

**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**

**Interagency Coordinating Committee on the
Validation of Alternative Methods**

**National Toxicology Program Interagency Center for the
Evaluation of Alternative Toxicological Methods**

**National Institute of Environmental Health Sciences
National Institutes of Health
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List of Abbreviations and Acronyms

%CV	Percent coefficient of variation
AMCP	Antimicrobial cleaning product
ATWG	Alternative Testing Working Group
BCOP	Bovine corneal opacity and permeability
BRD	Background review document
CFR	Code of Federal Regulations
CM	Cytosensor Microphysiometer
CPSC	U.S. Consumer Product Safety Commission
CV	Coefficient of variation
ECVAM	European Centre for the Validation of Alternative Methods
EEC	European Economic Community
EO	EpiOcular™
EPA	U.S. Environmental Protection Agency
ET ₅₀	Time needed to reduce cell viability by 50%
EU	European Union
FDA	U.S. Food and Drug Administration
FR	<i>Federal Register</i>
GHS	United Nations Globally Harmonized System of Classification and Labelling of Chemicals
HET-CAM	Hen's Egg Test Chorioallantoic Membrane
ICATM	International Cooperation on Alternative Test Methods
ICE	Isolated Chicken Eye
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IIVS	Institute for In Vitro Sciences, Inc.
ILS	Integrated Laboratory Systems, Inc.
IRE	Isolated Rabbit Eye
IVIS	<i>In vitro</i> irritancy score
JaCVAM	Japanese Center for the Validation of Alternative Methods
LVET	Low volume eye test
MRD ₅₀	Estimated concentration of a test substance needed to reduce the basal metabolic rate of L929 cells by 50%
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
NTP	National Toxicology Program
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development

OPP	Office of Pesticide Programs
OTWG	ICCVAM Ocular Toxicity Working Group
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SD	Standard deviation
SRD	Summary review document
TMER	Test method evaluation report
UN	United Nations
U.S.	United States
U.S.C.	United States Code

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Preface

Eye injury is a leading cause of visual impairment in the United States (U.S.) with 40,000 to 50,000 new cases of impaired vision reported each year.¹ Many eye injuries occur due to contact with workplace or household products or chemicals. Accidents involving common household products (e.g., oven cleaner and bleach) cause about 125,000 eye injuries each year.² These products often result in chemical burns and emergency room visits.³ Each day about 2,000 U.S. workers have a job-related eye injury that requires medical treatment. Although the majority of these eye injuries result from mechanical sources, chemical burns from industrial chemicals or cleaning products are common.⁴

In order to avoid eye injuries, regulatory agencies require testing to determine if chemicals and products have the potential to cause eye damage. This testing information is used to classify the ocular hazard and to determine appropriate labeling that must be used to warn consumers and workers of the potential hazard and how to avoid exposures that could result in damage to the eye, and what emergency procedures should be followed if there is accidental exposure.

Nearly all ocular safety testing has been conducted using the Draize rabbit eye test, although *in vitro* methods can now be used to identify whether substances cause severe irritation or permanent eye damage. The Draize rabbit eye test, originally described by Draize et al. (1944), involves instillation of 0.1 mL of the test substance into the conjunctival sac of one eye while the other eye serves as the untreated control. The eye is examined at least daily for up to 21 days. The presence and severity of any injuries to the cornea, conjunctiva, and iris (tissues inside the eye) are scored and the duration that the injuries persist is recorded.

In 2004, the U.S. Environmental Protection Agency (EPA) requested that the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluate an *in vitro* testing strategy that would meet their need to evaluate, categorize, and label antimicrobial cleaning products (AMCPs) for eye irritation. As part of this evaluation, ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) requested the submission of data and information on AMCPs (73 FR 18535).⁵

ICCVAM carefully compiled and assessed all available data and arranged an independent scientific peer review. ICCVAM and its Ocular Toxicity Working Group (OTWG) solicited and considered public comments and stakeholder involvement throughout the evaluation process. As part of their ongoing collaboration with ICCVAM, scientists from the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM) served as liaisons to the OTWG. ICCVAM, NICEATM, and the OTWG prepared a draft summary review document (SRD) describing the validation status of the AMCP testing strategy, including the reliability and accuracy of each of the three *in vitro* test methods in the AMCP testing strategy, and draft test method recommendations for usefulness and limitations. ICCVAM released this document to the public for comment on March 31, 2009, at which time ICCVAM also announced a meeting of the international independent scientific peer review panel (Panel) (74 FR 14556).⁶

The Panel met in public session on May 19–21, 2009, to review the ICCVAM draft AMCP SRD for completeness and accuracy. The Panel then evaluated (1) the extent to which the draft AMCP SRD addressed established validation and acceptance criteria and (2) the extent to which the draft AMCP

¹ Available at: http://www.preventblindness.org/resources/factsheets/Eye_Injuries_FS93.PDF

² Available at: <http://www.geteyesmart.org/eyesmart/injuries/home.cfm>

³ From the CPSC NEISS Database, 2007.

⁴ Available at: <http://www.cdc.gov/niosh/topics/eye/>

⁵ Available at <http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-6969.pdf>

⁶ Available at <http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/E9-7220.pdf>

SRD supported ICCVAM's draft test method recommendations. Before concluding their deliberations, the Panel considered written comments and comments made at the meeting by public stakeholders.

ICCVAM provided the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) with the draft AMCP SRD and draft test method recommendations, a summary of the conclusions and recommendations from the Panel meeting, and all public comments for discussion at their meeting on June 25-26, 2009, where public stakeholders were given another opportunity to comment. A detailed timeline of the evaluation is included with this report.

ICCVAM solicited and considered public comments and stakeholder involvement throughout the test method evaluation process. ICCVAM considered the SACATM comments, the conclusions of the Panel, and all public comments before finalizing the ICCVAM test method recommendations for each test method. The recommendations and the SRD, which is provided as an appendix to this report, are incorporated in this ICCVAM test method evaluation report. As required by the ICCVAM Authorization Act, ICCVAM will forward its recommendations to U.S. Federal regulatory agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving the ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM-ICCVAM website,⁷ and agency responses will also be made available on the website as they are received.

We gratefully acknowledge the many individuals who contributed to the preparation, review, and revision of this report. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. A. Wallace Hayes for serving as the Panel Chair and to Dr. Paul Bailey, Dr. Donald Sawyer, Dr. Kirk Tarlo, and Dr. Daniel Wilson for their service as Evaluation Group Chairs. We thank the OTWG for assuring a meaningful and comprehensive review. We especially thank Dr. Jill Merrill (U.S. Food and Drug Administration Center for Drug Evaluation and Research) and Dr. Karen Hamernik (EPA, to April 2009) for serving as Co-Chairs of the OTWG. Integrated Laboratory Systems, Inc., the NICEATM support contractor, provided excellent scientific support, for which we thank Dr. David Allen, Dr. Jonathan Hamm, Nelson Johnson, Dr. Brett Jones, Dr. Elizabeth Lipscomb, and James Truax. Finally, we thank European Centre for the Validation of Alternative Methods liaisons Dr. João Barroso, Dr. Thomas Cole, and Dr. Valerie Zuang and Japanese Center for the Validation of Alternative Methods liaison Dr. Hajime Kojima for their participation and contributions.

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⁷ <http://iccvam.niehs.nih.gov/methods/ocutox/AMCP.htm>

Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently evaluated the validation status of the antimicrobial cleaning product (AMCP) testing strategy, including the performance of three *in vitro* test methods: bovine corneal opacity and permeability (BCOP), Cytosensor[®] Microphysiometer (CM), and EpiOcular[™] (EO). This test method evaluation report (TMER) provides ICCVAM's recommendations regarding the usefulness and limitations of the AMCP testing strategy as well as recommendations for test method protocols, future studies, and performance standards. The report also includes ICCVAM's final summary review document (SRD) for the AMCP testing strategy.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), ICCVAM, and its Ocular Toxicity Working Group prepared the draft AMCP SRD and ICCVAM's draft test method recommendations. The drafts were provided to the public and an independent international scientific peer review panel (Panel) for comment. A detailed timeline of the ICCVAM evaluation process is appended to this report.

The Panel met in public session on May 19–21, 2009, to review and discuss the draft AMCP SRD and ICCVAM's draft test method recommendations. The Panel provided conclusions and recommendations on the validation status of the AMCP testing strategy. The Panel also reviewed how well the information contained in the draft SRD supported ICCVAM's draft test method recommendations. In finalizing this TMER and the AMCP SRD, ICCVAM considered (1) the conclusions and recommendations of the Panel, (2) comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods, and (3) public comments.

Specific ICCVAM Recommendations

Test Method Usefulness and Limitations

Given that none of the 228 AMCPs in the validation database has been tested in all three *in vitro* test methods (i.e., BCOP, CM, and EO), ICCVAM concludes that the data are insufficient to adequately demonstrate that the AMCP testing strategy using these test methods can identify all four U.S. Environmental Protection Agency (EPA) ocular hazard categories.

ICCVAM also concludes that the data are insufficient to support definitive recommendations on the alternate AMCP testing strategy, which uses only the BCOP and EO test methods to classify substances in all four EPA ocular hazard categories. Only 28 of the 228 AMCPs have been tested in both the BCOP and EO test methods. Of these, the Draize rabbit eye test classified only one as an EPA Category II substance and only four as EPA Category III substances.

Test Method Protocol

ICCVAM recommends using the updated ICCVAM protocols for the BCOP, CM, and EO test methods that are included as appendices to this report. In addition, all future studies intended to further characterize the usefulness and limitations of these test methods (i.e., BCOP, CM, and EO) should be conducted using the ICCVAM recommended protocols.

Future Studies

Given the limitations of the validation database, ICCVAM recommends a reference list of AMCPs for which high-quality Draize rabbit eye test data are available. These AMCPs should then be tested in each of the proposed test methods (i.e., BCOP, CM, and EO) to more thoroughly evaluate their usefulness and limitations. ICCVAM recommends that future test methods consider cells and tissue constructs from ocular tissues. In addition, ICCVAM encourages industry stakeholders to provide strategies and approaches that are currently used for corporate decisions on product safety. Users

should make available data gathered from future studies. The data could be used to further characterize the usefulness and limitations of an *in vitro* testing strategy.

Performance Standards

ICCVAM concludes that the development of performance standards for the AMCP testing strategy is not warranted at this time.

Validation Status of the AMCP Test Methods and Testing Strategies

The Bovine Corneal Opacity and Permeability Test Method

The validation database included 66 substances tested in both the BCOP test method and the Draize rabbit eye test. The accuracy of the overall EPA classification (EPA Category I, II, III, or IV) was 55%. While the BCOP test method correctly classified only 60% of the EPA Category II and 50% of the EPA Category III substances, it correctly identified 90% of the EPA Category I substances.

Intralaboratory repeatability for the BCOP test method (i.e., comparison of within-experiment runs of a test substance) was determined for 67 AMCPs as the mean percent coefficient of variation (%CV) for opacity (21%), permeability (25%), and *in vitro* irritancy score (IVIS) (18%). NICEATM also evaluated agreement with the ocular hazard classifications of the EPA and the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS). The EPA and GHS classification systems had 100% agreement in 63 of the 75 test runs (84%), 67% agreement in 11 of the 75 test runs (15%), and 60% agreement in one of the 75 test runs (1%).

NICEATM determined intralaboratory reproducibility (i.e., comparison of between-experiment runs of a test substance) for five AMCPs as the mean %CV for the IVIS. In two to six experiments, the mean %CV for the IVIS was 20%. These test substances were also evaluated for their agreement with the EPA and GHS ocular hazard classification systems. The evaluation found 100% agreement among the five test substances.

Interlaboratory reproducibility (i.e., comparison of runs of a test substance between different laboratories) for the BCOP test method could not be determined specifically for AMCPs because only one laboratory conducted the testing. However, three studies (3-12 laboratories each) determined interlaboratory reproducibility for non-AMCPs classified as severe or ocular corrosives by the BCOP test method (ICCVAM 2006a). The mean %CV for IVIS ranged from 25% to 36%.

The Cytosensor Microphysiometer Test Method

The validation database included 105 unique substances tested in both the CM test method and the low volume eye test (LVET). Three substances were tested twice for a total of 108 substances. The accuracy of the overall EPA classification (i.e., EPA Category I, II, III, or IV) was 30%. All nine of the EPA Category I substances were correctly identified. The CM test method overclassified the majority of substances classified by the LVET as EPA Category II, III, or IV substances (100% of the Category II substances, 67% of the Category III substances, and 89% of the Category IV substances).

Reliability of the CM test method could not be evaluated specifically for AMCPs due to insufficient data. However, NICEATM evaluated reliability of the test method in non-AMCPs. Intralaboratory repeatability was evaluated based on data from seven different studies of 1 to 35 substances. The mean %CV for the concentrations needed to reduce the basal metabolic rate of L929 cells by 50% (MRD₅₀ values) ranged from 6% to 25% for all materials tested.

Intralaboratory reproducibility for the CM test method was determined for 16 non-AMCP substances in one laboratory. The mean %CV for MRD₅₀ values for all substances tested was 25%.

Interlaboratory reproducibility for the CM test method was measured using results from two studies at two to four laboratories each. The mean %CV for MRD₅₀ values for all substances tested ranged from 17% to 51%.

The EpiOcular™ Test Method

Thirty substances were tested in both the EO test method and the Draize rabbit eye test. The accuracy of the overall EPA classification (i.e., EPA Category I, II, III, or IV) was 76%. All of the EPA Category I substances were correctly identified. Of the four EPA Category III substances, 75% were correctly identified by the EO test method. Forty-four percent of the nine EPA Category IV substances were correctly identified.

NICEATM determined intralaboratory repeatability for the EO test method in a subset of 15 AMCPs. The mean %CV for the times needed to reduce cell viability by 50% (ET₅₀ values) ranged from 0% to 62%. To evaluate the extent of agreement between the EPA and GHS ocular hazard classification systems, NICEATM analyzed intralaboratory reproducibility for three AMCPs that had been tested more than once at one laboratory. The three AMCPs had 100% agreement in both EPA and GHS classification systems. Intralaboratory reproducibility for the EO test method was also determined from repeat testing of a single substance (0.3% Triton X-100), which occurred at two different laboratories. The mean %CV for ET₅₀ values was approximately 20% in both laboratories.

Interlaboratory reproducibility for the EO test method was determined for non-AMCPs in a two-phase validation study of surfactants and surfactant-containing products. Mean %CVs ranged from 12% to 18%. However, this evaluation did not use a calculated ET₅₀ value to predict the ocular hazard classification category, as detailed in the AMCP background review document. Instead, it was based on an EO protocol that uses relative percent viability to classify irritancy (i.e., irritant vs. nonirritant).

These test substances were also evaluated for agreement in the EPA and GHS ocular hazard classification systems. Using either the EPA or GHS classification system in one phase of the validation study, 74% of the 19 substances had 100% agreement. In a subsequent study phase, 94% of the 54 substances had 100% agreement.

Original Testing Strategy Proposed in the AMCP Background Review Document: Combining the BCOP, CM, and EO Test Methods

The AMCP testing strategy (**Figure 1**) uses three *in vitro* test methods: BCOP, CM, and EO. For each test method, decision criteria have been developed to correspond to the four different categories of ocular irritation defined by the EPA hazard classification system.

The first test method used in the AMCP testing strategy depends on the chemical properties of the test substance. If the test substance is an oxidizer, which suggests that it will be an ocular corrosive or severe irritant, then the BCOP test method is used first. Test substances that produce an IVIS ≥ 75 in the BCOP would be classified as EPA Category I. If a test substance produces an IVIS < 75 , further assessment using histopathology evaluation of the affected tissue can then determine whether it meets the criteria for classification as EPA Category I, II, or III.

To determine whether the test substance is EPA Category III or IV, the test substance is subsequently tested in either the CM or EO test method. The choice of method again depends on the chemical properties of the test substance. If the test substance is water soluble, it can be tested in either the CM test method or the EO test method. If it is water insoluble, it must be tested in the EO test method to determine the final hazard classification.

None of the 228 substances in the validation database has been tested in all three of the *in vitro* test methods proposed for the AMCP testing strategy. Therefore, no data are available with which to characterize the actual performance of a testing strategy that includes the BCOP, CM, and EO test methods.

Alternate AMCP Testing Strategy: Combining the BCOP and EO Test Methods

As explained above, none of the 228 AMCPs included in the original testing strategy has been tested in all three of the *in vitro* test methods included in the AMCP testing strategy. However,

28 substances were tested in both the BCOP and EO test methods. ICCVAM also had concerns about the validation status of the low volume eye test, which was used as the *in vivo* reference test method for all of the CM test method data. Therefore, ICCVAM evaluated an alternate AMCP testing strategy (Figure 2) that included only the BCOP and EO test methods. The alternate AMCP testing strategy was evaluated using two approaches: (1) test in the BCOP test method first to identify EPA Category I and II substances and then test in the EO test method to identify EPA Category III and IV substances; or (2) test in the EO test method first to identify EPA Category III and IV substances and then in the BCOP test method to identify EPA Category I and II substances.

The alternate AMCP testing strategy performed the same regardless of which approach was used. It correctly classified 79% of the substances, identifying 100% of the EPA Category I substances, none of the EPA Category II substances, 100% of the EPA Category III substances, and 44% of the EPA Category IV substances.

Figure 1 Combining the BCOP, CM, and EO Test Methods into a Testing Strategy: AMCP Testing Strategy

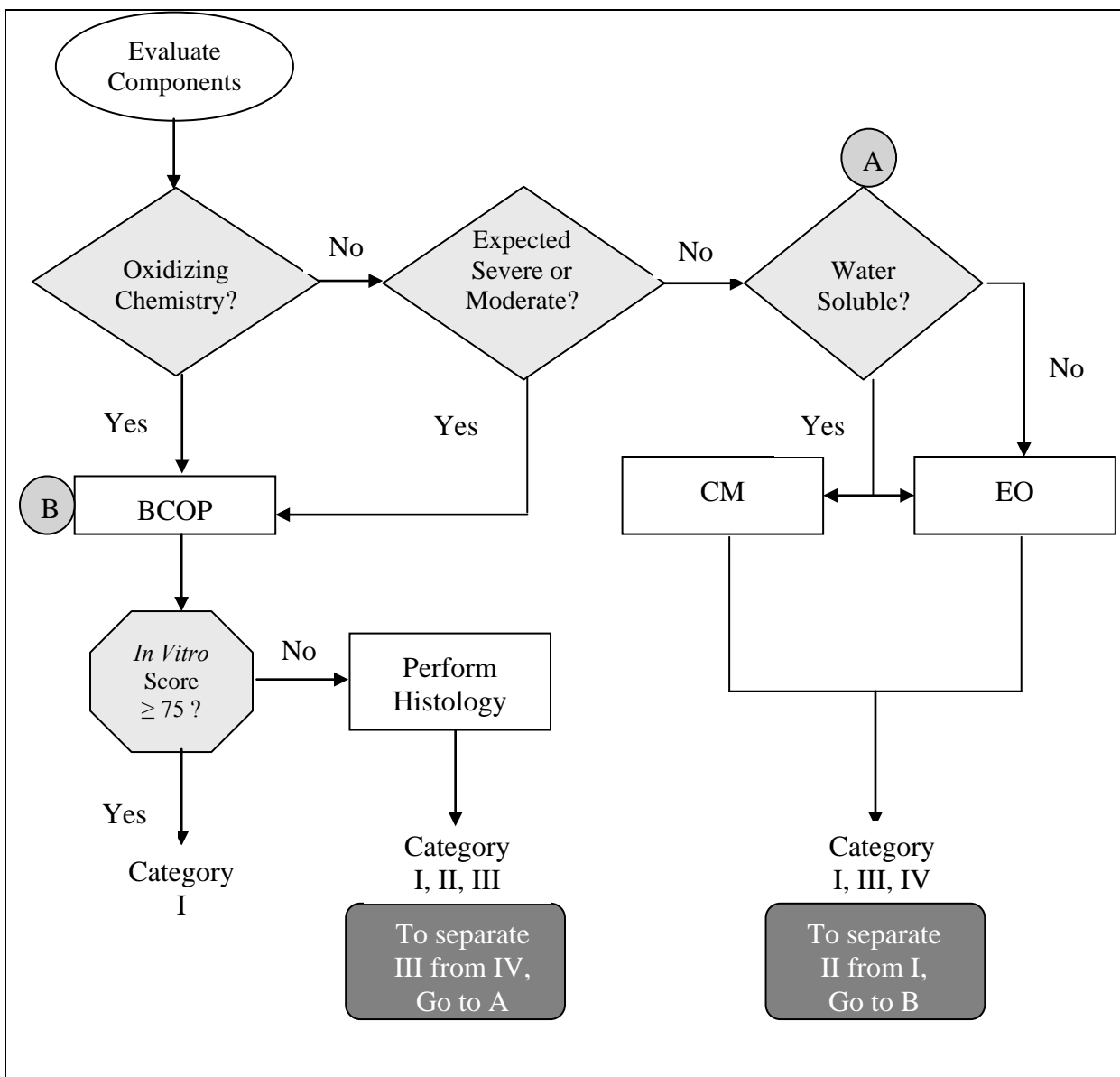
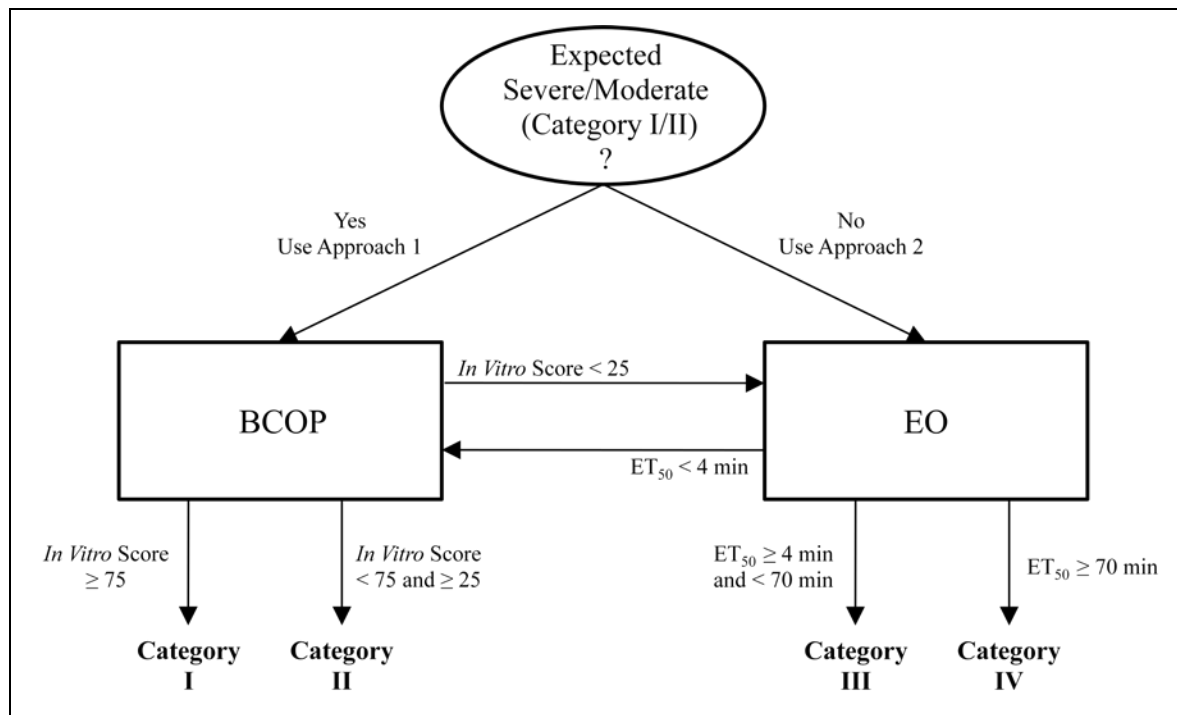


Figure 2 Combining the BCOP and EO Test Methods into a Testing Strategy: Alternate AMCP Testing Strategy



ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process provides numerous opportunities for stakeholder involvement. The public may submit written comments and provide oral comments at ICCVAM independent peer review panel meetings and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) meetings. From March 2005 to July 2009, there were nine opportunities for public comment on ICCVAM's evaluation of the validation status of alternative ocular safety testing methods and approaches. During this time, ICCVAM received 37 public comments, of which 25 pertained directly to the AMCP testing strategy or one of the three *in vitro* test methods (i.e., BCOP, CM, and EO) included in the AMCP testing strategy. SACATM reviewed and commented on the draft recommendations and associated conclusions of the Panel during their annual meeting in June 2009. ICCVAM considered public and SACATM comments in finalizing the test method recommendations provided in this report.

