: ALTERNATIVE EYE IRRITATION TESTING PROTOCOL GUIDANCE**

### **BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY**

#### 1.0 PURPOSE

The purpose of this study is to evaluate the potential ocular irritancy/toxicity of a test material as measured by the test material'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 Materials: 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 material 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

### 7.1 Reagents:

- 7.1.1 Hanks' Balanced Salt Solution with  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  (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  $32\pm 1^{\circ}\text{C}$ .

### 7.4 Sample Preparations

Liquid test materials will be tested neat whenever possible. When appropriate, test materials will be diluted or suspended in sterile deionized water. Samples should be diluted on a w/v basis.

### 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 material, negative control, or positive control.

### 7.6 Treatment of Corneas

Test materials defined as being a High Solvent (defined as having a solvent concentration of  $\geq 5\%$ ) will be tested as in 7.6.1 below, with the exception that the exposure time will be 3 minutes. For each assay, 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. For experimental groups, three corneas will be treated with each test material at each exposure time.

#### 7.6.1 Method A: Liquids

Liquids will be tested as intended to be marketed and/or sold. The pH of each liquid test material will be determined, if possible, and recorded. At least three corneas will be dosed per material.

Approximately seven hundred and fifty  $\mu\text{L}$  of test substance (test material, 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 material treated corneas will be exposed for 10 minutes at  $32\pm 1^\circ\text{C}$ . 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 material 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  $32\pm 1^\circ\text{C}$ . 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.

#### 7.6.2 Method B: Solids

Solid materials are generally tested as a 20% dilution (w/v) in sterile deionized water. Solids may also be tested neat with adequate scientific justification.

Seven hundred and fifty  $\mu\text{L}$  of test substance (test material, 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. 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 material 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  $32\pm 1^\circ\text{C}$ . The medium from the posterior chamber will be removed at the completion of the incubation period, and 360  $\mu\text{L}$  will be transferred to the appropriate wells of a prelabeled 96-well plate. Three hundred and sixty  $\mu\text{L}$  of fresh Complete MEM without phenol red will be added to the wells designated as blanks. The optical density at 490 nm ( $\text{OD}_{490}$ ) will be determined using a spectrophotometer. Samples reading 1.500 and above ( $\text{OD}_{490}$ ) will be diluted to bring the reading within the linear range of the plate reader 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.

## 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<sub>490</sub> value of each treatment group will be calculated by averaging the OD<sub>490</sub> 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:

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

### 9.2 Data Interpretation

Test materials having an *In Vitro* Score of  $\geq 75$  will be classified as an EPA Category I. Test materials having an *In Vitro* Score  $< 75$  are given a classification of EPA Category II.

## 10.0 REPORT

A report will be prepared by the applicant, be submitted according to PR notice 2011-3, 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 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.

## EpiOcular™ Human Cell Construct Assay

### 1.0 PURPOSE

The purpose of this study is to evaluate the potential ocular irritation of the test material 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 material.

### 2.0 SPONSOR

2.1 Name:

2.2 Address:

2.3 Representative:

### 3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Material(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 commercially available EpiOcular™ human cell construct can be used in this assay, or any other validated human cell construct. The EpiOcular™ construct offers features appropriate for an ocular irritation model. First, the model is composed of stratified human keratinocytes (neonatal foreskins) in a three-dimensional structure. Second, 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 material number, and the exposure time.

## 7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The ocular irritation potential of the test material is evaluated by the exposure time required to reduce tissue viability to 50% of controls (ET<sub>50</sub>). Viability is 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 material-treated cultures (Berridge, et al., 1996). Data are presented in the form of relative survival (relative MTT conversion) versus test material 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.

### 7.1 Media and Reagents

7.1.1 Assay Medium:

7.1.2 EpiOcular™ Tissue construct or other tissue construct as noted above in 6.0

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<sup>++</sup> and Mg<sup>++</sup> Free Dulbecco's Phosphate Buffered Saline (Ca<sup>++</sup>Mg<sup>++</sup>Free-DPBS)

7.1.7 Extraction Medium: Isopropanol

## 7.2 Preparation and Delivery of Test Article.

Test materials will be tested as intended to be sold and/or marketed. One hundred  $\mu\text{L}$  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<sup>®</sup> to bring the test article in contact with the tissue. When the test article must first be applied to a dosing device, approximately 30  $\mu\text{L}$  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 material under the actual experimental conditions will not be determined by the testing facility.

## 7.3 Route of Administration

The test material is administered by topical application to the construct.