1.0 Introduction

Commercial and household cleaning products must be labeled to indicate if they are hazardous to the consumer during handling or use. The U.S. Consumer Product Safety Commission (CPSC) typically regulates these products under the Federal Hazardous Substances Act (15 U.S.C. 1261 and 16 CFR 1500) and the Poison Prevention Packaging Act (16 CFR 1700). However, the Federal Insecticide, Fungicide, and Rodenticide Act (7 U.S.C. 136-136y, 40 CFR 161) requires that cleaning products with an antimicrobial claim register as antimicrobial pesticides with the U.S. Environmental Protection Agency (EPA) Office of Pesticide Products (OPP). To comply with EPA classification and labeling requirements for eye irritation (EPA 2003), a product manufacturer must provide Draize rabbit eye test data (Draize et al. 1944) (40 CFR 158; 40 CFR 161).

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285l-3) charged ICCVAM with coordinating the technical evaluation of new, revised, and alternative test methods that have regulatory applicability. The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) administers ICCVAM and provides scientific support for ICCVAM activities.

In June 2004, the EPA–OPP contacted NICEATM to request a technical assessment of an *in vitro* testing strategy that would meet their need to evaluate, categorize, and label antimicrobial cleaning products (AMCPs) for eye irritation. The AMCP testing strategy comprises three *in vitro* test methods: the bovine corneal opacity and permeability (BCOP), Cytosensor[®] Microphysiometer (CM), and EpiOcular[™] (EO) test methods. The Alternative Testing Working Group (ATWG), a consortium of seven consumer product companies (Clorox, Colgate-Palmolive, Dial, EcoLabs, JohnsonDiversey, Procter & Gamble, and SC Johnson), developed the AMCP testing strategy, coordinated by the Institute for In Vitro Sciences, Inc. (IIVS). IIVS performed additional testing to complete parallel sets of *in vivo* and *in vitro* data and described the AMCP testing strategy in a background review document (BRD). NICEATM received an initial draft of the AMCP BRD on December 27, 2007. A formal transmittal letter followed on January 8, 2008. **Appendix A** provides a detailed timeline of the ICCVAM evaluation. The ICCVAM recommended test method protocol for each test method are provided in **Appendix B**.

The EPA and the ATWG requested that NICEATM and ICCVAM assess the scientific validity of the AMCP testing strategy as described in the AMCP BRD. The EPA and the ATWG sought to determine whether the EPA could be reasonably certain that the testing strategy would be useful for making hazard classification and labeling decisions for AMCPs.

The ICCVAM Ocular Toxicity Working Group (OTWG) worked with NICEATM in evaluating the AMCP testing strategy. Drs. João Barroso, Thomas Cole, and Valerie Zuang represented the European Centre for the Validation of Alternative Methods (ECVAM). Dr. Hajime Kojima was the liaison from the Japanese Center for the Validation of Alternative Methods (JaCVAM). On March 17, 2008, after a preliminary review of the AMCP BRD, the OTWG requested additional documents from IIVS to fill essential information gaps noted in the original submission.

On April 4, 2008, NICEATM published a request for relevant data and nominations of individuals to serve on an independent international scientific peer review panel (Panel) (73 FR 18535). The request was also sent via the ICCVAM electronic mailing list and through direct requests to over 100 stakeholders. In response to these requests, 12 individuals or organizations nominated member to the Panel; however, no test method data were submitted.

The OTWG provided comments and requested additional information from IIVS on April 18, 2008. On June 23-24, 2008, the OTWG and ICCVAM assigned this activity a high priority after

considering comments from the public and ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM).

IIVS provided a revised AMCP BRD (**Appendix C, Annex I**) and AMCP BRD Supplement (**Appendix C, Annex II**) on July 21 and October 8, 2008, respectively.

To facilitate peer review, the OTWG and NICEATM prepared a draft AMCP summary review document (SRD). The AMCP SRD summarizes the available data and information regarding the validity of each of the three *in vitro* test methods, the AMCP testing strategy, and an alternate AMCP testing strategy.

On March 31, 2009, ICCVAM announced the availability of the ICCVAM draft documents and a public Panel meeting to review the validation status of the test methods (74 FR 14556¹). The ICCVAM draft AMCP SRD and draft test method recommendations were posted on the NICEATM-ICCVAM website. All of the information provided to the Panel and all public comments received before the Panel meeting were made available on the NICEATM-ICCVAM website.²

The Panel met in public session on May 19-21, 2009, to review the ICCVAM draft AMCP SRD for completeness and accuracy. The Panel then evaluated (1) the extent to which the draft AMCP SRD addressed established validation and acceptance criteria and (2) the extent to which the draft AMCP SRD supported ICCVAM's draft proposed test method recommendations. Interested stakeholders from the public commented at the Panel meeting. The Panel considered these comments, as well as those submitted previously, before concluding their deliberations. On July 13, 2009, ICCVAM posted the final report of the Panel's recommendations (**Appendix D**) on the NICEATM-ICCVAM website for public review and comment (74 FR 33444³).

ICCVAM provided SACATM with the draft AMCP SRD, and all public comments for discussion at their meeting on June 25-26, 2009, where public stakeholders were given another opportunity to comment.

After SACATM's meeting, ICCVAM and the OTWG considered the SACATM comments, the Panel report, and all public comments (**Appendix E**) before finalizing the ICCVAM test method evaluation report and the AMCP SRD provided in this report. As required by the ICCVAM Authorization Act, ICCVAM will make this test method evaluation report and the accompanying final SRD available to the public and to U.S. Federal agencies for consideration. The relevant U.S. Federal laws, regulations, guidelines, and recommendations for eye irritation/corrosion testing are summarized in **Appendix F**. Federal agencies must respond to ICCVAM within 180 days after receiving ICCVAM test method recommendations. Agency responses will be made available to the public on the NICEATM-ICCVAM website as they are received.

¹ <http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/E9-7220.pdf>

² <http://iccvam.niehs.nih.gov/methods/ocutox/PeerPanel09.htm>

³ http://iccvam.niehs.nih.gov/docs/ocutox_docs/OcularPRPrept2009.pdf

2.0 ICCVAM Recommendations for the AMCP Testing Strategy

2.1 ICCVAM Recommendations: Test Method Usefulness and Limitations

Given the limitations of the available database for the three *in vitro* test methods (i.e., BCOP, CM, and EO), there is currently insufficient data with which to adequately demonstrate that the AMCP testing strategy using these test methods can identify all four EPA ocular hazard categories.

Of the 228 AMCPs included in the validation database, none has been tested in all three *in vitro* test methods. There are a limited number of AMCPs (n=28) that have been tested in both the BCOP and EO test methods. However, of these, there is only one EPA Category II substance and only four EPA Category III substances (based on Draize rabbit eye test data). Therefore, although the performance of the alternate AMCP testing strategy using the BCOP and EO test methods appears to be useful for identifying EPA Category I substances using the BCOP test method and EPA Category IV substances using the EO test method, there is insufficient data with which to adequately demonstrate that this strategy can identify all four EPA ocular hazard categories.

Therefore, ICCVAM concludes that there are not enough data to support the AMCP testing strategy in terms of the proposed test method usefulness and limitations (i.e., the classification of substances in all four EPA ocular hazard categories). ICCVAM also concludes that there are insufficient available data on which to base definitive recommendations on the alternate AMCP testing strategy for classifying substances in all four EPA ocular hazard categories.

2.1.1 Independent Peer Review Panel Conclusions and Recommendations

The Panel concurred with ICCVAM's conclusion that there are not enough data to support the AMCP testing strategy in terms of the proposed test method usefulness and limitations (i.e., the classification of substances in all four EPA ocular hazard categories). Likewise, the Panel also concluded that there were insufficient available data on which to base definitive recommendations on the alternate AMCP testing strategy for classifying substances in all four EPA ocular hazard categories.

The Panel indicated that a retrospective evaluation of results in more than one test method can be considered adequate for the evaluation of test method performance. Retrospective studies must include an audit of the data to determine quality, comprehensiveness, and the number and severity of data errors. However, given the lack of available data for substances tested in more than one of the proposed test methods included in the strategy, the Panel concluded that any definitive recommendations should be based on prospective testing of a list of reference substances in each of the proposed *in vitro* test methods.

2.2 ICCVAM Recommendations: Test Method Protocol

The detailed test method protocols included in the AMCP BRD (**Appendix C, Annex I**) use a variety of endpoints to predict ocular irritation potential. While these test method protocols have not been adequately validated for use in the AMCP testing strategy, decision criteria have been developed to correspond to the four different categories of ocular irritation defined by the EPA hazard classification system (i.e., EPA Categories I, II, III, and IV).

Concurrent positive and negative controls should be included in each study. Additionally, ICCVAM recommends that appropriate benchmark controls should be defined for each hazard category. Periodic testing (i.e., at intervals ≤ 6 months) of these benchmark controls should be performed in laboratories that regularly conduct an *in vitro* testing strategy. Users should be aware that a negative study result will have ramifications on test substance results obtained in the interval between the last acceptable benchmark control study and the unacceptable benchmark control study. ICCVAM recommends using the updated ICCVAM protocols for the BCOP, CM, and EO test methods that are

included as appendices to this report (**Appendix B**). In addition, all future studies intended to further characterize the usefulness and limitations of these test methods (i.e., BCOP, CM, and EO) should be conducted using the ICCVAM recommended protocols.

2.2.1 Independent Peer Review Panel Conclusions and Recommendations

The Panel concluded that the available data supported the ICCVAM recommendations for the ocular test method procedures in terms of the proposed test method protocols.

2.3 ICCVAM Recommendations: Future Studies

Given the limitations in the validation database, a reference list of AMCPs (for which high quality Draize rabbit eye test data are available) should be tested prospectively in each of the proposed test methods (i.e., BCOP, CM, and EO) to allow for a more complete evaluation of the usefulness and limitations of the AMCP testing strategy.

The following additional recommendations are made:

- Future test methods should consider cells and tissue constructs of cornea/conjunctiva origins.
- Industry stakeholders are encouraged to provide strategies and approaches that are currently used for corporate decisions on product safety in an integrated decision strategy, including the various types of data and information and the respective qualitative and quantitative decision criteria.
- ICCVAM encourages users to provide all data that are generated from future studies, as they could be used to further characterize the usefulness and limitations of an *in vitro* testing strategy.

2.3.1 Independent Peer Review Panel Conclusions and Recommendations

The Panel concluded that additional testing would expand existing databases and could be used to optimize test method decision criteria. Additional studies recommended by the Panel are reflected in the ICCVAM recommendations detailed above. The Panel also concluded that additional studies should not focus on the use of the EO test method alone because it considered the use of an *in vitro* testing strategy more promising.

2.4 ICCVAM Recommendations: Performance Standards

Based on the available data and associated performance described in **Sections 3.2** and **3.4**, ICCVAM recommends that the development of performance standards for the AMCP testing strategy is not warranted at this time.

2.4.1 Independent Peer Review Panel Conclusions and Recommendations

The Panel concluded that the development of performance standards for the AMCP testing strategy was not warranted at this time.

3.0 Validation Status of the AMCP Testing Strategy

The information in the ICCVAM final AMCP summary review document (**Appendix C**) is summarized below. The SRD reviews the available data and information for the AMCP testing strategy. It describes the current validation status of the AMCP testing strategy, including what is known about its reliability and accuracy, the scope of the substances tested, and standardized protocols used for the validation study.

3.1 Test Method Description

3.1.1 AMCP Testing Strategy

The AMCP testing strategy (**Figure 3-1**) proposed in the AMCP BRD (**Appendix C, Annex I**) comprises three *in vitro* test methods: the BCOP, CM, and EO. Each test method includes decision criteria developed to correspond to the four ocular irritation categories defined in the EPA hazard classification system. The BCOP, CM, and EO test methods use a variety of endpoints to predict ocular irritation potential.

The two primary endpoints for the BCOP test method are the extent of corneal opacity and the permeability. Both are measured and used to calculate an *in vitro* irritancy score (IVIS).⁴

- $IVIS \geq 75$ = EPA Category I
- $IVIS \geq 25$ and < 75 = EPA Category II
- $IVIS < 25$ = EPA Category III

Because the data points from EPA Category III and Category IV overlap and it's impossible to assign a cutoff value, the AMCP BRD does not propose BCOP decision criteria for EPA Category IV. Histopathology evaluation of the affected tissue is an optional endpoint.

The endpoint for the CM test method is the estimated concentration of a test substance needed to reduce the basal metabolic rate of L929 cells by 50% (MRD₅₀).

- $MRD_{50} < 2$ mg/mL = EPA Category I
- $MRD_{50} \geq 2$ mg/mL and < 80 mg/mL = EPA Category III
- $MRD_{50} \geq 80$ mg/mL = EPA Category IV

The AMCP BRD does not propose CM decision criteria for EPA Category II because the data points from EPA Category I and Category II overlap making it impossible to assign a cutoff value.

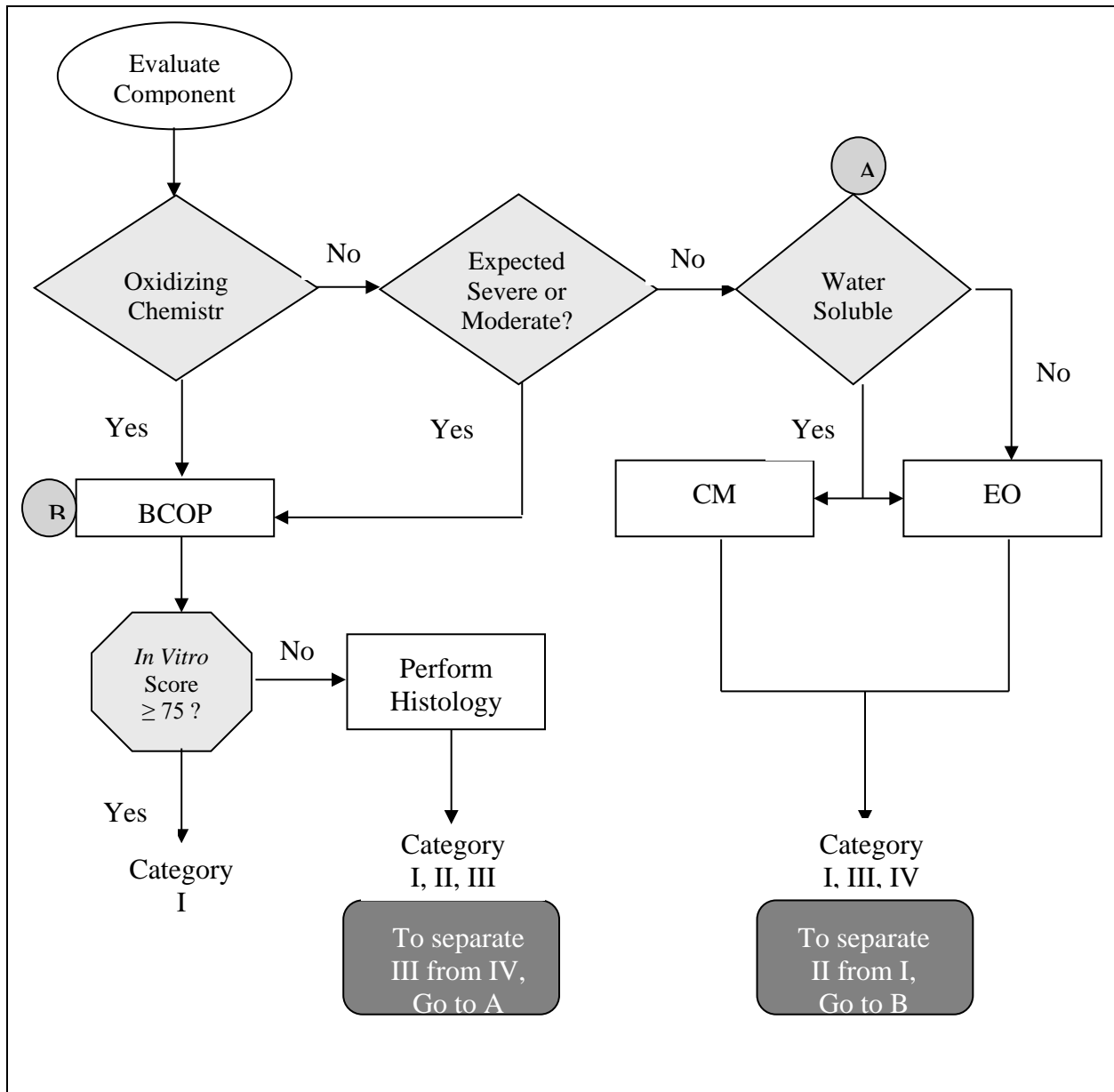
The endpoint for the EO test method is the time needed to reduce cell viability by 50% (ET₅₀).

- $ET_{50} < 4$ min = EPA Category I
- $ET_{50} \geq 4$ min and < 70 min = EPA Category III
- $ET_{50} \geq 70$ min = EPA Category IV

The AMCP BRD does not propose EO decision criteria for EPA Category II because the database includes only one EPA Category II substance.

⁴ The *in vitro* irritancy score (IVIS) is calculated as the sum of the mean corrected opacity value (\pm standard deviation [SD]) and 15 times the mean corrected permeability value (OD₄₉₀ units \pm SD).

Figure 3-1 Combining the BCOP, CM, and EO Test Methods into a Testing Strategy: AMCP Testing Strategy



In the AMCP testing strategy (**Figure 3-1**), the first test method used depends on knowledge of the chemical properties of the test substance. If the test substance is an oxidizer, which suggests that it will be an ocular corrosive/severe irritant, it is first tested in the BCOP test method. As noted above, test substances that produce an IVIS ≥ 75 would be classified as EPA Category I. If a test substance produces an IVIS < 75 , further assessment using histopathology evaluation can determine whether it meets the criteria for classification as EPA Category I, II, or III.

To determine whether the test substance is EPA Category III or IV, the test substance is subsequently tested in either the CM or EO test method. Selection of the CM or EO test method depends on the water solubility of the test substance. Water-soluble substances can be tested in either the CM test

method or the EO test method, but water-insoluble substances must be tested in the EO test method to determine their final hazard classification.

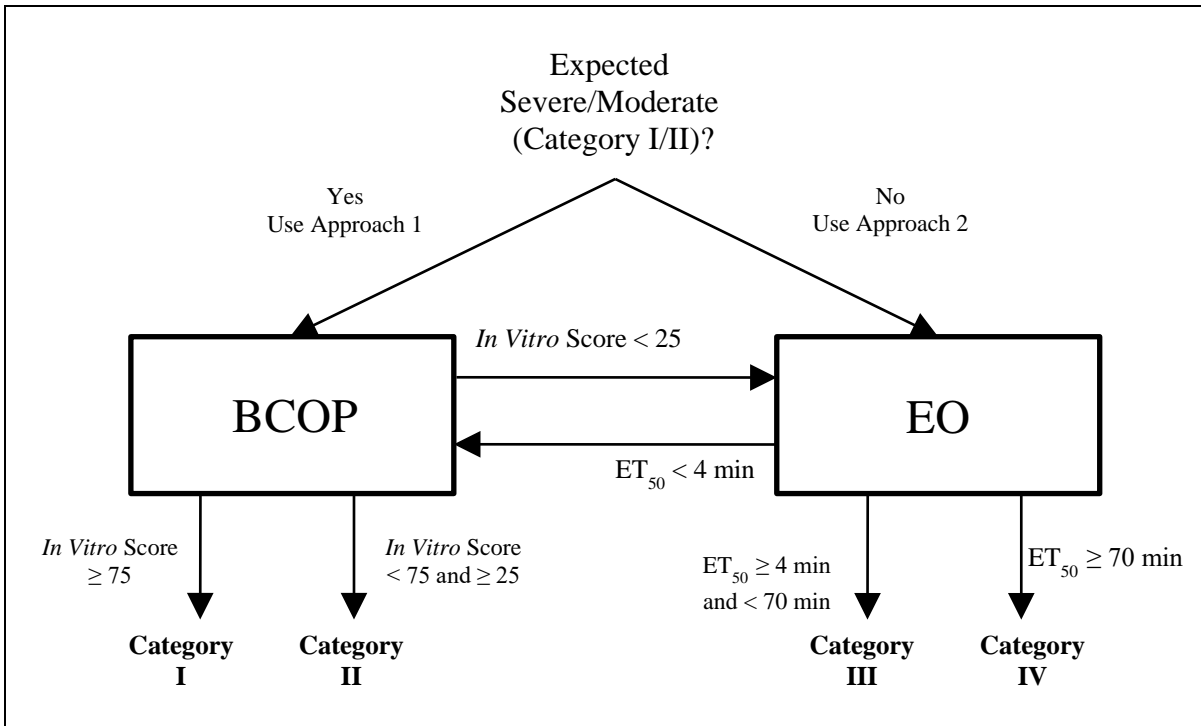
3.1.2 Combining the BCOP and EO Test Methods into a Testing Strategy: Alternate AMCP Testing Strategy

None of the 228 substances has been tested in all three of the *in vitro* test methods included in the AMCP testing strategy. There were also concerns about the validation status of the low volume eye test (LVET), which was used as the *in vivo* reference test method for all of the CM test method data. Therefore, ICCVAM evaluated an alternate AMCP testing strategy (**Figure 3-2**) that includes only the BCOP and EO test methods. In the alternate AMCP testing strategy, the BCOP test method would be used to identify EPA Category I or II substances, and the EO test method would be used to identify EPA Category III or IV substances.

ICCVAM evaluated two approaches in the alternate AMCP testing strategy: (1) test in the BCOP test method first and then in the EO test method or (2) test in the EO test method first and then in the BCOP test method. In the first proposed approach, the BCOP test method would classify all EPA Category I and II substances. The EO test method would then classify all other substances as either EPA Category III or IV.

In the second proposed approach, the EO test method would classify all EPA Category III and IV substances. All other substances would then be tested in the BCOP test method and classified as either EPA Category I or II.

Figure 3-2 Combining the BCOP and EO Test Methods into a Testing Strategy: Alternate AMCP Testing Strategy



3.2 Validation Database

3.2.1 Rationale for the Substances or Products Included in the AMCP Testing Strategy

The validation database for the AMCP BRD included 228 substances (**Appendix C, Annex I**). These include 68 substances tested in the BCOP test method, 105 substances tested in the CM test method, and 55 substances tested in the EO test method. None of the 228 substances has been tested in all three of the proposed *in vitro* test methods (i.e., BCOP, CM, and EO). It should be noted that, according to the submitter, “a minimum 28 of the materials are EPA registered antimicrobial cleaning products, with eight additional materials being in-use dilutions of concentrates which are EPA registered” (Rodger Curren, personal communication).

The distribution of product categories differed for each test method (**Table 3-1**). Most of the 105 substances tested in CM test method are surfactants (78% [82/105]) and solvents (17% [18/105]). The 68 substances tested in the BCOP test method and the 55 substances tested in the EO test method are relatively equally distributed among alkalis, oxidizers, solvents, and surfactants (approximately 20% to 30% each).

Table 3-1 Distribution of Product Categories Evaluated in the AMCP Testing Strategy

Product Categories	Substances Tested: Cytosensor Microphysiometer	Substances Tested: EpiOcular™	Substances Tested: BCOP	Substances Tested: Total
Solvents	18	10	12	39
Oxidizers	0	13	16	33
Surfactants	82	17	18	114
Acids	1	2	7	10
Bases	4	11	14	29
Others	0	2	1	3
Total	105	55	68	228

Abbreviations: AMCP = antimicrobial cleaning product; BCOP = bovine corneal opacity and permeability.

3.2.2 Rationale for the Substances or Products Included in the Alternate AMCP Testing Strategy

Only 28 substances tested in both the BCOP and EO and test methods were also tested in the Draize rabbit eye test. Therefore, ICCVAM limited its evaluation of the alternate AMCP testing strategy to these 28 substances. These substances included five surfactants, two acids, ten alkalis, four oxidizers, six solvents, and one “other” (or nonspecified) (**Table 3-2**). The Draize rabbit eye test classified only one as EPA Category II and only four as EPA Category III (**Table 3-2**).

Table 3-2 Distribution of Product Categories Evaluated in the Alternate AMCP Testing Strategy

Product Category	Number of Products Tested	<i>In Vivo</i> Draize Classification EPA Category I	<i>In Vivo</i> Draize Classification EPA Category II	<i>In Vivo</i> Draize Classification EPA Category III	<i>In Vivo</i> Draize Classification EPA Category IV
Surfactant	5	0	0	2	3
Acid	2	0	0	1	1
Alkali	10	9	1	0	0
Oxidizer	4	3	0	0	1
Solvent	6	2	0	1	3
Other	1	0	0	0	1
Total	28	14	1	4	9

Abbreviations: AMCP = antimicrobial cleaning product; EPA = U.S. Environmental Protection Agency

3.3 Reference Test Method Data

Varied test method protocols were used to generate the *in vivo* reference data for the 228 substances tested in the AMCP testing strategy (**Table 3-3**). Of the 68 substances tested in the BCOP test method, 85% (58/68) were tested using the traditional Draize rabbit eye test protocol (OECD 2002). Another 12% (8/68) were tested in a nontraditional protocol (i.e., application of 30 μ L instead of 100 μ L or application as an aerosol spray). The remaining 3% (2/68) were tested in the LVET. The LVET is a modification of the Draize rabbit eye test that involves application of 10 μ L of the test substance directly to the corneal surface rather than application of 100 μ L of the test substance into the conjunctival sac. All 105 substances tested in the CM test method were tested in the LVET. Of the 55 substances tested in EO test method, 55% (30/55) were tested in the Draize rabbit eye test. Forty-five percent (25/55) were tested in the LVET. None of the 228 substances was tested in both the Draize rabbit eye test and the LVET.

Table 3-3 Distribution of *In Vivo* Reference Data

Test Method	Number of AMCPs Tested	LVET	Traditional Draize ¹	Nontraditional Draize ¹	LVET and Draize
BCOP	68	2	58 ²	8 ³	0
CM	105	105	0	0	0
EO	55	25	30	0	0
Total	228	132	88	8	0

Abbreviations: AMCP = antimicrobial cleaning product; BCOP = bovine corneal opacity and permeability; CM = Cytosensor Microphysiometer; EO = EpiOcular™; LVET = low volume eye test

¹ The traditional Draize protocol involves instillation of 0.1 mL of test substance into the conjunctival sac of a rabbit eye. The nontraditional Draize protocol doses with 0.03 mL of test substance into the conjunctival sac of a rabbit eye.

² The dose volume for one substance was not provided. It was included in the traditional Draize total.

³ One of the substances was evaluated as an aerosol sprayed directly on the cornea for one second.

The alternate AMCP testing strategy is based on the results for the 28 substances that (1) were tested in both the BCOP and the EO test methods (see **Table 3-2**) and (2) were also tested in the Draize rabbit eye test and qualified for assignment of an EPA ocular hazard classification.

3.4 Test Method Accuracy

3.4.1 The Bovine Corneal Opacity and Permeability Test Method

The accuracy of the overall EPA classification was 55% (36/66) (**Table 3-4**) in the validation database of 66 substances tested in both the BCOP test method and the Draize rabbit eye test. Of these, the BCOP test method had only 60% (3/5) accuracy in identifying EPA Category II substances and 50% (6/12) accuracy in identifying EPA Category III substances. The BCOP correctly identified 90% (27/30) of the substances classified as EPA Category I by the Draize rabbit eye test.

Among the three EPA Category I substances that were underpredicted as EPA Category II by the BCOP test method, two were oxidizers and one was a base. It should be noted that the base would be correctly identified if the decision criteria were $IVIS \geq 55.1$, as recommended in the 2006 ICCVAM BRD (ICCVAM 2006a), instead of $IVIS \geq 75$ as proposed in the AMCP BRD (**Appendix C, Annex I**). However, such a change in decision criteria would also result in the overprediction of two EPA Category II substances (one oxidizer and one acid) and one EPA Category III substance (a base) as EPA Category I.

Among the EPA Category II substances that were incorrectly identified by the BCOP test method, one (a base) was underclassified as EPA Category III. One (an oxidizer) was overclassified as EPA Category I. The six EPA Category III substances incorrectly identified by the BCOP test method were overclassified as either EPA Category II (one solvent, one base, and one surfactant) or EPA Category I (two oxidizers and one base). Because the AMCP BRD does not propose BCOP decision criteria for EPA Category IV, the BCOP test method overpredicted 19 substances. The BCOP identified two as EPA Category II (both solvents) and 17 as EPA Category III (8 surfactants, 3 solvents, 3 acids, one base, one oxidizer, and one “other”).

To assess the use of histopathology evaluation, BCOP test method data with histopathology evaluation were compared to BCOP test method data only. Seventeen substances had BCOP test method data with histopathology evaluation. As noted in **Table 3-5**, the overall accuracy for EPA hazard classifications (i.e., EPA Category I, II, III, and IV) was reduced from 41% (7/17) to 35% (6/17) with histopathology evaluation. Using histopathology evaluation with the BCOP test method removed one of the EPA Category I false negatives, but added three EPA Category II false positives.

Table 3-4 Performance of AMCPs in the Bovine Corneal Opacity and Permeability, Cytosensor Microphysiometer, and EpiOcular™ Test Methods Compared to the Draize Rabbit Eye Test or the Low Volume Eye Test as Reported in the AMCP BRD Using the EPA Ocular Hazard Classification System

In Vitro Test Method	In Vivo Test Method	Overall Classification	Performance of the <i>In Vitro</i> Test Method Compared to the <i>In Vivo</i> Reference Test Method Using the EPA Ocular Hazard Classification System										
			Category I			Category II			Category III			Category IV	
			Actual	Under	Over	Actual	Under	Over	Actual	Under	Over	Actual	
BCOP ¹	Draize	55% (36/66)	90% (27/30)	10% (3/30)	20% (1/5)	60% (3/5)	20% (1/5)	50% (6/12)	50% (6/12)	0% (0/12)	100% (19/19)	0% (0/19)	
CM ²	LVET	30% (32/108)	100% (9/9)	0% (0/9)	100% (11/11)	0% (0/11)	0% (0/11)	67% (40/60)	33% (20/60)	0% (0/60)	89% (25/28)	11% (3/28)	
EO ³	Draize	76% (22/29)	100% (15/15)	0% (0/15)	0% (0/1)	0% (0/1)	100% (1/1)	25% (1/4)	75% (3/4)	0% (0/4)	56% (5/9)	44% (4/9)	
EO ⁴	LVET	44% (11/25)	100% (3/3)	0% (0/3)	100% (1/1)	0% (0/1)	0% (0/1)	33% (4/12)	67% (8/12)	0% (0/12)	100% (9/9)	0% (0/9)	

Abbreviations: AMCP = antimicrobial cleaning product; BCOP = bovine corneal opacity and permeability; CM = Cytosensor Microphysiometer; EO = EpiOcular™; EPA = U.S. Environmental Protection Agency; ET₅₀ = estimated time to decrease keratinocyte viability in the EO test method by 50%; IVIS = *in vitro* irritancy score; LVET = low volume eye test; MRD₅₀ = concentration of test substance that decreases the metabolic rate by 50% determined by a plot of the concentration-response curve.

- ¹ Classification of the BCOP data was based on IVIS ≥ 75 = EPA Category I; IVIS ≥ 25 and < 75 = EPA Category II; IVIS < 25 = EPA Category III. The BCOP test method was not proposed to identify EPA Category IV. All BCOP classifications, including high-solvent substances, used a 10-minute exposure time. The database comprised 66 substances tested in both the BCOP test method and the Draize rabbit eye test.
- ² Classification of the CM data was based on MRD₅₀ < 2 mg/mL = EPA Category I; MRD₅₀ ≥ 2mg/mL and < 80 mg/mL = EPA Category III; MRD₅₀ ≥ 80 mg/mL = EPA Category IV. The CM test method was not proposed to identify EPA Category II. The database consisted of 108 substances tested in both the CM test method and in the LVET (105 different substances because three substances were tested twice).
- ³ Classification of the EO data was based on ET₅₀ < 4 min = EPA Category I; ET₅₀ ≥ 4 min and < 70 min = EPA Category III; ET₅₀ ≥ 70 min = EPA Category IV. The EO test method was not proposed to identify EPA Category II. The database consisted of 29 substances tested in both the EO test method and the Draize rabbit eye test that qualified for EPA hazard classification (i.e., one substance producing a Draize score greater than 1 was not evaluated through day 21 as required by EPA).
- ⁴ Classification of the EO data was based on ET₅₀ < 4 min = EPA Category I; ET₅₀ ≥ 4 min and < 70 min = EPA Category III; ET₅₀ ≥ 70 min = EPA Category IV. The EO test method was not proposed to identify Category II. The database consisted of 25 substances tested in both the EO test method and the LVET.

Table 3-5 Comparison of the BCOP Test Method and the BCOP Test Method Using Histopathology Evaluation

EPA	Overall Classification	Draize Test Category I		Draize Test Category II			Draize Test Category III			Draize Test Category IV ¹	
		Actual	Under	Over	Actual	Under	Over	Actual	Under	Over	Actual
BCOP ² only	41% (7/17)	50% (3/6)	50% (3/6)	0% (0/4)	75% (3/4)	25% (1/4)	75% (3/4)	25% (1/4)	0% (0/4)	100% (3/3)	0% (0/3)
BCOP ² with histopathology	35% (6/17)	67% (4/6)	33% (2/6)	75% (3/4)	25% (1/4)	0% (0/4)	75% (3/4)	25% (1/4)	0% (0/4)	100% (3/3)	0% (0/3)

Abbreviations: BCOP = bovine corneal opacity and permeability

¹ The BCOP test method decision criteria do not propose to identify EPA Category IV substances.

² The BCOP test method was based on the use of decision criteria with a cutoff for corrosives/severe irritants of ≥ 75 tested with a 10-minute exposure time.

3.4.2 The Cytosensor Microphysiometer Test Method

The validation database included 108 substances tested in both the CM test method and the LVET (Table 3-4). Accuracy of the overall EPA classification (i.e., EPA Category I, II, III, and IV) was 30% (32/108). It should be noted that the database consisted of 105 unique substances because three substances were tested twice. The CM overclassified the majority of substances classified by the LVET as EPA Category II, III, and IV. Overclassification included 100% (11/11) of the EPA Category II substances, 67% (40/60) of the EPA Category III substances, and 89% (25/28) of the EPA Category IV substances. Among the 25 overclassified EPA Category IV substances, 16% (4/25; all surfactants) were classified by the CM test method as EPA Category I, and 84% (21/25; 6 solvents, 2 bases, and 13 surfactants) were classified by the CM test method as EPA Category III. Because decision criteria for the CM test method are not proposed in the AMCP BRD for EPA Category II, all EPA Category II or III substances that were overclassified by the CM test method were classified as EPA Category I. All but one of the 40 EPA Category III substances (a solvent) that were overclassified by the CM test method were surfactants. All 11 of EPA Category II substances that were overclassified by the CM test method were surfactants. All nine of the EPA Category I substances (all surfactants) were correctly identified. None of the irritant categories (i.e., EPA Category I, II, or III) were underpredicted by the CM test method.

3.4.3 The EpiOcular Test Method

Among the 55 substances tested in the EO test method (Table 3-4), 30 were also tested in the Draize rabbit eye test (29 qualified for EPA hazard classification [i.e., one substance producing a Draize score greater than 1 was not evaluated through day 21 as required by EPA]), and 25 were tested in the LVET. Based on the database of 29 substances tested in both the EO test method and the Draize rabbit eye test, accuracy of the overall EPA classification (i.e., EPA Category I, II, III, and IV) was 76% (22/29). Among the four EPA Category III substances, 75% (3/4) were correctly identified by the EO test method. The one substance incorrectly identified (a base) was overclassified as EPA Category I. Among the nine EPA Category IV substances, 44% (4/9) were correctly identified. Four of the five incorrectly identified substances were overclassified as EPA Category III (two solvents, one acid, and one surfactant). The remaining substance (a surfactant) was overclassified as EPA Category I. The EO test method correctly identified all of the EPA Category I substances (15/15, including 12 bases, two solvents, and one “other”).

The EO test method correctly classified 44% (11/25) of the 25 substances tested in both the EO test method and the LVET (**Table 3-4**). Among the 12 substances classified by the LVET as EPA Category III, the EO test method correctly identified 67% (8/12). The four substances incorrectly identified (two surfactants and two oxidizers) were overclassified as EPA Category I. The EO test method did not correctly identify any of the nine EPA Category IV substances. Forty-four percent (4/9: three surfactants and one solvent) were overclassified as EPA Category III, and 56% (5/9: three oxidizers and two solvents) were overclassified as EPA Category I. The EO test method correctly identified all of the EPA Category I substances (3/3: two oxidizers and one surfactant).

3.4.4 AMCP Testing Strategy

Table 3-4 summarizes the performance of each test method included in the AMCP testing strategy. None of the 228 substances included in the AMCP BRD was tested in all three of the proposed *in vitro* test methods. Therefore, no data are available with which to characterize the actual performance of the AMCP testing strategy that includes all three test methods: the BCOP, CM, and EO.

3.4.5 Alternate AMCP Testing Strategy

Twenty-eight substances with Draize rabbit eye test data were tested in both the BCOP and EO test methods. In the alternate AMCP testing strategy, the BCOP test method is intended to identify only EPA Category I and II substances. The EO test method is intended to identify only EPA Category III and IV substances. As described in **Section 3.1.2**, the alternate AMCP testing strategy could follow one of two approaches. The performance of the alternate AMCP testing strategy was the same (**Table 3-6**) regardless of which approach was used.

The alternate AMCP testing strategy correctly classified 79% (22/28) of the substances. Among these, it correctly identified all of the EPA Category I substances (14/14), all of the EPA Category III substances (4/4), and 44% (4/9) of the EPA Category IV substances. The one EPA Category II substance was underpredicted as EPA Category III. Furthermore, classification of the BCOP data using either the decision criteria in the AMCP BRD (**Appendix C, Annex I**) (IVIS \geq 75 for EPA Category I) or in the 2006 ICCVAM BRD (IVIS \geq 55 for EPA Category I) yielded identical results. All BCOP classifications, including high-solvent substances, used a 10-minute exposure time. When using 3-minute data for high solvents, the overall classification was 74% (17/23). Five high-solvent substances did not have 3-minute data; therefore, they cannot be considered in this analysis. It should be noted that, based on this limited database of 28 substances, the performance of the EO test method alone is the same as that of the alternate AMCP testing strategy.

Table 3-6 Performance of AMCPs Tested in Both the BCOP and EO Test Methods

EPA	Overall Classification	Draize									
		I		II			III		IV		
		Actual	Under	Over	Actual	Under	Over	Actual	Under	Over	Actual
Approach 1	79% (22/28)	100% (14/14)	0% (0/14)	0% (0/1)	0% (0/1)	100% (1/1)	0% (0/4)	100% (4/4)	0% (0/4)	56% (5/9)	44% (4/9)
Approach 2	79% (22/28)	100% (14/14)	0% (0/14)	0% (0/1)	0% (0/1)	100% (1/1)	0% (0/4)	100% (4/4)	0% (0/4)	56% (5/9)	44% (4/9)

Abbreviations: AMCP = antimicrobial cleaning product; BCOP = bovine corneal opacity and permeability; EO = EpiOcular™; EPA = U.S. Environmental Protection Agency.

Approach 1 = Test in the BCOP test method first to classify as either EPA Category I or II and then in the EO test method to identify EPA Category III and IV.

Approach 2 = Test in the EO test method first to classify as either EPA Category III or IV and then in the BCOP test method to identify EPA Category I and II.

3.5 Test Method Reliability

3.5.1 The Bovine Corneal Opacity and Permeability Test Method

Intralaboratory repeatability is determined by comparing within-experiment runs of a test substance. Intralaboratory repeatability for the BCOP test method was quantitatively determined for 67 AMCPs (four substances have repeat tests) as the mean %CV for opacity, permeability, and IVIS (AMCP BRD; **Appendix C, Annex I**). Because a very low IVIS significantly affects %CV, the overall mean %CV calculations did not include substances with an IVIS ≤ 10 (arbitrarily set in the AMCP BRD). The overall mean %CVs for opacity, permeability, and IVIS were 21%, 25%, and 18%, respectively.

These 67 test substances, tested in a total of 75 runs, were also qualitatively evaluated for their concordance using the EPA (EPA 2003) and GHS (UN 2007) ocular hazard classification systems (AMCP BRD Supplement; **Appendix C, Annex II**). For the EPA and GHS classification systems, there was 100% agreement among the corneas in 63 of the 75 runs (84%). There was 67% agreement in 11 of 75 runs (15%) and 60% agreement in one of 75 runs (1%). Of the 12 runs in which the test corneas were not in 100% agreement, seven had reactive chemistries, two were alkalis, two were surfactants, and one was an acid.

Intralaboratory repeatability for the BCOP test method has been quantitatively determined for non-AMCPs predicted as ocular corrosives/severe irritants in the BCOP test method in three studies (16-52 substances) (ICCVAM 2006a). The mean %CV for IVIS ranged from 39% to 71%.

Intralaboratory reproducibility is determined by comparing between-experiment runs of a test substance. For the BCOP test method, intralaboratory reproducibility was quantitatively determined for five AMCPs. For these five substances (2–6 experiments), the mean %CV for IVIS was 20% (see Section 7.3 of the AMCP BRD, **Appendix C, Annex I**).

These test substances were also qualitatively evaluated for their concordance using the EPA (EPA 2003) and GHS (UN 2007) ocular hazard classification systems (see Section 3.2 of the AMCP BRD Supplement, **Appendix C, Annex II**). The five test substances had 100% agreement in the EPA and GHS classification systems.

Intralaboratory reproducibility for the BCOP test method has been quantitatively determined for non-AMCPs predicted as ocular corrosives/severe irritants in the BCOP test method (ICCVAM 2006a). In one study composed of 25 surfactant-based personal care cleaning formulations, the mean %CV for permeability values was 33%. In the second study, the mean %CV for IVIS ranged from 13% to 15% for 16 test substances.

Interlaboratory reproducibility is determined by comparing between-laboratory runs of a test substance. Interlaboratory reproducibility for the BCOP test method could not be determined specifically for the AMCPs presented in the AMCP BRD (**Appendix C, Annex I**) because only one laboratory conducted the testing.

Interlaboratory reproducibility for the BCOP test method has been quantitatively determined for non-AMCPs predicted as ocular corrosives/severe irritants in the BCOP test method (ICCVAM 2006a). In three studies (3–12 laboratories each), the mean %CV for IVIS ranged from 25% to 36%. The study results were also qualitatively evaluated for their concordance using the EPA (EPA 2003), EU (EU 2001), and GHS (UN 2007) ocular hazard classification and labeling systems (ICCVAM 2006a).

3.5.2 The Cytosensor Microphysiometer Test Method

Reliability for the CM test method could not be evaluated specifically for AMCPs due to insufficient data. However, quantitative evaluations of reliability were conducted based on non-AMCPs tested in the CM test method (**Appendix C, Annexes I and II**).

Intralaboratory repeatability for the CM test method was quantitatively evaluated for non-AMCPs in seven studies of one to 35 test substances each (**Appendix C, Annexes I and II**). The mean % coefficient of variation (CV) for MRD₅₀ values for all materials tested, including surfactant and nonsurfactant materials, ranged from 6% to 25%.

Intralaboratory reproducibility was quantitatively determined for non-AMCPs in one laboratory (16 substances) (**Appendix C, Annex I**). The mean %CV for MRD₅₀ values for all materials tested, including surfactant and nonsurfactant materials, was 25%.

Interlaboratory reproducibility for the CM test method was quantitatively determined for non-AMCPs in two studies (2–4 laboratories each) (**Appendix C, Annex I and II**). The mean %CV for MRD₅₀ values for all materials tested, including surfactant and nonsurfactant materials, ranged from 17% to 51%. Nonsurfactant materials had a higher mean %CV in each study.

3.5.3 The EpiOcular Test Method

Intralaboratory repeatability for the EO test method was quantitatively determined specifically for a subset of 15 AMCPs presented in the AMCP BRD (**Appendix C, Annex I**). The mean %CV for ET₅₀ values ranged from 0% to 62%.