## 7.4 pH Determination

The pH of the neat liquid test material 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

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. Positive control cultures are treated with 0.3% (3 mg/mL) Triton<sup>®</sup>-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 material 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 materials) or 30 mg (solid test materials) 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% CO<sub>2</sub> 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 Assay materials

Upon receipt of the 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, 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 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<sup>®</sup> insert prior to opening the sealed package. Cultures with air bubbles under greater than 50% of the Millicell<sup>®</sup> 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 tissue construct 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<sub>2</sub>/95% air, and the bag will be sealed and stored at 2-8°C for subsequent use.

#### 7.8 Definitive MTT Assay

Exposure times will generally be 2, 15, 45 and 90 minutes. 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 material 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 material will be removed by extensively rinsing both sides of the culture with room temperature  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ -Free Dulbecco's Phosphate Buffered Saline ( $\text{Ca}^{++}\text{Mg}^{++}$ -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 material 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  $\mu\text{m}$  filter to remove undissolved crystals. Three hundred  $\mu\text{L}$  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  $3 \pm 0.1$  hours at standard culture conditions.

After  $3 \pm 0.1$  hours, the bottom of the tissue constructs will be blotted on absorbent paper, cleared of excess liquid, and transferred to a pre-labeled 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 ( $2-8^{\circ}\text{C}$ ) 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<sup>®</sup> insert will be decanted into the well from which it was taken. The extract solution will be mixed and 200  $\mu\text{L}$  transferred to the appropriate wells of a pre-labeled 96-well plate(s). Two hundred  $\mu\text{L}$  of isopropanol will be added to the wells designated as blanks. The absorbance at 550 nm ( $\text{OD}_{550}$ ) 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 material is shown to reduce MTT, only test materials that remain bound to the tissue after rinsing, resulting in a false MTT reduction signal, present a problem. To demonstrate that residual test material 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 material is acting to directly reduce the MTT, a freeze-killed control tissue is used. Freeze killed tissue is prepared by placing untreated tissue constructs in the  $-20^{\circ}\text{C}$  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 material reduction, killed tissues are treated with the test material in the normal fashion. Generally, each test material 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 material-treated killed control, the MTT reduction observed in the test material-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 material may be considered untestable in this system. The  $\text{OD}_{550}$  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  $\text{OD}_{550}$  of the blank control wells will be calculated. The corrected mean  $\text{OD}_{550}$  of the exposure time control(s) will be determined by subtracting the mean  $\text{OD}_{550}$  of the blank control from their mean  $\text{OD}_{550}$ s. The corrected  $\text{OD}_{550}$  of the individual test material exposure times and the positive control exposure times will be determined by subtracting the mean  $\text{OD}_{550}$  of the blank control from their respective  $\text{OD}_{550}$ s. When applicable, corrected  $\text{OD}_{550}$  values will be calculated for the control and test material-treated killed controls, as well. Generally, all calculations will be performed using a spreadsheet program similar to Microsoft Excel.

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

If killed controls (KC) are used, the following additional calculations will be performed to correct for the amount of MTT reduced directly by test material residues. The OD<sub>550</sub> value for the negative control killed control will be subtracted from the OD<sub>550</sub> values for each of the test material-treated killed controls (at each exposure time), to determine the net OD<sub>550</sub> values of the test material-treated killed controls.

Net OD<sub>550</sub> for each test material KC = Raw OD<sub>550</sub> test material KC – Raw OD<sub>550</sub> negative control KC

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

Final Corrected OD<sub>550</sub> = Corrected test material OD<sub>550</sub> (viable) – Net OD<sub>550</sub> test material (KC)

Finally, the following % of Control calculations are made:

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

The individual % of Control values are then averaged to calculate the mean % of Control per exposure time. Viability calculations for test materials treated in the long exposure time assay may be performed by comparing the corrected OD<sub>550</sub>s of each test material 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 material exposure time on the abscissa. The ET<sub>50</sub> will be interpolated from each plot. To determine the ET<sub>50</sub>, 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<sub>50</sub>, the equation will be solved for  $y = 50$ . If all of the exposure time points show greater than 50% survival, the ET<sub>50</sub> will be listed as greater than the longest exposure time. If all of the exposure times show less than 50% survival, the ET<sub>50</sub> 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<sub>50</sub> value.

## 8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The assay will be accepted if the positive control, 0.3% Triton<sup>®</sup>-X-100, causes an ET<sub>50</sub> within two standard deviations of the historical mean. The historical mean is updated every three months. The corrected mean OD<sub>550</sub> value for the minimum negative control exposure time must be within 20% of the corrected mean OD<sub>550</sub> 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<sub>50</sub> score of <4 minutes, it is classified as an EPA Category I. However, a BCOP should also be performed to confirm this result. If the antimicrobial product making a cleaning claim has an ET<sub>50</sub> 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<sub>50</sub> 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 applicant, be submitted according to PR notice 2011-3, and will accurately describe all methods used for generation and analysis of the data. A summary will be prepared reporting the ET<sub>50</sub> values for each test material 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 REFERENCES

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.