To evaluate concordance using the EPA (EPA 2003) and GHS (UN 2007) ocular hazard classification systems (AMCP BRD Supplement, **Appendix C, Annex II**), qualitative analyses were conducted for three AMCPs that were tested more than once at IIVS. There was 100% agreement for all three AMCPs in both classification systems.

Intralaboratory reproducibility for the EO test method was quantitatively determined from repeat testing of a single substance (0.3% Triton X-100). Data were presented as combined data from MatTek Corporation and IIVS (9-year period) and from IIVS only (8-year period). The mean %CV for ET₅₀ values was 21% and 22%, respectively.

Interlaboratory reproducibility for the EO test method cannot be determined specifically for the AMCPs presented in the AMCP BRD (**Appendix C, Annex I**) because only one laboratory conducted the testing. However, interlaboratory reproducibility for the EO test method was quantitatively determined for non-AMCPs in a phased validation study of surfactants and surfactant-containing products. The validation study is summarized in the AMCP BRD (**Appendix C, Annex I**). The mean %CVs ranged from 12% to 18%. However, it should be noted that this evaluation was based on an EO protocol that uses relative percent viability to assign an irritancy classification (irritant or nonirritant). It did not use a calculated ET₅₀ value to predict the EPA ocular hazard category. This protocol is included in the AMCP BRD.

These test substances were also qualitatively evaluated for their concordance using the EPA (EPA 2003) and GHS (UN 2007) ocular hazard classification systems (AMCP BRD Supplement; **Appendix C, Annex II**). Using either the EPA or GHS classification systems in Phase II of the validation study, there was 100% agreement for 14/19 (74%) substances, 75% agreement for 2/19 (11%) substances, and 50% agreement for 3/19 (16%) substances among four laboratories. In Phase III of the validation study using the EPA or GHS ocular hazard classification systems, there was 100% agreement for 51/54 (94%) substances and 0% agreement for 3/54 (6%) substances in two laboratories.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The AMCP testing strategy proposed in the AMCP BRD is a non-animal approach for classifying and labeling AMCPs, as is the alternate AMCP testing strategy.

Bovine eyes used in the BCOP test method are obtained post mortem from animals that are being used for food. The CM test method uses L929 cells, a commercially available mouse cell line. The EO test method uses primary human keratinocytes obtained from human donors during routine surgical procedures.

4.0 ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process incorporates a high level of transparency. This process is designed to provide numerous opportunities for stakeholder involvement, including submitting written public comments and providing oral comments at ICCVAM independent peer review panel meetings and SACATM meetings. **Table 4-1** lists the nine opportunities for public comments during the ICCVAM evaluation of the validation status of alternative ocular safety testing methods and approaches. The number of public comments received is also indicated. Thirty-seven comments were submitted. Comments received in response to or related to *Federal Register* notices (**Appendix E**) are also available on the NICEATM-ICCVAM website.⁵ The following sections, delineated by *Federal Register* notice, briefly discuss the public comments received.

Table 4-1 Opportunities for Public Comments

Opportunities for Public Comments	Date	Number of Public Comments Received
70 FR 13512: Request for Data on Non-Animal Methods and Approaches for Determining Skin and Eye Irritation Potential of Antimicrobial Cleaning Product Formulations; Request for Nominations for an Independent Expert Panel	March 21, 2005	0
72 FR 26396: Request for Data on the Use of Topical Anesthetics and Systemic Analgesics for <i>In Vivo</i> Eye Irritation Testing	May 9, 2007	1
72 FR 31582: Request for Ocular Irritancy Test Data From Human, Rabbit, and <i>In Vitro</i> Studies Using Standardized Testing Methods	June 7, 2007	0
73 FR 18535: Non-Animal Methods and Approach for Evaluating Eye Irritation Potential for Antimicrobial Cleaning Products (AMCPs): Request for Nominations for an Independent Expert Panel and Submission of Relevant Data	April 4, 2008	12
74 FR 14556: Announcement of an Independent Scientific Peer Review Panel on Alternative Ocular Safety Testing Methods; Availability of Draft Background Review Documents (BRD); Request for Comments	March 31, 2009	8
74 FR 19562: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	April 29, 2009	2
Independent Scientific Peer Review Panel Meeting: Alternative Ocular Safety Testing Methods	May 19–21, 2009	12
SACATM Meeting, Arlington Hilton, Arlington, VA	June 25–26, 2009	2
74 FR 33444: Independent Scientific Peer Review Panel Report: Evaluation of the Validation Status of Alternative Ocular Safety Testing Methods and Approaches; Notice of Availability and Request for Public Comments	July 13, 2009	0

⁵ Available at <http://ntp-apps.niehs.nih.gov/iccvambp/searchPubCom.cfm>

**4.1 Public Comments in Response to 70 FR 13512 (March 21, 2005)
Request for Data on Non-Animal Methods and Approaches for Determining
Skin and Eye Irritation Potential of AMCP Formulations; Request for
Nominations for an Independent Expert Panel**

NICEATM requested (1) submission of data that would assist in evaluating the validation status of non-animal methods and approaches used for determining the skin and eye irritation potential of AMCP formulations to meet regulatory hazard classification and labeling purposes and (2) nominations of expert scientists to serve as members of an independent peer review panel.

No data or nominations were received in response to this *Federal Register* notice.

**4.2 Public Comments in Response to 72 FR 26396 (May 9, 2007)
Request for Data on the Use of Topical Anesthetics and Systemic Analgesics for
In Vivo Eye Irritation Testing**

NICEATM requested submission of (1) data and information on the use of topical anesthetics and systemic analgesics for alleviating pain and distress in rabbits during eye irritation testing and (2) information about other procedures and strategies that may reduce or eliminate pain and distress associated with *in vivo* eye irritation methods.

In response to this *Federal Register* notice, NICEATM received one comment. This comment was not relevant to the AMCP testing strategy or the three *in vitro* test methods (i.e., BCOP, CM, and EO) included in the AMCP testing strategy.

**4.3 Public Comments in Response to 72 FR 31582 (June 7, 2007)
Request for Ocular Irritancy Test Data From Human, Rabbit, and *In Vitro*
Studies Using Standardized Test Methods**

NICEATM requested data on substances tested for ocular irritancy in humans, rabbits, and/or *in vitro* to be used to:

- Review the state of the science in regard to the availability of accurate and reliable *in vitro* test methods for assessing the range of potential ocular irritation activity, including whether ocular damage is reversible or not
- Expand NICEATM's high-quality ocular toxicity database. *In vitro* test methods for which data are sought include but are not limited to (1) the bovine corneal opacity and permeability test, (2) the isolated rabbit eye test, (3) the isolated chicken eye test, and (4) the hen's egg test–chorioallantoic membrane.

No data or information was received in response to this *Federal Register* notice.

**4.4 Public Comments in Response to 73 FR 18535 (April 4, 2008)
Non-Animal Methods and Approach for Evaluating Eye Irritation Potential for
Antimicrobial Cleaning Products: Request for Nominations for an Independent
Expert Panel and Submission of Relevant Data**

NICEATM requested the following:

- Nominations of expert scientists to serve as members of an independent peer review panel
- Submission of relevant data and information on AMCPs or related substances obtained from (1) human testing or experience, including reports from accidental exposures, and (2) rabbit testing using the standard eye test or the LVET

- *In vitro* ocular safety test methods such as the bovine corneal opacity and permeability test method, the Cytosensor Microphysiometer test method, and the EpiOcular test method, including data supporting the accuracy and reproducibility of these methods

In response to this *Federal Register* notice, NICEATM received 12 comments, including nominations of 20 potential panelists. The nominees were included in the database of experts from which the Panel was selected. No additional data were received.

4.5 Public Comments in Response to 74 FR 14556 (March 31, 2009) Announcement of an Independent Scientific Peer Review Panel on Alternative Ocular Safety Testing Methods; Availability of Draft Background Review Documents; Request for Comments

NICEATM requested public comments on the draft BRDs, SRDs, and draft ICCVAM test method recommendations that were provided to an independent scientific peer review panel meeting (May 19–21, 2009). These documents summarized the current validation status of several test methods and testing strategies for identifying potential ocular irritants. The test methods and testing strategies included the following:

- A testing strategy that proposes the use of three *in vitro* test methods to assess the eye irritation potential of AMCPs
- Four *in vitro* test methods for identifying moderate (EPA Category II, UN Globally Harmonized System of Classification and Labelling of Chemicals [GHS] Category 2A) and mild (EPA Category III, GHS Category 2B) ocular irritants and substances not classified as ocular irritants (EPA Category IV, GHS Not Classified)
- The *in vivo* LVET
- A proposal for the routine use of topical anesthetics, systemic analgesics, and earlier humane endpoints to avoid and minimize pain and distress during *in vivo* ocular safety testing

NICEATM received 20 comments in response to this *Federal Register* notice. Eight written comments were received before the Panel meeting, and 12 oral comments were provided at the Panel meeting.

Public Responses (written)

Two of the written comments were related to the AMCP testing strategy or one of the three *in vitro* test methods (i.e., BCOP, CM, and EO) included in the AMCP testing strategy.

Comment:

One commenter acknowledged that replacement of the Draize rabbit eye test will require combinations of *in vitro* test methods and welcomed further discussions to develop these approaches, in particular in the context of the recently established International Cooperation on Alternative Test Methods (ICATM).

ICCVAM Response:

ICCVAM is fully committed to ICATM and welcomes any discussions that would promote harmonization of approaches for validation of *in vitro* test methods. ICCVAM is working to identify integrated testing strategies that could be applied to ocular toxicity testing.

Comment:

One commenter provided comments to support the value of the EO test method and outlined a proposal for an improved testing strategy for use of the BCOP and EO test methods for determination of EPA hazard classification of AMCPs. Specifically, the commenter summarized data from the AMCP SRD to indicate that the EO test method can identify EPA Categories I, II, or IV as a stand-

alone test method and that combining the BCOP and EO test methods did not provide any benefit to results obtained with the EO test method alone.

ICCVAM Response:

As noted in **Section 2.3**, ICCVAM recommends that a reference list of AMCPs (for which high-quality Draize rabbit eye test data are available) should be tested in each of the proposed test methods (i.e., BCOP, CM, and EO) to allow more complete evaluation of the usefulness and limitations of an *in vitro* testing strategy. The Panel agreed with the recommendation, having concluded that additional studies should not focus on the use of the EO test method alone. The Panel considered the use of an *in vitro* testing strategy more promising.

Public Responses, Oral

Twelve oral public comments were provided at the Panel meeting (May 19-21, 2009). Seven of these comments were related to the AMCP testing strategy or one of the three *in vitro* test methods (i.e., BCOP, CM, and EO) included in the AMCP testing strategy.

Comment:

One commenter indicated that the performance of the BCOP test method was unlikely to improve based on the lack of reproducibility with the Draize rabbit eye test in the mild and moderate categories. The commenter stated that results from Weil and Scala (1971) show that the extremes (i.e., corrosives/severe irritants and substances not labeled as irritants) are reproducible, but the mild and moderate levels of ocular irritation are highly variable. The commenter referenced the AMCP BRD, which includes an analysis of the impact on the ocular hazard category when the results of a six-rabbit Draize test are randomly sampled for a 3-rabbit test.

ICCVAM Response:

The Draize rabbit eye test (Draize et al. 1944) has a long history of demonstrated protection of public health; therefore, U.S. and international regulatory agencies currently use this test to identify potential ocular hazards. Alternatives are accepted only when they demonstrate the ability to provide equal or better protection than the reference test method. Given the uncertainty of the results associated with the BCOP test method for substances in the mild/moderate irritancy range, the BCOP test method cannot be considered a complete replacement at this time.

Comment:

One commenter stated that damaged eyes are quickly removed and excluded from the BCOP test method and that Gautheron et al. (1992) used both fresh eyes and eyes maintained at 4°C and found no differences in results. The commenter also asked the Panel to reconsider the use of a histopathology evaluation in the BCOP test method.

ICCVAM Response:

The final ICCVAM recommendations state that a histopathological evaluation of the corneal tissue, using standardized procedures, should be included when the BCOP test method is conducted. Such data will allow for development of decision criteria and future assessments on the usefulness of this endpoint for classifying and labeling substances, especially those that may otherwise produce borderline or false negative results.

Comment:

One commenter discussed the “top-down” (i.e., screening for corrosives/severe irritants) and “bottom-up” (i.e., screening for substances not labeled as irritants) approaches using the ICE and BCOP test methods. The commenter stated that ECVAM is developing a paper to recommend the use of these testing strategies for both ICE and BCOP. Substances could be tested in the BCOP or ICE test methods to identify corrosives/severe irritants or substances not labeled as irritants without using an animal test.

ICCVAM Response:

ICCVAM previously recommended the ICE and BCOP test methods for use in a tiered-testing strategy, where positive substances can be classified as ocular corrosives/severe irritants without the need for animal testing (ICCVAM 2006b). Based on the current evaluation of available data and corresponding performance, the original ICCVAM recommendations for the use of the BCOP and ICE test methods to identify substances as ocular corrosives/severe irritants remains unchanged.

Comment:

One commenter questioned the need for performance standards for the CM test method, given that the Panel did not recommend performance standards for the BCOP and ICE test methods.

ICCVAM Response:

The final ICCAM recommendations state that the development of performance standards for the CM test method is not warranted at this time.

Comment:

One commenter indicated that it was appropriate to include EO data that used a different protocol as a measure of test method reproducibility.

ICCVAM Response:

As stated in the AMCP SRD, ICCVAM notes that the reproducibility of the EO test method is based on an EO protocol that uses relative percent viability to assign an irritancy classification (irritant or nonirritant). It does not use a calculated ET_{50} value to predict multiple ocular hazard categories (i.e., EPA Categories I–IV). The latter is the protocol included in the AMCP BRD.

Comment:

One commenter noted that a small change in classification is seen when the BCOP test method decision criterion is changed from 55 to 75. ECVAM considers 55 the best cut-off for their intended purpose.

ICCVAM Response:

ICCVAM notes that using alternative decision criteria to identify ocular corrosives/severe irritants does not improve BCOP test method performance (i.e., $IVIS \geq 75$, proposed in the AMCP BRD, instead of $IVIS \geq 55.1$, per the ICCVAM-recommended BCOP protocol).

Comment:

One commenter responded to the concern about the limited number of AMCPs tested, stating that most industrial-strength cleaners are severe irritants and household cleaners are mostly mild irritants. Very few AMCPs are in the moderate range.

ICCVAM Response:

As outlined in the final AMCP SRD, only 28 AMCPs have been tested in both the BCOP and EO test methods. Of these, Draize rabbit eye test data classified only one as an EPA Category II substance and only four as EPA Category III substances. Therefore, ICCVAM concludes that although the performance of the alternate AMCP testing strategy using the BCOP and EO test methods appears useful for identifying EPA Category I substances using the BCOP test method and EPA Category IV substances using the EO test method, the data are not sufficient to adequately demonstrate that this strategy can identify all four EPA ocular hazard categories.

4.6 Public Comments in Response to 74 FR 19562 (April 29, 2009) Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting (June 25–26, 2009) and requested written and public oral comments on the agenda topics.

NICEATM received four comments. Two written comments were received before the meeting, and two oral comments were provided at the SACATM meeting.

Public Responses (written)

Two written public comments were relevant to the AMCP testing strategy or one of the three *in vitro* test methods (i.e., BCOP, CM, and EO) included in the AMCP testing strategy.

Comment:

One commenter strongly supported the EPA's implementation of a pilot program for ocular safety labeling for AMCPs. The commenter suggested reserving ICCVAM reviews for tests/strategies with multi-agency applicability and adopting a streamlined approach to agency acceptance of methods/strategies deemed scientifically valid in other regions of the world.

ICCVAM Response:

ICCVAM encourages industry to generate more data using alternative *in vitro* test methods. Thus, EPA's pilot program for ocular safety labeling for AMCPs, which encourages industry to generate and submit data using the test methods in the AMCP testing strategy, should produce important data for use in future evaluations.

Comment:

One commenter commented on (1) the reason for the extensive peer review of the AMCP submission and lack of communication with the consortium regarding this evaluation, (2) the review of the validation status of the LVET, and (3) the need to change the scoring system of the LVET to replicate the Draize rabbit eye test results.

ICCVAM Response:

NICEATM requested additional information and communicated issues and data gaps to representatives of the consortium on several occasions before the Panel meeting. Because the LVET is not a validated *in vivo* reference test method, ICCVAM felt it necessary to evaluate the LVET for this purpose before using it as the basis for evaluating the validation status of the CM test method, where *in vitro* results for AMCPs were compared exclusively to LVET data. The Panel stated that the currently utilized Draize scoring system is not considered relevant to the LVET because it uses 10% of the volume. In this regard, the Panel highly recommended development of a more appropriate scoring/classification system for the LVET. However, the Panel recommended using existing data for a statistical analysis to develop such a classification system.

Public Responses, Oral

Two oral public comments were relevant to the AMCP testing strategy or one of the three *in vitro* test methods (i.e., BCOP, CM, and EO) included in the AMCP testing strategy.

Comment:

One commenter indicated that there was no need for the substances to be tested in all three of the *in vitro* test methods in the AMCP testing strategy. The commenter also suggested that test method developers be allowed greater interaction with the Panel.

ICCVAM Response:

Given the limitations of the available database for the three *in vitro* test methods, both ICCVAM and the Panel concluded that the data were not sufficient to support the AMCP testing strategy in terms of the proposed test method usefulness and limitations (i.e., the classification of substances in all four EPA ocular hazard categories). The agenda for the public peer review panel meeting included 10 opportunities for public comment, after which the Panel was asked if it had any questions for the commenter. As explained during the Panel orientation session before the meeting, the Panel Chair has the prerogative to invite additional discussion between the Panel and public commenters/invited experts.

Comment:

One commenter questioned the reason for the extensive peer review of the AMCP submission, including review of the validation status of the LVET and other test methods, when the EPA nominated only the AMCP testing strategy.

ICCVAM Response:

The charge to the Panel was clearly communicated, including the specific charge that the EPA and the consortium requested of NICEATM-ICCVAM. Given that convening a Panel meeting is a very expensive, time-consuming process, NICEATM-ICCVAM wanted to take advantage of this international Panel of experts to review other related test methods. It resulted in an aggressive agenda, but the Panel was very thorough and took the time for a careful, comprehensive review that has benefited the entire effort in this area.

SACATM Response

In general, SACATM was pleased overall with the Panel report. One SACATM member expressed the need for harmonization in the assessment of performance standards. Another SACATM member said the focus should be on the GHS system since it will ultimately be adopted. Another SACATM member expressed concern regarding the availability of the CM instrument.

**4.7 Public Comments in Response to 74 FR 33444 (July 13, 2009)
Independent Scientific Peer Review Panel Report: Evaluation of the Validation
Status of Alternative Ocular Safety Testing Methods and Approaches: Notice of
Availability and Request for Public Comments**

NICEATM requested submission of written public comments on the independent scientific peer review panel report.

No public comments were received.

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Appendix A
ICCVAM Evaluation Timeline

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ICCVAM Evaluation Timeline

June 4, 2004	The U.S. Environmental Protection Agency (EPA) requested that the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) conduct a technical review of the antimicrobial cleaning product (AMCP) testing strategy when finalized.
March 21, 2005	<i>Federal Register</i> Notice (70 FR 13512) – Request for Data on Non-Animal Methods and Approaches for Determining Skin and Eye Irritation Potential of Antimicrobial Cleaning Product Formulations; Request for Nominations for an Independent Expert Panel.
December 27, 2007	Background Review Document titled <i>In Vitro</i> Approach for EPA Toxicity Labeling of AMCPs received from the Institute for In Vitro Sciences, Inc.
April 4, 2008	<i>Federal Register</i> Notice (73 FR 18535) – Non-Animal Methods and Approaches for Evaluating Eye Irritation Potential for Antimicrobial Cleaning Products (AMCPs): Request for Nominations for an Independent Expert Panel and Submission of Relevant Data.
March 31, 2009	<i>Federal Register</i> Notice (74 FR 14556) – Announcement of an Independent Scientific Peer Review Panel Meeting on the Evaluation of the Validation Status of Alternative Ocular Safety Testing Methods and Approaches; Availability of Draft Background Review Documents (BRDs); Request for Comments.
May 19-21, 2009	Independent Scientific Peer Review Panel holds a public meeting, with opportunity for public comments, at the U.S. Consumer Product Safety Commission Headquarters in Bethesda, MD. The Panel was charged with reviewing the current validation status of alternative ocular safety testing methods and strategies, and commenting on the extent to which the information in the draft BRDs and Summary Review Documents (SRDs) supported the draft ICCVAM test method recommendations.
June 25-26, 2009	Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) public meeting, SACATM and public comments on the draft Panel conclusions and recommendations.
July 13, 2009	<i>Federal Register</i> Notice (74 FR 33444) – Independent Scientific Peer Review Panel Report: Evaluation of the Validation Status of Alternative Ocular Safety Testing Methods and Approaches: Notice of Availability and Request for Public Comments.
October 29, 2009	ICCVAM endorses the Test Method Evaluation Report, which includes the final SRD.

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Appendix B

ICCVAM-Recommended Test Method Protocols

B1	ICCVAM-Recommended Bovine Corneal Opacity And Permeability (BCOP) Test Method Protocol	B-3
B2	ICCVAM-Recommended Cytosensor Microphysiometer (CM) Test Method Protocol	B-19
B3	ICCVAM-Recommended EpiOcular™ (EO) Test Method Protocol	B-29

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Appendix B1

ICCVAM-Recommended Bovine Corneal Opacity and Permeability (BCOP) Test Method Protocol

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ICCVAM-Recommended Protocol for Future Studies Using the Bovine Corneal Opacity and Permeability (BCOP) Test Method

PREFACE

This proposed protocol for measuring corneal damage was developed following a comprehensive test method evaluation process conducted by ICCVAM, which included an international independent scientific peer review of the validation status and scientific validity of the BCOP (ICCVAM 2006a,b). It is based primarily on information obtained from 1) the Institute for In Vitro Sciences, Inc. (IIVS), a nonprofit foundation that has performed the BCOP assay since 1997 in a Good Laboratory Practice (GLP)-compliant testing facility; and 2) INVITTOX Protocol 124 (1999), which represents the protocol used for the European Community sponsored prevalidation study of the BCOP assay conducted in 1997-1998. Both of these protocols are based on the BCOP assay methodology first reported by Gautheron et al. (1992). Future studies using the BCOP test method could include further characterization of the usefulness or limitations of the BCOP in a weight-of-evidence approach for regulatory decision-making. Users should be aware that the proposed test method protocol could be revised based on any additional optimization and/or validation studies that are conducted in the future. ICCVAM recommends that test method users consult the NICEATM-ICCVAM website (<http://iccvam.niehs.nih.gov/>) to ensure use of the most current test method protocol.

1.0 Purpose and Applicability

The purpose of this protocol is to describe the procedures used to evaluate the potential ocular corrosivity or severe irritancy of a test substance as measured by its ability to induce opacity and increase permeability in an isolated bovine cornea. Effects are measured by: 1) decreased light transmission through the cornea (opacity); 2) increased passage of sodium fluorescein dye through the cornea (permeability); and 3) evaluation of fixed and sectioned tissue at the light microscopic level, if applicable. The opacity and permeability assessments of the cornea following exposure to a test substance are considered individually and also combined to derive an *In Vitro* Irritancy Score, which is used to classify the irritancy level of the test substance. Histological evaluation of the corneas can be useful for identifying damage in tissue layers that does not produce significant opacity or permeability.

The focus of this protocol is on the use of the BCOP test method for the detection of ocular corrosives and severe irritants, as defined by the U.S. Environmental Protection Agency (EPA; EPA 2003a), European Union (EU; EU 2001), and United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2007). Substances other than ocular corrosives and severe irritants (e.g., substances not labeled as irritants and mild/moderate ocular irritants) have been tested using this protocol; however, the BCOP test method is not currently considered to be adequately validated for these classes of ocular irritancy as defined by EPA (2003a), EU (2001), and GHS (UN 2007).

2.0 Safety and Operating Precautions

All procedures with bovine eyes and bovine corneas should follow the institution's applicable regulations and procedures for handling animal substances, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions are recommended, including the use of laboratory coats, eye protection, and gloves. If available, additional precautions required for specific study substances should be identified in the Material Safety Data Sheet for that substance.

3.0 Materials, Equipment, and Supplies

3.1 Source of Bovine Eyes

Eyes from cattle are obtained from an abattoir located within close proximity of the testing facility. The cattle type (breed not specified) can be cows, heifers, steers, or bulls. Because cattle have a wide range of weights depending on breed, age, and sex, there is no recommended weight for the animal at the time of sacrifice.

Eyes from very old cattle are not recommended because the corneas tend to have a greater horizontal corneal diameter and vertical corneal thickness that could affect assay performance (Doughty et al. 1995; Harbell J, personal communication). Additionally, eyes from calves are not recommended since their corneal thickness and corneal diameter are considerably less than that of eyes from adult cattle.

3.2 Equipment and Supplies

- Corneal holders¹
- Dissection equipment (scissors, scalpels, forceps)
- Electric screwdriver
- Falcon tubes (50 mL)
- Incubator or water bath
- Liquinox (or equivalent)
- Microplate reader or UV/VIS spectrophotometer
- Micropipettors and pipette tips
- Opacitometer
- Petri dishes
- Plastic containers for collection and transport of eyes
- Sample tubes (5 mL, glass) for permeability determination
- Spatula
- Specialized window-locking ring screwdriver
- Standard tissue culture and laboratory equipment
- Sterile deionized water
- Syringes (10 mL) and blunt tip needles (19 Gauge)
- Vacuum pump
- Volumetric flasks
- 96 well plates (polystyrene) or cuvettes of an appropriate size for UV/VIS spectrophotometer

3.3 Chemicals

- Ethanol (200 proof, absolute, anhydrous, ACS/USP grade)
- Imidazole
- Penicillin
- Sodium chloride
- Sodium fluorescein
- Streptomycin

¹ Users should be aware of a proposed corneal holder developed by Ubels et al. (2002). The ICCVAM Test Method Evaluation Report (2006b) recommends, “Studies should be conducted to evaluate the impact of using a corneal holder that maintains normal curvature (e.g., the corneal mounting system designed by Ubels et al. 2002) on accuracy and/or reliability of the BCOP test method.”

3.4 Solutions

Follow the manufacturer's recommendations with regard to storage temperature and shelf life of stock solutions. Prepare assay solutions volumetrically.

- 0.9% (w/v) NaCl in sterile deionized water (saline)
- 1X Hanks' Balanced Salt Solution with Ca^{++} and Mg^{++} (HBSS) containing 100 IU/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin
- Dulbecco's Phosphate Buffered Saline (DPBS)
- Eagle's Minimum Essential Medium without phenol red containing 1% (v/v) Fetal Bovine Serum (complete MEM), warmed to 32°C
- Eagle's Minimum Essential Medium with phenol red containing 1% Fetal Bovine Serum (complete MEM with phenol red, used only for rinsing test substances), warmed to 32°C
- Sodium fluorescein (Na-fluorescein) diluted in DPBS to 4 mg/mL for liquid test articles or 5 mg/mL for solid test articles

4.0 Test Substance Preparation

All test substance solutions should be prepared fresh on the day of use.

4.1 Nonsurfactant Liquid Test Substances

Liquid test substances are usually tested undiluted. However, if prescribed, dilutions of aqueous soluble test substances should be prepared in 0.9% sodium chloride.

4.2 Nonsurfactant Solid Test Substances

Nonsurfactant solid test substances should be prepared as 20% (w/v) solutions or suspensions in 0.9% sodium chloride.

4.3 Surfactants

Solid and concentrated liquid surfactants should be prepared and tested as a 10% (w/v, v/v) dilution or suspension in 0.9% sodium chloride.

4.4 Surfactant Preparations

Surfactant-based preparations (e.g., product formulations) are usually tested neat, or can be diluted in 0.9% sodium chloride, with justification of the selected dilution.

5.0 Controls

5.1 Negative Control

When testing a liquid substance at 100%, a concurrent negative control (e.g., 0.9% sodium chloride) is included to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.2 Solvent/Vehicle Control

When testing a diluted liquid, surfactant, or solid, a concurrent solvent/vehicle control is included to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.3 Positive Control

A known ocular irritant is included as a concurrent positive control in each experiment to verify that an appropriate response is induced. As the BCOP assay is being used to identify corrosive or severe irritants, ideally the positive control should be a reference substance that induces a severe response in

this test method. However, to ensure that variability in the positive control response across time can be assessed, the magnitude of irritant response should not be excessive.

Examples of positive controls for liquid test substances are 10% sodium hydroxide or dimethylformamide. An example of a positive control for solid test substances is 20% (weight to volume) imidazole in 0.9% sodium chloride solution.

5.4 Benchmark Substances (if appropriate)

Benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative irritancy potential of an ocular irritant within a specific range of irritant responses. Appropriate benchmark substances should have the following properties:

- A consistent and reliable source(s)
- Structural and functional similarity to the class of the substance being tested
- Known physical/chemical characteristics
- Supporting data on known effects in the *in vivo* rabbit eye test
- Known potency in the range of the desired response

6.0 Experimental Design

6.1 Collection and Transport Conditions of Bovine Eyes

Bovine eyes are typically obtained from a local cattle abattoir, where the eyes are excised as soon as possible after sacrifice. Care should be taken to avoid damaging the cornea during the enucleation procedure. Eyes are collected in a suitable container in which they are immersed in HBSS containing the antibiotics penicillin (100 IU/mL) and streptomycin (100 µg/mL). The container is maintained on ice at all times throughout collection of the eyes and transportation to the testing facility (NOTE: antibiotics may not be necessary if the eyes are kept below 4°C throughout transport). The eyes are used within five hours of sacrifice.

Under conditions where contamination of the bovine eyes with yeast occurs, immersion of the eyes in HBSS containing fungizone should be evaluated.

6.2 Preparation of Corneas

- a. Carefully examine all eyes macroscopically. Those exhibiting unacceptable defects, such as opacity, scratches, pigmentation, and neovascularization are rejected.
- b. Carefully remove the cornea from each selected eye by making an incision with a scalpel 2 to 3 mm outside the cornea, then by cutting around the cornea with dissection scissors, leaving a rim of sclera to facilitate handling. Carefully peel off the iris and lens, ensuring no fragments of these tissues are remaining on the cornea. Take care to avoid damaging the corneal epithelium and endothelium during dissection.
- c. Store the isolated corneas in a petri dish containing HBSS until they are mounted in holders. Examine the corneas before use, and discard those with defects.
- d. Mount the corneas in holders (one cornea per holder), by placing the endothelial side of the cornea against the O-ring of the posterior chamber. Place the anterior chamber over the cornea and join the chambers together by tightening the chamber screws. Care should be taken not to shift the two chambers to avoid damaging the cornea.
- e. Fill both chambers with fresh complete MEM (about 5 mL), always filling the posterior chamber first to return the cornea to its natural curvature. Care should be taken when

adding or removing liquid from the posterior chamber to avoid the formation of bubbles and to minimize shear forces on the corneal endothelium.

- f. Seal each chamber with plugs provided with the holders.
- g. Incubate the holders in a vertical position at $32 \pm 1^\circ\text{C}$ for at least 60 minutes.
- h. At the end of the initial 1-hour incubation period, examine each cornea for defects, such as tears or wrinkling. Discard corneas with any observed defects.

6.3 Control Cornea Selection and Opacity Reading

- a. After the 1-hour incubation period, remove the medium from both chambers of each holder (anterior chamber first) and replace with fresh complete MEM.
- b. Take and record an initial opacity reading for each cornea, using an opacitometer or equivalent instrument that has been appropriately calibrated according to the manufacturer's specifications. This initial opacity reading will be used to calculate the final opacity value for each cornea. The testing facility should ensure the opacitometer is functioning properly each day it is used.
- c. Calculate the average opacity value for all corneas.
- d. Select a minimum of three corneas with opacity values close to the average value for all corneas as negative (or solvent/vehicle) control corneas.
- e. Corneas that display an initial opacity reading significantly greater (+ 2 standard deviations [SDs]) than the average opacity for all corneas in the batch of eyes collected the day of testing should not be used in the assay.

6.4 Treatment Groups

A minimum of three corneas is treated with each test substance solution or suspension. In addition, three corneas per assay are treated with the positive control and three corneas per assay are treated with the negative control. If a benchmark substance is used the day of testing, three corneas should be treated with the benchmark.

Different treatment methods are used depending on the physical nature and chemical characteristics (liquid or surfactant versus nonsurfactant solid) of the test substance. The controls used depend on which method is used.

6.5 Treatment of Corneas and Opacity Measurements

6.5.1 Closed chamber method for nonviscous to slightly viscous liquid test substances

- a. Record the initial opacity readings and label each chamber with the appropriate control or test substance identification. Just prior to treatment, remove the medium from the anterior chamber through the dosing holes using an appropriate aspiration technique (e.g., blunt needle attached to a vacuum pump).
- b. Add 0.75 mL of the control or test substance to the anterior chamber through the dosing holes using a micropipettor. The dosing holes are then resealed with the chamber plugs.
- c. Rotate the holders such that the corneas are in a horizontal position. The holders should be gently tilted back and forth to ensure a uniform application of the control or test substance over the entire cornea.
- d. Incubate the holders in a horizontal position at $32 \pm 1^\circ\text{C}$ for 10 ± 1 minutes. If other exposures times are used, justification must be provided.

- e. Remove the control or test substance from the anterior chamber through the dosing holes and rinse the epithelium at least three times with approximately 2 to 3 mL of fresh complete MEM with phenol red. Perform one last rinse of the epithelium using fresh complete MEM. If it is not possible to remove all visible signs of the test substance, document the observation in the study notebook. Refill the anterior chamber with fresh complete MEM.
- f. Perform a post-treatment opacity reading for each cornea and record the results. Observe each cornea visually and, if applicable, record pertinent observations (e.g., dissimilar opacity patterns, tissue peeling or residual test article).
- g. Incubate the holders in a vertical (anterior chamber facing forward) position at $32 \pm 1^\circ\text{C}$ for 120 ± 10 minutes. If other post-exposure incubation times are used, justification should be provided.
- h. Record a post-incubation opacity reading for each cornea, which will be used to calculate the final corneal opacity value. Observe each cornea visually and record pertinent observations in the study notebook. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test substance, etc.

6.5.2 Open chamber method for semiviscous and viscous liquid test substances and surfactant preparations

- a. Record the initial opacity readings and label each chamber with the appropriate control or test article identification. Just prior to treatment, remove the medium from the anterior chamber through the dosing holes.
- b. Remove the window-locking ring and glass window from all appropriate anterior chambers and place the holders into a horizontal position (anterior chamber facing up).
- c. Add test substance to each chamber successively at a constant rate of 15 to 30 seconds between each chamber. Apply approximately 0.75 mL of the control or test substance (or enough test substance to completely cover the cornea) directly to the epithelial surface of the cornea using a micropipettor or other appropriate device, such as a spatula. Maintain the holders in a horizontal position (anterior chamber up).
- d. If necessary, to aid in filling the pipette with substances that are viscous, the test article may first be transferred to a syringe. Insert the pipette tip of the positive displacement pipette into the dispensing tip of the syringe, so that the substance can be loaded into the displacement tip under pressure. Simultaneously, depress the syringe plunger as the pipette piston is drawn upwards. If air bubbles appear in the pipette tip, the test article should be expelled and the process repeated until the tip is filled without air bubbles. This method should be used for any substances that cannot be easily drawn into the pipette (e.g., gels, toothpastes, and face creams).
- e. If necessary, immediately upon dosing, slightly tilt the holders to achieve a uniform application of the test article over the entire cornea.
- f. After all of the chambers are dosed, replace the glass windows and window-locking rings.
- g. Incubate the holders in a horizontal position at $32 \pm 1^\circ\text{C}$ for 10 ± 1 minutes. If other exposure incubation times are used, justification should be provided.
- h. Prior to the end of the exposure period, remove the window-locking ring and glass window from each appropriate chamber.

- i. At the completion of the exposure period, successively rinse each cornea in the exposure group according to the intervals that they were dosed. Using a syringe, add fresh complete MEM with phenol red to the inside wall of the anterior chamber creating a “whirlpool or vortex effect”, which causes the test article to be rinsed off the cornea. Take special care not to spray the medium directly onto the cornea. Residual test article that cannot be removed from the cornea by the “whirlpool method” is removed by placing a layer of medium over the cornea (added to the inside wall of the chamber). Spray a gentle stream of medium through the medium layer, directing it towards the residual test article. If after several tries the test article cannot be removed, document this in the study notebook, and proceed to the next step.
- j. Once each cornea is completely rinsed of test article, replace the glass window and window-locking ring. Continue rinsing as stated previously for the “closed chamber method” (see **Section 6.5.1, step e**).
- k. Perform a post-treatment opacity reading for each cornea and record the results. Observe each cornea visually and, if applicable, record pertinent observations (e.g., dissimilar opacity patterns, tissue peeling or residual test article).
- l. Incubate the holders in a vertical (anterior chamber facing forward) position at $32 \pm 1^\circ\text{C}$ for 120 ± 10 minutes. If other post-exposure incubation times are used, justification should be provided.
- m. Record a post-incubation opacity reading for each cornea, which will be used to calculate the final corneal opacity value. Observe each cornea visually and record pertinent observations in the study notebook. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test substance, etc.

6.5.3 Solid and liquid surfactant test substances

Surfactant test substances are administered following one of the previously described procedures, with one exception, which is noted below:

- Surfactant test substances are tested on the cornea as a 10% (w/v) solution or suspension prepared in an appropriate solvent/vehicle (e.g., sterile deionized water).

6.5.4 Solid nonsurfactant test substances

Solid nonsurfactant test substances are administered following one of the previously described procedures, with a few exceptions, which are noted below:

- Solid test substances are tested on the cornea as a 20% (w/v) solution or suspension prepared in an appropriate solvent/vehicle (e.g., sterile deionized water).
- Solid test substances are incubated at $32 \pm 1^\circ\text{C}$ for 240 ± 10 minutes.
- There is no post-treatment incubation period. Thus, immediately following the rinsing process, both chambers are refilled (posterior chamber first) with fresh complete MEM, and the post-treatment opacity readings are taken. During the post-treatment opacity reading, visual observations are performed for each cornea and, if necessary, are recorded in the workbook. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test article, etc. Immediately following these opacity readings and visual observations, the permeability experiment is performed.

6.6 Application of Sodium Fluorescein

Following the final opacity measurement, permeability of the cornea to Na-fluorescein is evaluated. The Na-fluorescein solution is applied to the cornea by one of two methods, depending on the nature of the test substance:

- a. Liquid and surfactant test substances and surfactant preparations: Remove the medium from both chambers (anterior chamber first). Fill the posterior chamber with fresh complete MEM, and add 1 mL of a 4 mg/mL Na-fluorescein solution to the anterior chamber using a micropipettor. Reseal the dosing holes in the top of both chambers with the chamber plugs.
- b. Solid nonsurfactant test substances: Remove the medium from the anterior chamber only and replace with 1 mL of a 5 mg/mL Na-fluorescein solution. Reseal the dosing holes in the top of both chambers with the chamber plugs.

6.7 Permeability Determinations

- a. After adding the Na-fluorescein to the anterior chamber and sealing the chambers, rotate the holders into a horizontal position with the anterior chamber facing up. Tilt the holders slightly, if necessary, to achieve a uniform application of the Na-fluorescein over the entire cornea. Incubate the holders in a horizontal position for 90 ± 5 minutes at $32 \pm 1^\circ\text{C}$.
- b. After the 90-minute incubation period, remove the medium in the posterior chamber of each holder and place into sample tubes pre-labeled according to holder number. It is important to remove most of the medium from the posterior chamber and mix it in the tube so that a representative sample can be obtained for the OD₄₉₀ determination.
- c. After completing the Na-fluorescein penetration steps, the corneas should be fixed in an appropriate fixative (e.g., 10% neutral buffered formalin) at room temperature for at least 24 hours, so that the tissues are available if histology is necessary or requested at a later time. It is important that the corneas not be allowed to dry between transfer from the holders and fixation (submersion in the fixative).
- d. If using a microplate reader to measure optical density, transfer 360 μL of the medium from each sample tube into its designated well on a 96-well plate. The standard plate map provides two wells for each cornea. The first well receives an undiluted sample from each cornea tested. When all of the media samples have been transferred onto the plate, measure and record their OD₄₉₀. Any OD₄₉₀ value (of a control or test substance sample) that is 1.500 or greater must be diluted to bring the OD₄₉₀ into the acceptable range. A dilution of 1:5 is generally sufficient but higher dilutions may be required. Prepare the dilution from the original sample of medium and transfer 360 μL into the second well designated for that cornea. Reread the plate and record the data from both the undiluted and diluted OD₄₉₀ values. Use the values from this second reading in all calculations. The OD₄₉₀ values of less than 1.500 will be used in the permeability calculation.
- e. Note: The linear range of absorbance of different microplate readers can vary. Thus, each laboratory must determine the upper limit of absorbance (in the linear range) for the microplate reader used in its facility.
- f. If using a UV/VIS spectrophotometer to measure optical density, adjust the spectrophotometer to read at OD₄₉₀, and zero the spectrophotometer on a sample of complete MEM. Prior to reading samples from the BCOP assay, prepare and read two quality control samples of Na-fluorescein solution to ensure the Na-fluorescein calibration curve (see note below) conducted for the spectrophotometer is still acceptable.

If the average of the quality control samples does not fall within the accepted range of the Na-fluorescein calibration curve, then prepare a Na-fluorescein calibration curve prior to running samples from the BCOP assay. If the average of the quality control samples falls within the accepted range of the calibration curve, then proceed to read samples from the BCOP assay. Transfer an aliquot of the mixed medium from the posterior chamber of the BCOP holder into a cuvette, then take and record an absorbance reading using the spectrophotometer. Any solutions giving an OD₄₉₀ beyond the linear range of the spectrophotometer must be diluted in complete MEM, and another reading taken, repeating these steps until the OD₄₉₀ is within the linear range of the spectrophotometer. Repeat these procedures for each sample from the BCOP assay, rinsing the cuvette(s) thoroughly between each sample, until all samples have been read and results recorded.

Note: If conducting this assay for the first time, a calibration curve for the spectrophotometer must be performed, using a series of dilutions of Na-fluorescein solution in complete MEM. A calibration curve should be prepared and used to determine the linear range of the spectrophotometer and thus determine the upper limit of absorbance.

6.8 Histopathology

A histopathological evaluation of the corneal tissue might be useful when the standard BCOP endpoints (i.e., corneal opacity and permeability) produce borderline results. A standardized scoring scheme using the formal language of pathology to describe any effects should be used.

6.9 Maintenance of the Corneal Holders

Following completion of the assay, clean the disassembled parts of each holder as follows:

- a. Soak the posterior and anterior chambers in a solution of warm tap water and a dime-size or greater amount of Liquinox (or equivalent).
- b. Soak the chamber plugs, O-rings, and handle screws in 70% ethanol. Rinse the chamber plugs, O-rings, and handle screws thoroughly in hot tap water, and air dry prior to reassembling the chambers.
- c. Clean the interior and exterior surfaces of each pre-soaked posterior and anterior chamber by using a scrubbing sponge. Rinse each posterior and anterior chamber thoroughly in warm tap water and air dry prior to reassembling the chambers.
- d. Match up each numbered posterior chamber with its corresponding anterior chamber; insert an O-ring into the appropriate place; attach a chamber handle screw to the anterior chamber; and finally insert the chamber screws into the anterior chamber.

7.0 Evaluation of Test Results

Results from the two test method endpoints, opacity and permeability, should be combined in an empirically derived formula that generates an *In Vitro* Irritancy Score for each test substance.

7.1 Opacity

- a. Calculate the change in opacity for each individual cornea (including the negative control) by subtracting the initial opacity reading from the final post-treatment opacity reading. Then calculate the average change in opacity for the negative control corneas.
- b. Calculate a corrected opacity value for each treated cornea, positive control, and solvent/vehicle control (if applicable) by subtracting the average change in opacity of the negative control corneas from the change in opacity of each treated, positive control, or solvent/vehicle control cornea.

- c. Calculate the mean opacity value of each treatment group by averaging the corrected opacity values of the treated corneas for each treatment group.

7.2 Permeability

Microplate Reader Method

- a. Calculate the mean OD₄₉₀ for the blank wells (plate blanks). Subtract the mean blank OD₄₉₀ from the raw OD₄₉₀ of each well (blank corrected OD₄₉₀).
- b. If a dilution has been performed, correct the OD₄₉₀ for the plate blank before the dilution factor is applied to the reading. Multiply each blank corrected OD₄₉₀ by the dilution factor (e.g., a factor of 5 for a 1:5 dilution).
- c. Calculate the final corrected OD₄₉₀ value for each cornea by subtracting the mean OD₄₉₀ value for the negative control corneas from the OD₄₉₀ value of each treated cornea.
- d. **Final Corrected OD₄₉₀** = (raw OD₄₉₀ – mean blank OD₄₉₀) – mean blank corrected negative control OD₄₉₀
- e. Calculate the mean OD₄₉₀ value for each treatment group by averaging the final corrected OD₄₉₀ values of the treated corneas for a particular treatment group.

UV/VIS Spectrophotometer Method

- a. Calculate the corrected OD₄₉₀ value of each treated, positive control, or solvent/vehicle control cornea by subtracting the average value of the negative control corneas from the original OD₄₉₀ value for each cornea.

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

- b. Calculate the mean OD₄₉₀ value for each treatment group by averaging the final corrected OD₄₉₀ values of the treated corneas for a particular treatment group.