## 6.0 TEST SYSTEM

L929 cells will be used in the study. Cells can be obtained commercially or constructed in the testing laboratory. Any new model should be validated. An isolated population of L929 cells is exposed to increasing concentrations of test material, starting at the lowest concentration. The concentration of test material that causes a 50% decrease in the acidification rate (MRD<sub>50</sub>) is 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<sub>2</sub>) 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, the determination of the pH of the neat test material if possible, the determination of the pH at the highest concentration of test material in the medium if possible, a dose range finding assay and at least two definitive assay trials. 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 material concentrations. The concentration of test material which causes a 50% reduction in the acidification rate is calculated and termed the MRD<sub>50</sub> (Metabolic Rate Decrement 50%). The MRD<sub>50</sub> 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  $\text{Ca}^{++}\text{Mg}^{++}$ -Free Phosphate Buffered Saline (PBS)

8.1.5 0.05% Trypsin in  $\text{Ca}^{++}\text{Mg}^{++}$ -Free- Hanks' Balanced Salts Solution

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

## 8.2 Preparation and Delivery of Test Material

The test material will be dissolved in Low-Buffered DMEM. Other solvent systems will be used only with adequate scientific justification, but should generally be avoided. 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 material dosing solutions will be administered directly to the cells. Cells will be exposed to each concentration of test material for approximately 810 sec, after which time the test material 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  $\text{MRD}_{50}$  point has been surpassed or until the highest concentration has been dosed.

## 8.4 pH Determination

The pH of the neat liquid test material 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.

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 material 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  $5 \pm 1\%$   $\text{CO}_2$  in air. L929 cells will be seeded onto capsule cups at approximately  $6.0 \times 10^5$  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  $75\text{cm}^2$  of growth surface. The cells are trypsinized with approximately 3 mL of trypsin (for each  $75\text{cm}^2$  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  $75\text{cm}^2$  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  $6.0 \times 10^5$  cells per each capsule cup (0.5 mL of a  $1.2 \times 10^6$  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  $5 \pm 1\%$   $\text{CO}_2$  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 ± 1°C and 5 ± 1% CO<sub>2</sub> in air.

## 8.7 Dose Range Finding Assay

A dose range finding assay will always be performed to establish an appropriate test material 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.

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.

<b>DILUTION #</b>	<b>CONCENTRATION</b>
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 material 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 material or positive control prior to exposure. After the baseline data points have been taken, the exposure cycle will begin with the lowest test material concentration. From these baseline data points, the spreadsheet will compute the mean baseline value used in the MRD<sub>50</sub> calculation. Each exposure cycle will take 20 minutes.

The maximum solvent concentration (other than Low-Buffered DMEM) will be 10.

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

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

The test material doses for the definitive assay are chosen so that generally seven doses (spaced as three-fold dilutions) are available for the determination of the  $\text{MRD}_{50}$ . Generally, three concentrations are chosen to result in expected survivals lower than 50%, one concentration is chosen to result in an expected survival of approximately 50%, and three or more concentrations are chosen to result in expected survivals greater than 50%. If a test material 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 is performed in the same manner as the dose range finding assay, with the exception that if the  $\text{MRD}_{50}$  value from the dose range finding assay is  $> 10$  mg/mL, higher doses of test material 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  $\text{MRD}_{50}$  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  $\text{MRD}_{50}$  could be determined.

## 8.9 Data Analysis

The acidification rates which occur after exposure to each test material 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 is 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<sub>50</sub>. MRD<sub>50</sub> data are 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<sub>50</sub> 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<sub>50</sub> 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 pesticide product making a cleaning claim has an MRD<sub>50</sub> 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<sub>50</sub> 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 applicant, submitted according to PR notice 2011-3, and will accurately describe all methods used for the generation and analysis of the data. For each test material, the individual MRD<sub>50</sub> values from each assay trial, and the average MRD<sub>50</sub> value from at least two valid definitive trials will be presented. The MRD<sub>50</sub> value from the dose range finding assay will be included in the calculation of the average MRD<sub>50</sub>, if one can be determined. A separate summary will be prepared reporting the MRD<sub>50</sub> values for each assay with each test material as well as the positive control data.

## 12.0 REFERENCES

Botham, P.A., Osborne, R., Atkinson, K., Carr, G., Cottin, M., and Van Buskirk, R.G. (1997) IRAG working group 3: cell function-based assays. In: Eye Irritation Testing: Practical Applications of Non-Whole Animal Alternatives. Food and Chemical Toxicology 37:67-77.

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.