7.3 In Vitro Irritancy Score

Use the mean opacity and mean permeability values (OD₄₉₀) for each treatment group to calculate an *in vitro* score for each treatment group:

$$\text{In Vitro Irritancy Score} = \text{mean opacity value} + (15 \times \text{mean OD}_{490} \text{ value})$$

Additionally, the opacity and permeability values should be evaluated independently to determine whether a test substance induced irritation through only one of the two endpoints.

8.0 Criteria for an Acceptable Test

A test is acceptable if the positive control gives an *In Vitro* Irritancy Score that falls within two SDs of the current historical mean, which is to be updated at least every three months. In the BCOP, 100% ethanol induces a moderate to severe response (*in vitro* score = 39.9 -65.4 at IIVS [n = 632]; mean = 52.7, standard deviation [SD] = 6.4), while 20% (w/v) imidazole induces a severe response (*in vitro* score = 69.7 -136.2 at IIVS [n=125]; mean = 103, SD = 16.6). The negative or solvent/vehicle control responses should result in opacity and permeability values that are less than the established upper limits for background opacity and permeability values for bovine corneas treated with the respective negative or solvent/vehicle control.

9.0 Data Interpretation

The following classification system was established by Sina et al. (1995) based on studies with pharmaceutical intermediates exposed for 10 minutes (liquids) or 4 hours (solids).

In Vitro Score: 55.1 and above = severe irritant

While this classification system provides a good initial guide to interpretation of these *in vitro* data, these specific ranges may not be applicable to all classes of substances. For example, the Sina et al. (1995) scoring scale is not appropriate for anionic and nonionic surfactants since they produce appreciable permeability while inducing little direct opacity.

For these and other substances that produce significant permeability with minimal opacity, it is recommended that permeability values > 0.600 be considered severe. Benchmark substances are recommended for assaying the responses of test substances of different product or chemical classes. Histological evaluation of the corneas may be instrumental in identifying additional changes (e.g., peroxide-induced stromal damage).

10.0 Study Report

The test report should include the following information, if relevant to the conduct of the study:

Test and Control Substances

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known
- The CAS Registry Number (RN), if known
- Purity and composition of the substance or preparation (in percentage[s] by weight), to the extent this information is available
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study
- Treatment of the test/control substances prior to testing, if applicable (e.g., warming, grinding)
- Stability, if known

Information Concerning the Sponsor and the Test Facility

- Name and address of the sponsor, test facility, and study director
- Identification of the source of the eyes (i.e., the facility from which they were collected)
- Storage and transport conditions of eyes (e.g., date and time of eye collection, time interval prior to initiating testing, transport media and temperature conditions, any antibiotics used)
- If available, specific characteristics of the animals from which the eyes were collected (e.g., age, sex, strain, weight of the donor animal)

Justification of the Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time (e.g., periodic testing of proficiency substances, use of historical negative and positive control data)

Criteria for an Acceptable Test

- Acceptable concurrent positive and negative control ranges based on historical data
- If applicable, acceptable concurrent benchmark control ranges based on historical data

Test Conditions

- Description of test system used
- Type of corneal holder used
- Calibration information for devices used for measuring opacity and permeability (e.g., opacitometer and spectrophotometer)
- Information on the bovine corneas used, including statements regarding their quality
- Details of test procedure used
- Test substance concentration(s) used
- Description of any modifications of the test procedure
- Reference to historical data of the model (e.g., negative and positive controls, proficiency substances, benchmark substances)
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples (e.g., opacity and OD_{.490} values and calculated *in vitro* irritancy score for the test substance and the positive, negative, and benchmark controls [if included], reported in tabular form, including data from replicate repeat experiments as appropriate, and means \pm the standard deviation for each experiment)
- Description of other effects observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement indicates all inspections made during the study, and the dates any results were reported to the study director. This statement also serves to confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003b, 2003c; FDA 2003) should be followed.

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Appendix B2

ICCVAM-Recommended Cytosensor Microphysiometer (CM) Test Method Protocol

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ICCVAM-Recommended Protocol for Future Studies Using the Cytosensor Microphysiometer (CM) Test Method

PREFACE

This proposed protocol for ocular toxicity is based primarily on information obtained in INVITTOX Protocol 102 derived from the standard operation procedure used in the Home Office UK/EEC Validation Study for Alternatives to the Draize Test. The information contained within INVITTOX 102 was modified based upon the COLIPA protocol (Brantom et al., 1997; Harbell et al., 1999). Future studies using the CM test method could include further characterization of the usefulness and limitations of the CM test method in a weight-of-evidence approach for regulatory decision-making. Users should be aware that the proposed test method protocol could be revised based on any additional optimization and/or validation studies that are conducted in the future. ICCVAM recommends that test method users consult the NICEATM–ICCVAM website (<http://iccvam.niehs.nih.gov/>) to ensure use of the most current test method protocol.

1.0 Purpose and Applicability

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

The focus of this protocol is on the use of the CM test method for the detection of ocular corrosives and severe irritants and substances not labeled as irritants as defined by the U.S. Environmental Protection Agency (EPA; EPA, 2003a), the European Union (EU; EU, 2001), and United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2007). Mild/moderate ocular irritants have been tested using this protocol; however, the CM test method is not currently considered to be adequately validated for these classes of ocular irritancy as defined by EPA (2003a), EU (2001), and GHS (UN 2007).

2.0 Safety and Operating Precautions

All procedures with L929 cells should follow the institution's applicable regulations and procedures for handling human or animal substances, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions are recommended, including the use of laboratory coats, eye protection, and gloves. If available, additional precautions required for specific study substances should be identified in the Material Safety Data Sheet for that substance.

3.0 Materials, Equipment, and Supplies

3.1 Equipment and Supplies

- Aspirator
- Balance
- Beakers, disposable
- Capsules, eight with L-929 cells grown to be <80% confluent at time of use (confluent monolayer could interfere with accurate CM readings) in DMEM. To prepare these, load $5-6 \times 10^5$ cells about 18 hr prior to use and incubate in complete DMEM with 1% calf serum under standard culture conditions.

- Cell culture equipment for preparation of cells
- Cytosensor System with eight sterilized chambers, set up in the injection loop mode - Molecular Devices Corporation, Menlo Park, California, USA
- Cytosoft and the following Cytosoft protocols for toxicity testing:
 - Tox Maintenance (ii) Routine Tox 003 (4x2) (both supplied by MDC)
 - A statistics program capable of MRD₅₀
- Pipettors, rack, etc., for preparation of dilutions
- Refrigerator
- Statistical program for calculation of MRD₅₀
- Tubes, 15 ml, for preparation of dilutions (4 dilutions per test sample).
- Tube racks
- Syringes, 4 x 5 ml and a 30 ml
- Water bath

3.2 Media and Reagents

- Assay Medium: DMEM complete with 1% Fetal Bovine Serum, 5.0 µg/ml gentamicin, 2.0 mM L-glutamine, and 1.0 mM sodium pyruvate
- Growth Medium: Dulbecco's modified Eagle's medium (DMEM) (1mg/ml glucose) complete with 10% Fetal Bovine Serum, 2.0 mM L-glutamine, and 1.0 mM sodium pyruvate
- Positive Control: sodium lauryl sulfate (SLS) 10% in water (stock)
- Treatment medium: Serum-free, Sodium Bicarbonate-free, DMEM with 5.0 µg/ml gentamicin, 2.0 mM L-glutamine, and additional NaCl for consistent osmolarity (MDMEM). 11.1 ml of 4 M NaCl is required per liter
- Trypsin, 0.05% in Ca⁺² and Mg⁺²-free Hank's Balanced Salt Solution

4.0 Test Substance Preparation

- The test article will be dissolved in MDMEM. It is essential that the test material be in a single-phase solution in the highest dose used (300 mg/mL) to prepare the subsequent dilutions. If the substance cannot form a single phase solution/suspension at a concentration of 33.3 mg/mL, the test sample cannot be tested by the CM using standard techniques
- The stability of the test article under the actual experimental conditions will not be determined by the testing laboratory

5.0 Controls

5.1 Negative Control

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

5.2 Solvent/Vehicle Control

Untreated controls are recommended when solvents/vehicles other than 0.9% sodium chloride or distilled water are used to dissolve test substances, in order to demonstrate that the solvent/vehicle is not interfering with the test system.

5.3 Positive Control

When the 8-channel Cytosensor is used, a positive control assay will be performed with each definitive trial of the assay. When the 4-channel machine is used, a concurrent positive control trial will be performed with at least one of the definitive trials for each test material. The positive control substance is SLS prepared from a 10% stock in water.

5.4 Benchmark Substances (if appropriate)

Benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative irritancy potential of an ocular irritant within a specific range of irritant responses. Appropriate benchmark substances should have the following properties:

- A consistent and reliable source(s)
- Structural and functional similarity to the class of the substance being tested
- Known physical/chemical characteristics
- Supporting data on known effects in the *in vivo* rabbit eye test
- Known potency in the range of the desired response

6.0 Experimental Design

6.1 Filling the Workstations with Medium

Put 8 x 50 ml tubes, each having at least 20 ml of MDMEM on the Cytosensor and fill the injection loops with MDMEM, using a 30 ml syringe. Using the "Front Panel" controls, set the flow rate to 90-100% to fill the lines, and then set the flow rate back to idle (5%).

6.2 Checking Out the Equipment

Empty Sterilant from the sensor chambers, wash them by repeated filling with, and aspiration of, distilled water, and then add about 2 ml of low-buffer DMEM to each chamber. Put them on the Cytosensor. Set flow rate to High (90-100% of max) and clear obvious bubbles. Run Cytosoft default protocol ("New") to see that system sets up and the background rate in the absence of cells settles within 10 minutes to between +5 and -5 microvolts/sec. This gives the opportunity to attend to any equipment problems before starting to use cells.

6.3 Checking Out the Cells

Exit "New" protocol and set flow rate to Normal (approx. 50%) using "Front Panel" controls. To at least 8 cell-containing cell capsules in a culture tray containing Low-Buffered DMEM, add spacers and inserts as described in the Manual. Move the tray to the Cytosensor and use forceps to transfer the completed capsules to the sensor chambers, lifting the gantries and raising the plungers one set at a time. When all the capsules are in place, set the flow rate to High and clear obvious bubbles again.

6.4 Cell Culture Maintenance and Preparation of the Capsule Cups

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

Flasks of L929 cells to be passaged or seeded are selected at or near confluency. The size of flasks used will depend on the number of cells needed. The Growth Medium is decanted and the cell sheet washed twice with approximately 10 mL of PBS for each 75cm^2 of growth surface. The cells are trypsinized with approximately 3 mL of 0.05% 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, which contains approximately 5mL of Seeding Medium for each 75cm² 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 10⁵ cells per each capsule cup (0.5 mL of a 1.2 x 10⁶ cell suspension) with 1.5 mL of Seeding Medium added to each outside well. The plate will be incubated at 37 ± 1°C and 5 ± 1% CO₂ 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% CO₂ in air.

6.5 Dose Range Finding Assay

A dose range finding assay will be performed to establish an appropriate test article dose range for the definitive CM 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. 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 MRD₅₀ calculation. Each exposure cycle will take 20 minutes.

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

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

The exposure cycle will repeat with increasing test article concentrations until either the highest test article concentration is reached or until the MRD₅₀ 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 MRD₅₀ 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 MRD₅₀. 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 CM assay, the maximum dose will generally be 270 mg/mL, or less based on its solubility/workability.

6.6 Definitive Assay

The definitive assay will be performed in the same manner as the dose range finding assay, with the exception that if the MRD₅₀ 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 MRD₅₀ 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 MRD₅₀ could be determined. The results from additional definitive assays may also be incorporated into the calculation of the final MRD₅₀.

7.0 Evaluation of Test Results

The acidification rates that occur after exposure to each test article concentration are calculated by the CM software (Cytosoft) and compared to the mean acidification rate (base acidification rate) of the same cells prior to exposure to a test material. The percent of control acidification rate will be determined by comparing the dose response acidification rate to the base acidification rate. The dose response curve will be plotted with the percent of control acidification rates on the ordinate and the test article concentration on the abscissa. The concentration of the test material that results in a fifty percent reduction in acidification is interpolated from the curve and referred to as the MRD₅₀. These calculations can be performed using the Excel spreadsheet program provided for this study.

8.0 Criteria for an Acceptable Test

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

9.0 Data Interpretation

Interpretation of MRD₅₀ values is done according to the decision criteria provided in Background Review Document: Existing Methods for Eye Irritation Testing: Silicon Microphysiometer and Cytosensor Microphysiometer (ECVAM 2008), as follows:

For the EU system (EU 2001) the proposed PM is:

	MRD ₅₀
R41	<2 mg/mL
R36	<10 mg/mL; >2 mg/mL
Not classified	>10 mg/mL

For the GHS system (UN 2007) the proposed PM is:

	MRD ₅₀
1	<2 mg/mL
2A or 2B	<10 mg/mL; >2 mg/mL
No Label	>10 mg/mL

For the EPA system (EPA 2003a) the proposed PM is:

	MRD ₅₀
I	<2 mg/mL
III	<80 mg/mL; >2 mg/mL
Not classified	>80 mg/mL

10.0 Study Report

The test report should include the following information, if relevant to the conduct of the study:

Test and Control Substances

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known
- The CAS Registry Number (RN), if known
- Purity and composition of the substance or preparation (in percentage(s) by weight), to the extent this information is available
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study
- Treatment of the test/control substances prior to testing, if applicable (e.g., warming, grinding)
- Stability, if known

Information Concerning the Sponsor and the Test Facility

- Name and address of the sponsor

- Name and address of the test facility
- Name and address of the Study Director

Justification of the Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time (e.g., periodic testing of proficiency substances, use of historical negative and positive control data)

Criteria for an Acceptable Test

- Acceptable concurrent negative control ranges based on historical data
- Acceptable concurrent positive control ranges based on historical data
- If applicable, acceptable concurrent benchmark control ranges based on historical data

Test Conditions

- Description of test system used
- Calibration information for measuring device used
- Details of test procedure used
- Test concentration(s) used
- Description of any modifications of the test procedure
- Reference to historical data of the model (e.g., negative and positive controls, proficiency substances, benchmark substances)
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

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Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement indicates all inspections made during the study, and the dates any results were reported to the Study Director. This statement also serves to confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003b, 2003c; FDA 2003) should be followed.

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Appendix B3

ICCVAM-Recommended EpiOcular™ (EO) Test Method Protocol

ICCVAM recommends this EpiOcular™ test method protocol for nonregulatory, validation, or optimization studies to facilitate collection of consistent data and expand the available database. Exceptions and/or changes to the test method protocol should be accompanied by a scientific rationale.

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ICCVAM-Recommended Protocol for Future Studies Using the EpiOcular™ (EO) Test Method

PREFACE

This proposed protocol for ocular toxicity is based primarily on information obtained from the Institute for In Vitro Sciences, Inc. (IIVS). Future studies using the EO test method could include further characterization of the usefulness and limitations of the EO test method in a weight-of-evidence approach for regulatory decision-making. Users should be aware that the proposed test method protocol could be revised based on any additional optimization and/or validation studies that are conducted in the future. ICCVAM recommends that test method users consult the NICEATM-ICCVAM website (<http://iccvam.niehs.nih.gov>) to ensure use of the most current test method protocol.

1.0 Purpose and Applicability

The purpose of this protocol is to evaluate the potential ocular irritation of the test substance by measuring 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye conversion by the EO human tissue construct, a proprietary three-dimensional epithelial construct available from MatTek Corporation, following topical exposure to the test substance.

The focus of this protocol is on the use of the EO test method for the detection of substances not labeled as irritants, as defined by the U.S. Environmental Protection Agency (EPA; EPA, 2003a), European Union (EU; EU, 2001), and United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2007). However, the EO test method is not currently considered to be adequately validated for classification of ocular irritancy as defined by EPA (2003a), EU (2001), and GHS (UN 2007).

ICCVAM recommends this EO test method protocol for nonregulatory, validation, or optimization studies to facilitate collection of consistent data and expand the available database. Exceptions and/or changes to the test method protocol should be accompanied by a scientific rationale.

2.0 Safety and Operating Precautions

All procedures with the EO test method should follow the institution's applicable regulations and procedures for handling human or animal substances, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions are recommended, including the use of laboratory coats, eye protection, and gloves. If available, additional precautions required for specific study substances should be identified in the Material Safety Data Sheet for that substance.

3.0 Materials, Equipment, and Supplies

3.1 Assay Medium and Reagents

- Assay medium is provided with the Standard EpiOcular™ kit (OCL-200). The base medium is Dulbecco's Modified Eagle's Medium (DMEM) containing epidermal growth factor, insulin, hydrocortisone, and other proprietary stimulators of non-keratinizing epithelial differentiation, gentamycin (5 µg/mL), phenol red, and amphotericin B (0.25 µg/mL). Alternative kits with various medium components removed (e.g., antibiotic-free, phenol red-free, antifungal-free) are also available.
- Dulbecco's Phosphate Buffered Saline, Ca²⁺- and Mg²⁺-Free (Ca²⁺Mg²⁺-Free-DPBS)
- Isopropanol, for extraction

- MTT Addition Medium is DMEM containing 2mM L-glutamine by Quality Biological (or equivalent)
- Sterile deionized water by Quality Biological (or equivalent)

3.2 EpiOcular™ Three-Dimensional Epithelial Tissue Construct

- Standard EpiOcular™ kit (OCL-200) is available for purchase from MatTek Corporation, Ashland, MA (<http://www.mattek.com>). Each kit consists of 24 tissues, each tissue 9 mm in diameter. Half kit (i.e., 12 tissues) and six tissue kits are also available. The tissues are screened using PCR and negative for HIV, hepatitis B, and hepatitis C. The tissues are shipped at 4°C on medium-supplemented agarose gels.
- The use of EO tissues offers features appropriate for a model for ocular irritation. First, the model is composed of stratified human keratinocytes in a three-dimensional structure. Secondly, test materials can be applied topically to the model so that water-insoluble materials may be tested.

3.3 Chemicals

- Isopropanol, reagent-grade

3.4 Solutions

Follow the manufacturer's recommendations with regard to storage temperature and shelf life of stock solutions. Prepare assay solutions volumetrically.

- Triton® X-100, 0.3%

4.0 Test Substance Preparation

Test articles will generally be tested neat. End use concentrations or other forms may be used. 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 (e.g., gels, toothpastes, mascaras, and face creams) and solid test articles that are creamed like lipsticks and antiperspirants/deodorant sticks. Dry powders will be ground with a mortar and pestle and passed through a #40 copper sieve, if needed. Materials that are too viscous to spread over the tissue will first be spread onto the flat end of a dosing device. When the test article must first be applied to a dosing device, approximately 30 µL or 30 mg of material will be applied to the dosing device so as to cover the dosing surface. The sample should be spread to form a relatively smooth even layer on the surface of the dosing device to maximize uniform tissue contact. Solids such as lipsticks or antiperspirant/deodorant sticks can be pre-softened by creaming a portion in a weigh boat. The softened portion can be transferred to a syringe affixed with a three way stopcock attached to a second syringe. The sample is pushed from syringe to syringe until it is of a consistency, which can be pipetted. The exact exposure conditions used for other test article forms will be determined.

Controls

5.1 Negative Control

A negative control (e.g., sterile deionized water or other solvent as appropriate) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.2 Positive Control

A known ocular irritant is included in each experiment to verify that an appropriate response is induced. For the EO test method, the positive control is 0.3% Triton[®] X-100. The selection of positive control test substances should be based on the availability of high quality *in vivo* data.

5.3 Benchmark Substances (if appropriate)

Benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative irritancy potential of an ocular irritant within a specific range of irritant responses. Appropriate benchmark substances should have the following properties:

- A consistent and reliable source(s)
- Structural and functional similarity to the class of the substance being tested
- Known physical/chemical characteristics
- Supporting data on known effects in the *in vivo* rabbit eye test
- Known potency in the range of the desired response

6.0 Experimental Design

6.1 Test System

The EO human cell construct described in **Section 3.2** is used. According to the manufacturer, the EO tissue construct is derived from normal, human epidermal keratinocytes derived from the neonatal foreskin of a single donor and cultured using serum-free media to form a stratified squamous epithelium similar to that found in the cornea. The tissue construct consists of highly organized basal cells that progressively flatten out, as the apical surface of the tissue is approached, analogous to the normal *in vivo* corneal epithelium. EO is mitotically and metabolically active and releases many of the pro-inflammatory agents (cytokines) known to be important in ocular irritation and inflammation. Substances are applied topically, which permits the use of solid or semi-solid materials (e.g., gels) in addition to water-soluble materials.

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.

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

One of two exposure time ranges may be used. The standard exposure time range extends up to four hours and is used for most substances to be tested. For extremely mild substances, such as those that might be applied around or in the eyes, a long exposure assay might be used. For the long exposure study, exposure times of up to 24 hours could be used. In general, the standard exposure range will be used, unless the available information (e.g., test substance chemical class or physicochemical properties necessitates an alternative exposure time range).

6.2 Collection and Transport Conditions of EO Tissues

Including time in transit, tissues may be stored at 4°C for up to four days prior to use. However, extended storage periods are not recommended unless absolutely necessary. In addition, the best

reproducibility will be obtained if tissues are used consistently on the same day (e.g., Tuesday afternoon or following overnight storage at 4°C on Wednesday morning).

6.3 Administration of Test Substances

Test substances will generally be tested neat. End-use concentrations or other forms may be used as needed. One hundred μ 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 (i.e., topical administration). Powders will be placed directly onto the culture at approximately 30 mg/culture. For viscous substances applied with the aid of a dosing device, the dosing device is placed into the Millicell[®] to bring the test substance in contact with the tissue. All exposure conditions must be documented.

6.4 pH Determination

The pH of the neat liquid test substance (and/or dosing solution as appropriate) will be determined, if possible. The pH will be determined using pH paper (e.g., 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 unit.

6.5 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 substance 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 substance or positive control exposure time (whichever is longer, up to 240 minutes). On occasion, the second negative control exposure time may be selected to fit the longest test substance exposure time of a test substance run concurrently, but from an independent study. For the long exposure assay (exposures of greater than 240 minutes), multiple negative control exposure times may be selected to fit the range of test substance exposure times. A single negative control exposure-time may be used if all exposure times are one hour and less. Additional negative control exposure times may be selected if needed. 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.

6.6 Assessment of Direct Reduction of MTT by the Test Substance

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

6.7 Receipt of the EpiOcular[™] Tissue

Upon receipt of the EO 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, EO assay medium will be warmed to approximately 37°C. Nine tenths (0.9) mL of Assay Medium will be aliquoted into the appropriate wells of pre-labeled 6-well plates. The 6-well plates will be labeled with the test substance(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 EO tissues will be incubated at standard culture conditions for at least one hour. The medium will be aspirated and 0.9 mL of fresh Assay Medium will be aliquoted into each assay well below the tissue. Upon opening the bag, any unused tissues remaining on the shipping agar at the time of tissue transfer will be briefly gassed with an atmosphere of 5% CO₂/95% air, and the bag will be sealed and stored at 2-8°C for subsequent use.

6.8 Definitive MTT Assay

Four to five exposure times will be tested for each test substance. The exposure times should be chosen to maximize the response based on knowledge of the test substance (e.g., chemical class or physicochemical characteristics). In the short-term exposure assay, if the expected range of toxic response is unknown, a 20-minute exposure time may be performed first to determine the remaining exposure durations. The maximum exposure time will be 240 minutes unless there is a need to extend it, based on knowledge of the test substance.

Two tissues will be treated for each test substance at each control exposure time. The dosing procedure will be determined as indicated in **Section 6.5**. 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 substances up to 240 minutes.

At the end of the treatment time, the test substance will be removed by extensively rinsing both sides of the culture with room temperature Ca²⁺ and Mg²⁺-Free Dulbecco's Phosphate Buffered Saline (Ca²⁺Mg²⁺-Free-DPBS). The process will be performed until the culture appears free from test substance. If it is not possible to remove all of the visible test material, this will be noted.

After rinsing, the tissue will be transferred to 5 mL of Assay Medium for a 10 to 20 minute incubation period at room temperature. This rinse is intended to remove any test substance 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 μ m filter to remove undissolved crystals. Three hundred μ L 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 \pm 0.1 hours at standard culture conditions.

After 3 \pm 0.1 hours, the bottom of the EO tissue constructs will be blotted on absorbent paper, cleared of excess liquid, and transferred to a prelabeled 24-well plate containing 2.0 mL of isopropanol in each designated well. The plates will be sealed with parafilm and stored in the refrigerator (2-8°C) until the last exposure time is harvested. The plates are then 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 μ L transferred to the appropriate wells of a prelabeled 96-well plate(s). Two hundred μ L of isopropanol will be added to the wells designated as blanks. The absorbance at 550 nm (OD₅₅₀) of each well will be measured with a Molecular Devices Vmax plate reader.

6.9 Killed Controls for Assessment of Residual Test Substance Reduction of MTT

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

To determine whether residual test substance is acting to directly reduce the MTT, a freeze-killed control tissue is used. Freeze-killed tissue is prepared by placing untreated EO constructs in the -20°C freezer at least overnight, thawing to room temperature, and then refreezing. Once refrozen, the tissue may be stored indefinitely in the freezer. To test for residual test substance reduction, killed tissues are treated with the test substances in the normal fashion. Generally, each test substance 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 substance-treated killed control, the MTT reduction observed in the test substance-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 substance may be considered untestable in this system. The OD₅₅₀ values from the killed controls will be analyzed as described in **Section 7.1**.

7.0 Evaluation of Test Results

7.1 Data Evaluation and Interpretation

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

$$\text{Corrected test substance exposure time OD}_{550} = \text{Test substance exposure time OD}_{550} - \text{Blank mean OD}_{550}$$

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

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

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

corrected OD₅₅₀ values will be used to determine the percent of control viabilities at each exposure time as follows:

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

Finally, the following percent of control calculations will be made as follows:

$$\text{Percent of Control} = (\text{Corrected OD}_{550} \text{ of each test substance or positive control exposure time} / \text{Corrected mean OD}_{550} \text{ of negative control}) \times 100$$

The individual % of control values are averaged to calculate the mean percent of control per exposure time. Viability calculations for test substances treated in the long exposure time assay may be performed by comparing the corrected OD₅₅₀'s of each test substance 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 substance exposure time on the abscissa. Other plot forms may be used as needed. The ET₅₀ will be interpolated from each plot. To determine the ET₅₀, two adjacent points will be selected, one that shows greater than 50% survival and one that shows less than 50% survival. The two selected points will be used to determine the slope and the y-intercept for the equation $y = m(x) + b$. Finally, to determine the ET₅₀, the equation will be solved for $y = 50$. If all of the exposure time points show greater than 50% survival, the ET₅₀ will be listed as greater than the longest exposure time. If all of the exposure times show less than 50% survival, the ET₅₀ will be presented as less than the shortest exposure time. Additional assays may be performed as needed to produce the final ET₅₀ value.

8.0 Criteria for an Acceptable Test

The assay will be accepted if the positive control, 0.3% Triton-X-100, causes an ET₅₀ within two standard deviations of the historical mean. The historical mean is updated every three months. The corrected mean OD₅₅₀ value for the minimum negative control exposure time must be within 20% of the corrected mean OD₅₅₀ value for the maximum negative control exposure time (up to 240 minutes).

9.0 Data Interpretation

Test substances with an ET₅₀ ≥ 70 minutes are identified as substances not labeled as irritants.

10.0 Study 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 ET₅₀ values for each test substance 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.

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

The test report should include the following information, if relevant to the conduct of the study:

Test and Control Substances

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known
- The CAS Registry Number (RN), if known

- Purity and composition of the substance or preparation (in percentage(s) by weight), to the extent this information is available
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study
- Treatment of the test/control substances prior to testing, if applicable (e.g., warming, grinding)
- Stability, if known

Information Concerning the Sponsor and the Test Facility

- Name and address of the Sponsor
- Name and address of the test facility
- Name and address of the Study Director

Justification of the Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time (e.g., periodic testing of proficiency substances, use of historical negative and positive control data)

Criteria for an Acceptable Test

- Acceptable concurrent negative control ranges based on historical data
- Acceptable concurrent positive control ranges based on historical data
- If applicable, acceptable concurrent benchmark control ranges based on historical data

Test Conditions

- Description of test system used
- Calibration information for instrument used for optical density measurements (e.g., spectrophotometer)
- Details of test procedure used
- Test concentration(s) used
- Description of any modifications of the test procedure
- Reference to historical data of the model (e.g., negative and positive controls, proficiency substances, benchmark substances)
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples (e.g., ET₅₀)

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement indicates all inspections made during the study, and the dates any results were reported to the Study Director. This statement also serves to confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

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Appendix C

Summary Review Document

Strategy for U.S. Environmental Protection Agency Ocular Hazard Classification and Labeling of Antimicrobial Cleaning Products Using *In Vitro* Alternative Methods

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Summary Review Document
Strategy for U.S. Environmental Protection Agency Ocular
Hazard Classification and Labeling of Antimicrobial Cleaning
Products Using *In Vitro* Alternative Test Methods

Interagency Coordinating Committee on the
Validation of Alternative Methods

National Toxicology Program Interagency Center for the
Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

2010

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List of Abbreviations and Acronyms

%CV	Percent coefficient of variation
AMCP	Antimicrobial cleaning product
ATWG	Alternative Testing Working Group
BCOP	Bovine corneal opacity and permeability
BRD	Background review document
CFR	Code of Federal Regulations
CM	Cytosensor [®] Microphysiometer
COLIPA	European Cosmetic, Toiletry and Perfumery Association
CPSC	U.S. Consumer Product Safety Commission
CTFA	Cosmetic, Toiletry and Fragrance Association
CV	Coefficient of variation
EC/HO	European Commission/British Home Office
ECVAM	European Centre for the Validation of Alternative Methods
EO	EpiOcular [™]
EPA	U.S. Environmental Protection Agency
ESAC	European Centre for the Validation of Alternative Methods Scientific Advisory Committee
ET ₅₀	Time needed to reduce cell viability by 50%
FDA	U.S. Food and Drug Administration
FHSA	Federal Hazardous Substances Act
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FR	<i>Federal Register</i>
GHS	United Nations Globally Harmonized System of Classification and Labelling of Chemicals
GLP	Good Laboratory Practice
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems, Inc.
ISO	International Organization for Standardization
IVIS	<i>In vitro</i> irritancy score
JaCVAM	Japanese Center for the Validation of Alternative Methods
LVET	Low volume eye test
MAS	Maximum average score
MRD ₅₀	Estimated concentration of a test substance needed to reduce the basal metabolic rate of L929 cells by 50%
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NTP	National Toxicology Program

OECD	Organisation for Economic Co-operation and Development
OPP	Office of Pesticide Programs
OPPTS	Office of Prevention, Pesticides and Toxic Substances
OSHA	Occupational Safety and Health Administration
OTWG	Ocular Toxicity Working Group
PPDC	Pesticide Product Dialog Committee
REACH	Registration, Evaluation, and Authorisation of Chemicals
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SD	Standard deviation
SM	Silicon Microphysiometer
SRD	Summary review document
TG	Test guideline
TNO	TNO Nutrition and Food Research Institute (Netherlands)
TSCA	Toxic Substances Control Act
U.K.	United Kingdom
U.N.	United Nations
U.S.	United States
U.S.C.	United States Code
w/v	Weight-to-volume ratio

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Preface

Commercial and household cleaning products require labeling to indicate if they are hazardous to the consumer and have the potential to cause injury during handling or use, including possible ingestion by children. The U.S. Consumer Product Safety Commission (CPSC) typically regulates these cleaning products. However, inclusion of an antimicrobial claim in such cleaning products necessitates their registration as antimicrobial pesticides with the U.S. Environmental Protection Agency (EPA). Accordingly, to comply with EPA classification and labeling requirements for eye irritation (EPA 2003a), a product manufacturer must test these cleaning products in the Draize rabbit eye test (Draize et al. 1944) to adequately characterize their ocular hazard potential.

In June 2004, the EPA contacted the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), which administers the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and provides scientific support for ICCVAM activities, to seek the assistance in a technical assessment of an *in vitro* testing strategy that would meet their need to evaluate, categorize, and label antimicrobial cleaning products (AMCPs) for eye irritation. Subsequently, the Alternative Testing Working Group (ATWG), a consortium of seven consumer product companies (Clorox, Colgate-Palmolive, Dial, EcoLabs, JohnsonDiversey, Procter & Gamble, and SC Johnson), developed a testing strategy that is comprised of three *in vitro* test methods (i.e., bovine corneal opacity and permeability [BCOP], Cytosensor[®] Microphysiometer [CM], and EpiOcular[™] [EO]) for this limited group of products. The Institute for In Vitro Sciences, Inc., which coordinated the ATWG collaboration, performed additional testing to complete parallel sets of *in vivo* and *in vitro* data and described the testing strategy in a background review document (BRD). The EPA and the ATWG requested that NICEATM and ICCVAM use the information in the AMCP BRD to conduct a technical review of the scientific validity of the AMCP testing strategy. The EPA and the ATWG sought to determine whether EPA could be assured with a reasonable degree of certainty that the AMCP testing strategy would be useful for making hazard classification and labeling decisions for AMCPs in order to appropriately inform users. A *Federal Register* (FR) notice (70 FR 13512) issued on March 21, 2005, requested relevant data and nominations for potential peer review panel members.

NICEATM received an initial draft of the AMCP BRD from the Institute for In Vitro Sciences, Inc., on December 27, 2007; formal transmittal letters were received from the Institute for In Vitro Sciences, Inc., and the EPA on January 8 and 10, 2008, respectively. On March 17, 2008, following a preliminary review of the AMCP BRD, the ICCVAM Ocular Toxicity Working Group (OTWG) requested additional information and data from the Institute for In Vitro Sciences, Inc. The additional data, which were necessary to complete an evaluation, were received on April 4, 2008.

On April 4, 2008, *Federal Register* notice (73 FR 18535) requested relevant data and nominations for potential peer review panel members. On June 23–24, 2008, the OTWG and ICCVAM assigned this activity a high priority following consideration of comments from the public and ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). The Institute for In Vitro Sciences, Inc. submitted a final revised AMCP BRD on July 21, 2008. A supplement to the AMCP BRD, which included reliability analyses for the *in vitro* test methods (i.e., BCOP, CM, and EO), was submitted on October 8, 2008.

The OTWG worked with NICEATM to prepare this summary review document (SRD), which summarizes the current validation status of the AMCP testing strategy based on information in the AMCP BRD and other related information and data obtained by NICEATM. This AMCP SRD also provides similar information for an alternate AMCP testing strategy. This AMCP SRD summarizes the information from the AMCP BRD needed to evaluate the validation status of each of the *in vitro* test methods, the AMCP testing strategy, and the alternate AMCP testing strategy and forms the basis for the ICCVAM test method recommendations.

An independent international scientific peer review panel met in public forum on May 19–21, 2009, to develop conclusions and recommendations for the AMCP testing strategy. The Panel included expert scientists nominated by the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM). The Panel considered this AMCP SRD and evaluated the extent to which the available information supported the draft ICCVAM test method recommendations. ICCVAM considered the conclusions and recommendations of the Panel, along with comments received from the public and the SACATM, before finalizing this AMCP SRD and test method recommendations.

We gratefully acknowledge the organizations and scientists who provided data and information for this document. We also acknowledge the efforts of those individuals who helped prepare this AMCP SRD. These include Dr. Jill Merrill (U.S. Food and Drug Administration Center for Drug Evaluation and Research) and Dr. Karen Hamernik (EPA, to April 2009) for serving as Co-chairs of the OTWG and ICCVAM representatives who reviewed and provided comments throughout the evaluation process. We also acknowledge the following staff from the NICEATM support contractor, Integrated Laboratory Systems, Inc.: Dr. David Allen, Dr. Jonathan Hamm, Nelson Johnson, Dr. Brett Jones, Dr. Elizabeth Lipscomb, and James Truax. Finally, we thank ECVAM liaisons Dr. João Barroso, Dr. Thomas Cole, and Dr. Valerie Zuang and JaCVAM liaison Dr. Hajime Kojima for their participation.

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Executive Summary

The Alternative Testing Working Group, a consortium of consumer product companies, developed a testing approach for antimicrobial cleaning products (AMCPs). In 2007, the Institute for In Vitro Sciences, Inc. (IIVS), described the approach in a background review document (BRD). The AMCP testing strategy consists of three *in vitro* test methods: bovine corneal opacity and permeability (BCOP), Cytosensor[®] Microphysiometer (CM), and EpiOcular[™] (EO). The AMCP BRD includes a detailed protocol for each test method. Decision criteria were developed for each test method to correspond to the four ocular hazard categories in the U.S. Environmental Protection Agency (EPA) classification system (EPA Category I, II, III, and IV [EPA 2003a]). These test methods use a variety of endpoints to predict the potential of test substances to cause eye irritation.

The AMCP Testing Strategy: Combining the BCOP, CM, and EO Test Methods

The BCOP includes two primary endpoints, opacity and permeability. Opacity and permeability measurements are used to calculate an *in vitro* irritancy score (IVIS).¹ Histopathology evaluation of the affected tissue is an optional endpoint. Substances with an IVIS ≥ 75 are classified as EPA Category I; those with an IVIS ≥ 25 and < 75 are EPA Category II; and substances with an IVIS < 25 are EPA Category III. If a test substance produces an IVIS < 75 , further assessment using histopathology evaluation can determine whether it meets the criteria for classification as EPA Category I, II, or III. Because the data points from EPA Category III and Category IV overlap and it's impossible to assign a cutoff value, the AMCP BRD does not propose BCOP decision criteria for EPA Category IV.

The endpoint for the CM test method is the estimated concentration of a test substance needed to reduce the basal metabolic rate of L929 cells by 50% (the MRD₅₀). Substances with an MRD₅₀ value < 2 mg/mL are classified as EPA Category I; those with an MRD₅₀ ≥ 2 mg/mL and < 80 mg/mL are EPA Category III; and substances with an MRD₅₀ ≥ 80 mg/mL are classified as EPA Category IV. The AMCP BRD does not propose CM decision criteria for EPA Category II because the data points from EPA Category I and Category II overlap making it impossible to assign a cutoff value.

The endpoint for the EO test method is the time needed to reduce cell viability by 50% (ET₅₀). Substances with an ET₅₀ < 4 minutes are classified as EPA Category I; those with an ET₅₀ ≥ 4 minutes and < 70 minutes are EPA Category III; and substances with an ET₅₀ ≥ 70 minutes are classified as EPA Category IV. The AMCP BRD does not propose decision criteria for the EO test method for EPA Category II because the database includes only one EPA Category II substance.

The AMCP BRD proposes starting with different test methods depending on the chemical properties of the test substance. If the test substance is an oxidizer, which suggests that it will be an ocular corrosive or severe irritant, it is first tested in the BCOP test method. As noted above, test substances that produce an IVIS ≥ 75 would be classified as EPA Category I. If a test substance produces an IVIS < 75 , further assessment using histopathology evaluation can determine whether it meets the criteria for classification as EPA Category I, II, or III.

To determine whether the test substance is EPA Category III or IV, the test substance is subsequently tested in either the CM or EO test method to determine the final hazard category. The choice of test method depends on the chemical properties of the test substance. If the test substance is water soluble, it can be tested in either the CM test method or the EO test method. If it is water insoluble, it must be tested in the EO test method to determine the final hazard classification.

¹The *in vitro* irritancy score (IVIS) is the sum of the mean corrected opacity value (\pm standard deviation [SD]) and 15 times the mean corrected permeability value (OD₄₉₀ units \pm SD).

Alternate AMCP Testing Strategy: Combining the BCOP and EO Test Methods

None of the 228 substances in the validation database has been tested in all three of the *in vitro* test methods included in the AMCP testing strategy. ICCVAM also had concerns about the validation status of the low volume eye test (LVET),² which was used as the *in vivo* reference test method for all of the CM test method data. Therefore, the Interagency Coordination Committee on the Validation of Alternative Methods (ICCVAM) evaluated an alternate AMCP testing strategy that included only the BCOP and the EO test methods. In this alternate AMCP testing strategy, the BCOP test method would be used to identify EPA Category I and II substances and the EO test method would be used to identify EPA Category III and IV substances.

Testing in the alternate AMCP testing strategy could proceed by one of two approaches: (1) test in the BCOP test method first and then in the EO test method or (2) test in the EO test method first and then in the BCOP test method. Using the first approach, the BCOP test method would classify all EPA Category I and II substances. All other substances would then be tested in the EO test method and classified as either EPA Category III or IV. Using the second approach, substances would first be tested in the EO test method, which would classify all EPA Category III and IV substances. All other substances would then be tested in the BCOP test method and classified as either EPA Category I or II.

Validation Database

A total of 228 substances were included in the validation database for the AMCP BRD. These include 68 substances tested in the BCOP test method, 105 substances tested in the CM test method, and 55 substances tested in the EO test method. None of the 228 substances has been tested in all three *in vitro* test methods. According to the submitter, “a minimum 28 of the materials are EPA registered AMCPs, with eight additional materials being in-use dilutions of concentrates which are EPA registered” (Rodger Curren, IIVS, Inc., personal communication).

The distribution of product categories differed among the test methods. Most of the 105 substances tested in the CM test method are surfactants (78%). The substances tested in the BCOP and EO test methods are relatively equally distributed among alkalis, oxidizers, solvents, and surfactants (approximately 20% to 30% each).

Only 28 AMCPs have been tested in both the BCOP and EO test methods.

In Vivo Reference Data

The test method protocol used to generate the *in vivo* reference data varied among the 228 substances. Among the 68 substances tested in the BCOP test method, 85% were also tested in the traditional Draize rabbit eye test protocol (i.e., OECD TG 405 [OECD 2002]). Another 12% were tested with a nontraditional protocol (i.e., application volume of 30 μL instead of 100 μL or application as an aerosol spray). The remaining 3% were tested in the LVET.

Among the 55 substances tested in the EO test method, 55% were tested in the Draize rabbit eye test, and 45% were tested in the LVET. All 105 of the substances tested in the CM test method were tested in the LVET.

²The LVET is a modification to the rabbit eye test that involves application of 10 μL of the test substance directly to the corneal surface instead of 100 μL of the test substance applied into the conjunctival sac.

Test Method Accuracy

The Bovine Corneal Opacity and Permeability Test Method

The validation database of 66 substances tested in both the BCOP test method and the Draize rabbit eye test showed 55% accuracy (36 of 66 tests agreed in overall EPA classification) (**Table 1**). The BCOP test method correctly classified only 60% as EPA Category II and 50% as EPA Category III. However, the BCOP test method correctly identified 90% of the EPA Category I substances. Because the AMCP BRD does not propose BCOP decision criteria for EPA Category IV, all 19 substances were overpredicted.

The Cytosensor Microphysiometer Test Method

The validation database includes 105 unique substances tested in both the CM test method and the LVET (**Table 1**). Three substances were tested twice for a total of 108 tests. These tests had 30% accuracy (32 of 108 tests agreed in overall classification of EPA Category I, II, III, or IV). The CM test method overclassified the majority of EPA Category II, III, and IV substances in the database: 100% of the EPA Category II substances, 67% of the EPA Category III substances, and 89% of the EPA Category IV substances. Because the AMCP BRD does not propose CM test method decision criteria for EPA Category II, the CM test method overclassified all EPA Category II and III substances as EPA Category I.

The EpiOcular Test Method

Among the 55 substances tested in the EO test method (**Table 1**), 30 were also tested in the Draize rabbit eye test (29 qualified for EPA hazard classification) and 25 were tested in the LVET. Those tested in both the EO test method and the Draize rabbit eye test had 76% accuracy (22 of 29 tests agreed in overall classification of EPA Category I, II, III, or IV). The EO test method correctly identified three (75%) of the four substances categorized as EPA Category III by the Draize rabbit eye test. The EO test method correctly identified 44% of the nine EPA Category IV substances. Four of the five substances incorrectly identified by the EO test method were overclassified as EPA Category III. The EO test method overclassified the remaining substance as EPA Category I. All of the EPA Category I substances were correctly identified.

Among the 25 substances tested in both the EO test method and the LVET (**Table 1**), the EO test method correctly classified 44%. The EO test method correctly identified 67% of the 12 substances classified as EPA Category III by the LVET. None of the nine EPA Category IV substances was correctly identified; 44% were overclassified as EPA Category III; and 56% were overclassified as EPA Category I. The EO test method correctly identified all three of the substances classified as EPA Category I by the LVET.

AMCP Testing Strategy: Combining the BCOP, CM, and EO Test Methods

As explained above, none of the 228 substances included in the AMCP BRD was tested in all three of the *in vitro* test methods proposed for the AMCP testing strategy. Therefore, no data are available to characterize the actual performance of a testing strategy that includes the BCOP, CM, and EO test methods.

Alternate AMCP Testing Strategy: Combining the BCOP and EO Test Methods

The BCOP and EO test methods were both used to test 28 substances for which Draize rabbit eye test data were available. This suggested an alternate AMCP testing strategy in which the BCOP test method might be used to identify EPA Category I or Category II substances and the EO test method might be used to identify EPA Category III or Category IV substances. ICCVAM evaluated the data based on two approaches: (1) test in the BCOP test method first and then in the EO test method or (2) test in the EO test method first and then in the BCOP test method.

Table 1 Performance of AMCPs in the Bovine Corneal Opacity and Permeability, Cytosensor Microphysiometer, and EpiOcular Test Methods Compared to the Draize Rabbit Eye Test or the Low Volume Eye Test as Reported in the AMCP BRD Using the EPA Classification System

In Vitro Test Method	In Vivo Test Method	Overall Classification	Performance of the <i>In Vitro</i> Test Method Compared to the <i>In Vivo</i> Reference Test Method Using the EPA Classification System									
			I		II		III			IV		
			Actual	Under	Over	Actual	Under	Over	Actual	Under	Over	Actual
BCOP ¹	Draize	55% (36/66)	90% (27/30)	10% (3/30)	20% (1/5)	60% (3/5)	20% (1/5)	50% (6/12)	50% (6/12)	0% (0/12)	100% (19/19)	0% (0/19)
CM ²	LVET	30% (32/108)	100% (9/9)	0% (0/9)	100% (11/11)	0% (0/11)	0% (0/11)	67% (40/60)	33% (20/60)	0% (0/60)	89% (25/28)	11% (3/28)
EO ³	Draize	76% (22/29)	100% (15/15)	0% (0/15)	0% (0/1)	0% (0/1)	100% (1/1)	25% (1/4)	75% (3/4)	0% (0/4)	56% (5/9)	44% (4/9)
EO ⁴	LVET	44% (11/25)	100% (3/3)	0% (0/3)	100% (1/1)	0% (0/1)	0% (0/1)	33% (4/12)	67% (8/12)	0% (0/12)	100% (9/9)	0% (0/9)

Abbreviations: AMCP = antimicrobial cleaning product; BCOP = bovine corneal opacity and permeability; CM = Cytosensor Microphysiometer; EO = EpiOcular; EPA = U.S. Environmental Protection Agency; ET₅₀ = estimated time to decrease keratinocyte viability in the EO test method by 50%; IVIS = *in vitro* irritancy score; LVET = low volume eye test; MRD₅₀ = concentration of test substance that decreases the metabolic rate by 50% determined by a plot of the concentration-response curve.

- ¹ Classification of the BCOP data was based on IVIS ≥75 = EPA Category I; IVIS ≥25 and <75 = EPA Category II; IVIS <25 = EPA Category III. The BCOP test method was not proposed to identify EPA Category IV. All BCOP classifications, including high-solvent substances, used a 10-minute exposure time. The database comprised 66 substances tested in both the BCOP test method and the Draize rabbit eye test.
- ² Classification of the CM data was based on MRD₅₀ <2 mg/mL = EPA Category I; MRD₅₀ ≥2mg/mL and <80 mg/mL = EPA Category III; MRD₅₀ ≥80 mg/mL = EPA Category IV. The CM test method was not proposed to identify EPA Category II. The database consisted of 108 substances tested in both the CM test method and in the LVET (105 different substances because three substances were tested twice).
- ³ Classification of the EO data was based on ET₅₀ <4 min = EPA Category I; ET₅₀ ≥4 min and <70 min = EPA Category III; ET₅₀ ≥70 min = EPA Category IV. The EO test method was not proposed to identify EPA Category II. The database consisted of 29 substances tested in both the EO test method and the Draize rabbit eye test that qualified for EPA hazard classification (i.e., one substance producing a Draize score greater than 1 was not evaluated through day 21 as required by EPA).
- ⁴ Classification of the EO data was based on ET₅₀ <4 min = EPA Category I; ET₅₀ ≥4 min and <70 min = EPA Category III; ET₅₀ ≥70 min = EPA Category IV. The EO test method was not proposed to identify Category II. The database consisted of 25 substances tested in both the EO test method and the LVET.

For the first approach, ICCVAM evaluated the BCOP test method's ability to identify substances as either EPA Category I or Category II. All 15 substances that were classified as EPA Category I or II in the BCOP test method were removed from the database. The remaining 13 substances were then evaluated in the EO test method for identifying EPA Category III or IV substances. The reverse was done for the second approach: the EO test method was evaluated for its ability to classify substances as either EPA Category III or IV. All 13 substances that had been classified as EPA Category III or IV by the EO test method were removed from the database. The remaining 15 substances were then evaluated in the BCOP test method for identifying EPA Category I or II substances.

The alternate AMCP testing strategy performed the same regardless of which approach was used (**Table 2**). The alternate AMCP testing strategy correctly classified 79% of the substances, which included all 14 of the EPA Category I substances, all four of the EPA Category III substances, and four of the nine (44%) EPA Category IV substances. The one EPA Category II substance was underpredicted as EPA Category III.

Test Method Reliability

The Bovine Corneal Opacity and Permeability Test Method

In the AMCP BRD, intralaboratory repeatability for the BCOP test method (i.e., comparison of within-experiment runs of a test substance) was determined for 67 AMCPs (four substances have repeat tests) as the mean percent coefficient of variation (%CV) for opacity, permeability, and IVIS. Because scores in the very low range significantly affect %CVs, the mean %CVs for materials with an IVIS ≤ 10 (arbitrarily set in the AMCP BRD) were excluded from the overall mean %CV calculations. The overall mean %CVs for opacity, permeability, and IVIS were 21%, 25%, and 18%, respectively.

These 67 test substances, tested in a total of 75 runs, were also evaluated for their agreement in the EPA (EPA 2003a) and Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN 2007) ocular hazard classification systems. The EPA and GHS classification systems had 100% agreement in 84% (63 of 75) of test runs, 67% agreement in 15% (11 of 75) of test runs, and 60% agreement in 1% (1 of 75) of test runs. Among the 12 test runs that did not have 100% agreement, seven substances had reactive chemistries, two were alkalis, two were surfactants, and one was an acid.

Intralaboratory repeatability for the BCOP test method was determined for non-AMCPs classified as severe or ocular corrosives in three BCOP studies, which tested from 16 to 52 substances (ICCVAM 2006a). The mean %CVs for IVIS ranged from 39% to 71%.

Intralaboratory reproducibility for the BCOP test method (i.e., comparison of between-experiment runs of a test substance) was determined for five AMCPs as the mean %CV for IVIS. In two to six experiments, the mean %CV for IVIS was 20%. The agreement in the EPA (EPA 2003a) and GHS (UN 2007) ocular hazard classification systems for these five test substances was 100%.

Intralaboratory reproducibility for the BCOP test method was also determined for non-AMCPs classified as severe ocular irritants or ocular corrosives by the BCOP test method (ICCVAM 2006a). One of the two studies consisted of 25 surfactant-based personal-care cleaning formulations. The mean %CV for permeability values in that study was 33%. In the second study of 16 substances, the mean %CV for IVIS ranged from 13% to 15%.

Interlaboratory reproducibility for the BCOP test method (i.e., comparison of runs of a test substance between different laboratories) cannot be specifically determined for AMCPs in the BRD because only one laboratory conducted the testing.

Table 2 AMCPs Tested in Both the BCOP and EO Test Methods: Performance Using the Alternate AMCP Testing Strategy

EPA	Overall Classification	Draize									
		I		II			III		IV		
		Actual	Under	Over	Actual	Under	Over	Actual	Under	Over	Actual
Approach 1	79% (22/28)	100% (14/14)	0% (0/14)	0% (0/1)	0% (0/1)	100% (1/1)	0% (0/4)	100% (4/4)	0% (0/4)	56% (5/9)	44% (4/9)
EPA	Overall Classification	Draize									
		I		II			III		IV		
		Actual	Under	Over	Actual	Under	Over	Actual	Under	Over	Actual
Approach 2	79% (22/28)	100% (14/14)	0% (0/14)	0% (0/1)	0% (0/1)	100% (1/1)	0% (0/4)	100% (4/4)	0% (0/4)	56% (5/9)	44% (4/9)

Abbreviations: AMCP = antimicrobial cleaning product; BCOP = bovine corneal opacity and permeability; EO = EpiOcular; EPA = U.S. Environmental Protection Agency.

Approach 1 = test in the BCOP test method first to identify EPA Category I or II, and then in the EO test method to identify EPA Category III or IV.

Approach 2 = test in the EO test method first to identify EPA Category III or IV, and then in the BCOP test method to identify EPA Category I or II.

Three studies (3–12 laboratories each) were used to determine interlaboratory reproducibility in non-AMCPs classified as severe or ocular corrosives by the BCOP test method (ICCVAM 2006a). The mean %CV for IVIS ranged from 25% to 36%. These test substances were also evaluated (ICCVAM 2006a) for their agreement with the EPA (EPA 2003a), GHS (UN 2007), and European Union (EU 2001) ocular hazard classification systems.

The Cytosensor Microphysiometer Test Method

Reliability for the CM test method could not be evaluated specifically for AMCPs due to insufficient data. However, the reliability of the CM test method was evaluated in non-AMCPs.

Intralaboratory repeatability for the CM test method was evaluated for non-AMCPs in seven studies of 1 to 35 test substances each. The mean %CV for MRD₅₀ values for all materials tested, including surfactant and nonsurfactant materials, ranged from 6% to 25%.

The intralaboratory reproducibility of the CM test method for non-AMCPs in one laboratory (16 substances). The mean %CV for MRD₅₀ values for all materials tested, including surfactant and nonsurfactant materials, was 25%.

Interlaboratory reproducibility for this test method was determined for non-AMCPs in two studies at two to four laboratories each. The mean %CV for MRD₅₀ values for all materials tested, including surfactant and nonsurfactant materials, ranged from 17% to 51%, with nonsurfactant materials having a higher mean %CV in each study.

The EpiOcular Test Method

Intralaboratory repeatability for the EO test method was determined specifically for a subset of 15 AMCPs presented in the AMCP BRD. The mean %CV for ET₅₀ values ranged from 0% to 62%.

The extent of agreement between the EPA and GHS ocular hazard classification systems (EPA 2003a; UN 2007) was evaluated for three AMCPs that were tested more than once by IIVS. All three AMCPs had 100% agreement for both hazard classification systems.

Intralaboratory reproducibility for the EO test method was also determined from repeat testing of a single substance, 0.3% Triton X-100. Data were presented as combined data from MatTek Corporation and IIVS (9-year period) and from IIVS only (8-year period). The mean %CVs for ET₅₀ values were 21% and 22%, respectively.

Interlaboratory reproducibility for the EO test method cannot be determined specifically for the AMCPs presented in the AMCP BRD because only one laboratory conducted the testing. However, interlaboratory reproducibility for this test method has been determined for non-AMCPs in a multiphase validation study of surfactants and surfactant-containing products (73 substances). The study is summarized in the AMCP BRD. Mean %CVs ranged from 12% to 18%. It should be noted, however, that this reproducibility evaluation did not use a calculated ET₅₀ value to predict the ocular hazard classification (i.e., EPA Category I, II, III, and IV), as specified in the protocol included in the AMCP BRD. Instead, it is based on an EO protocol that uses relative percent viability to classify irritancy (i.e., irritant vs. nonirritant).

These same non-AMCP test substances were also evaluated for agreement with the EPA and GHS ocular hazard classification systems (EPA 2003a; UN 2007). This analysis is summarized in a supplement to the AMCP BRD. Using the EPA and GHS classification systems in Phase II of the validation study, four laboratories produced 100% agreement for 74% of the 19 substances, 75% agreement for 11% of the substances, and 50% agreement for 16% of the substances. In Phase III at two laboratories, 94% of the 54 substances had 100% agreement, and the remaining 6% (3 substances) had 0% agreement.

Animal Welfare Considerations

Both of the AMCP testing strategies are non-animal approaches for the classification and labeling of AMCPs. Bovine eyes used in the BCOP test method are obtained post mortem from animals being used for food. The CM test method uses a mouse cell line that can be purchased. The EO test method uses primary human keratinocytes obtained from human donors during routine surgical procedures.

Practical Considerations

The BCOP test method can be completed in one day, but histopathology evaluation may require an additional four weeks.

The CM test method, including multiple runs of the test material, can be completed in a single workday. However, the instrument for the CM test method has been discontinued.

The EO test method uses tissue that is commercially available from MatTek Corporation (Ashland, MA). The cost of the EO test method is similar to or less than that of a Draize rabbit eye test. Although it may take several weeks to procure tissue from the MatTek Corporation, the EO test method may be run in less time than the Draize rabbit eye test or the LVET.

1.0 Introduction and Rationale for the Use of a Testing Strategy for U.S. Environmental Protection Agency Classification and Labeling of Antimicrobial Cleaning Products

1.1 Historical Background of *In Vitro* Ocular Corrosion and Irritation Test Methods and the Rationale for Their Development

Over the years, legislative statutes have been enacted that enable government agencies to regulate a variety of substances that pose a potential risk to ocular health. **Table 1-1** provides a synopsis of current U.S. regulatory laws that pertain to ocular corrosion and irritation.

Table 1-1 Summary of Current U.S. Legislation Related to Ocular Health¹

Legislation (Year of Initial Enactment)	Agency	Substance
Food, Drug and Cosmetic Act (1938)	FDA	Pharmaceuticals and cosmetics
FIFRA (1947) and Federal Environmental Pesticide Control Act (1972)	EPA	Pesticides
FHSA (1964)	CPSC	Household products
FHSA (1964) and TSCA (1976)	Department of Agriculture and EPA	Agricultural and industrial chemicals
Occupational Safety and Health Act (1970)	OSHA	Occupational materials
Clean Air Act Amendments (1990)	Chemical Safety and Hazard Investigation Board and EPA	Accidentally released chemicals and air pollutants

Abbreviations: CPSC = U.S. Consumer Product Safety Commission; EPA = U.S. Environmental Protection Agency; FDA = U.S. Food and Drug Administration, FHSA = Federal Hazardous Substances Act; FIFRA = Federal Insecticide, Fungicide, and Rodenticide Act; OSHA = Occupational Safety and Health Administration; TSCA = Toxic Substances Control Act.

¹ Adapted from Wilhelmus (2001).

Exposing rabbit eyes to a test substance is the primary method for assessing the ocular hazard potential of substances that may come near or in contact with the eye of a human. The test method currently accepted by U.S. Federal and international regulatory agencies (CPSC 1995; EPA 1998; OECD 2002) is the Draize rabbit eye test (Draize et. al. 1944). In the Draize rabbit eye test, a test substance is applied to the lower conjunctival sac of one eye of a rabbit and compared to the contralateral eye, which serves as a negative control. The eyes of each rabbit are examined for adverse corneal (i.e., opacity and area of involvement), iridal, or conjunctival (i.e., redness, chemosis, and discharge) effects for a period up to 21 days after exposure to the test substance.

The Draize rabbit eye test can identify both irreversible (corrosive) and reversible ocular effects. The wide ranges used for scoring a majority of these lesions permit categorization of the severity of reversible effects as moderate, mild, or nonirritant (see U.S. Environmental Protection Agency [EPA] Ocular Classification System discussed below). Current EPA ocular testing guidelines and the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN 2007) indicate that if serious ocular damage is anticipated (e.g., irreversible adverse effects on day 21), then a test on a single animal may be considered. If serious damage is observed, then no further

animal testing is necessary (EPA 1998; UN 2007). If no serious damage is observed, additional test animals (1 or 2 rabbits) may be evaluated sequentially until concordant responses are observed (UN 2007).

The ocular classification systems vary depending on the regulatory agency's legislative mandate and goals for protecting human health (**Table 1-2**). The EPA classification system and testing guidelines (EPA 1998, 2003a) are based on the most severe response in one animal in a group of three or more animals. This classification system considers the kinds of ocular effects produced, as well as the reversibility and the severity of the effects. The EPA classifies substances into four ocular irritant categories (i.e., EPA Category I, II, III, and IV) (**Table 1-2**) (EPA 2003a). The EPA defines Category I substances as corrosive or severe irritants, while classification in EPA Category II, III, or IV is based on decreasing severity of ocular lesions, as well as the time required for the ocular lesions to clear. Irritation that clears in 8 to 21 days is classified as EPA Category II, while irritation that clears within 7 days is classified as EPA Category III. For EPA Category IV substances, irritation clears within 24 hours.

To harmonize the classification of ocular irritants internationally, the GHS classification system (UN 2007) includes two categories (**Table 1-2**), one for irreversible effects on the eye/serious damage to the eye (GHS Category 1) and one for reversible effects on the eye (GHS Category 2). Classification is based on the severity of the lesions and/or the duration of their persistence. Reversible effects are further classified based on the duration as GHS Category 2A (“irritating to eyes” referring to an effect that reverses within 21 days) and GHS Category 2B (“mildly irritating to eyes” referring to an effect that reverses within 7 days).

The U.S. Federal Hazardous Substances Act (FHSA; FHSA 1964) (CPSC 1995) and the European Union (EU; EU 2001) also have classification criteria for ocular irritation. However, because this evaluation focuses on ocular hazard classification according to the EPA and GHS systems, the criteria for the FHSA and EU systems will not be discussed. Additional details on these systems can be found in the BCOP BRD (ICCVAM 2006a).

Recently, the EPA requested that the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluate a non-animal strategy to classify and label antimicrobial cleaning products (AMCPs). This testing strategy was developed by the Alternative Testing Working Group (ATWG), composed of seven consumer product companies (Clorox, Colgate-Palmolive, Dial, EcoLabs, JohnsonDiversey, Procter & Gamble, and SC Johnson). The AMCP testing strategy includes three *in vitro* test methods (bovine corneal opacity and permeability [BCOP], Cytosensor Microphysiometer [CM]), and EpiOcular [EO]). *In vitro* data were paired with *in vivo* data obtained in either the Draize rabbit eye test or the low volume eye test (LVET).

On behalf of the ATWG, the Institute for In Vitro Sciences, Inc. submitted an AMCP background review document (BRD) (**Annex I**) and AMCP BRD Supplement (**Annex II**), which provided additional information on the reliability for each *in vitro* test method, to ICCVAM for review of the validation status of the AMCP testing strategy. The EPA and the ATWG requested that NICEATM and ICCVAM use information within the AMCP BRD to conduct a technical review of the AMCP testing strategy to determine whether ICCVAM could assure the EPA with a reasonable degree of certainty that the AMCP testing strategy would help the EPA determine AMCP labeling that would appropriately inform users.

This AMCP summary review document (SRD) summarizes the available data and information regarding the usefulness and limitations of the AMCP testing strategy as described in the AMCP BRD and an alternate AMCP testing strategy that uses only the BCOP and EO test methods.

Table 1-2 Ocular Toxicity Classification Systems

Regulatory Agency (Authorizing Act)	Number of Animals	Observation Days (after treatment)	Mean Score Taken?	Positive Response	Classification Criteria
EPA (FIFRA, Federal Insecticide, Fungicide, and Rodenticide Act, and TSCA)	At least 3	1 hr, 1, 2, 3, 7, and 21	No	Maximum score in an animal used for classification Opacity or Iritis ≥ 1 or Redness or Chemosis ≥ 2	One or more positive animals needed for classification in categories below. <u>Category:</u> I = Corrosive, corneal involvement, or irritation persisting more than 21 days II = Corneal involvement or irritation clearing in 8–21 days III = Corneal involvement or irritation clearing in 7 days or less IV = Minimal effects clearing in less than 24 hours Definition of Full Reversal: Opacity and Iritis scores = 0 and Redness and Chemosis scores ≤ 1
GHS: Irreversible Eye Effects	3	1, 2, 3 (observation until day 21)	Yes	Mean animal values (over days 1, 2, and 3) of: Opacity ≥ 3 and/or Iritis ≥ 1.5	At least 2 positive response animals = Eye Irritant Category 1 At least 1 animal with Opacity, Chemosis, Redness, or Iritis scores > 0 on day 21 = Eye Irritant Category 1 Definition of Full Reversal: Opacity, Iritis, Redness, and Chemosis scores = 0
GHS: Reversible Eye Effects	3	1, 2, 3 (observation until day 21)	Yes	Mean animal values (over days 1, 2, and 3) of: Opacity or Iritis ≥ 1 or Redness or Chemosis ≥ 2 and the effect fully reverses in 7 or 21 days	At least 2 positive response animals and the effect fully reverses in 21 days = Eye Irritant Category 2A At least 2 positive response animals and effect fully reverses in 7 days = Eye Irritant Category 2B Definition of Full Reversal: Opacity, Iritis, Redness, and Chemosis scores = 0

Abbreviations: EPA = U.S. Environmental Protection Agency; FIFRA = Federal Insecticide, Fungicide, and Rodenticide Act; GHS = Globally Harmonised System of Classification and Labelling of Chemicals; TSCA = Toxic Substances Control Act.

1.2 Regulatory Rationale and Applicability

The U.S. Consumer Product Safety Commission (CPSC) typically regulates commercial and household cleaning products. However, inclusion of an antimicrobial claim in such cleaning products necessitates their registration as antimicrobial pesticides with the EPA. Currently, the EPA requires AMCPs to be tested in the Draize rabbit eye test in order to adequately characterize their ocular hazard potential.

2.0 Testing Strategies for Ocular Hazard Classification and Labeling of Antimicrobial Cleaning Products

2.1 AMCP Testing Strategy

The testing strategy (**Figure 2-1**) described in the AMCP BRD (**Annex I**) is based on the use of three *in vitro* test methods: BCOP, CM, and EO. Each test method includes decision criteria developed to correspond to the four categories of ocular irritation defined by the EPA classification system (i.e., EPA Category I, II, III, and IV [EPA 2003a]). These test methods use a variety of endpoints to predict ocular irritation potential.

The BCOP includes two primary endpoints (i.e., corneal opacity and permeability) that are measured quantitatively and used to calculate an *in vitro* irritancy score (IVIS).³ An IVIS ≥ 75 = EPA Category I; IVIS ≥ 25 and < 75 = EPA Category II; IVIS < 25 = EPA Category III. The AMCP BRD does not propose decision criteria for EPA Category IV for the BCOP test method because the data points from EPA Category III and IV overlap and it is not possible to assign a cut-off value. Histopathology evaluation of the affected tissue is an optional endpoint for the BCOP test method. If a test substance produces an IVIS < 75 , further assessment using histopathology evaluation can determine whether it meets the criteria for classification as EPA Category I, II, or III.

The endpoint for the CM test method is the estimated concentration of a test substance needed to reduce the basal metabolic rate of L929 cells by 50% (the MRD₅₀). An MRD₅₀ < 2 mg/mL = EPA Category I; MRD₅₀ ≥ 2 mg/mL and < 80 mg/mL = EPA Category III; MRD₅₀ ≥ 80 mg/mL = EPA Category IV. The rationale for the use of L929 cells, a mouse fibroblast cell line, in the CM test method is provided in Section 2.2.1 of the AMCP BRD (**Annex I**). The AMCP BRD does not propose decision criteria for EPA Category II for the CM test method because the data points from EPA Category I and II overlap and it is not possible to assign a cut-off value.

The endpoint for the EO test method is the time needed to reduce cell viability by 50% (ET₅₀). An ET₅₀ < 4 minutes = EPA Category I; ET₅₀ ≥ 4 minutes and < 70 minutes = EPA Category III; ET₅₀ ≥ 70 minutes = EPA Category IV. The EO test method uses a proprietary tissue (i.e., EO tissue, MatTek Corporation, Ashland, MA) derived from normal human neonatal foreskin keratinocytes (see Section 2.2.2 of the AMCP BRD, **Annex I**). The keratinocytes are grown under standardized conditions to produce a highly uniform and reproducible cornea-like tissue. The AMCP BRD does not propose decision criteria for EPA Category II for the EO test method because only one EPA Category II substance is present in the database.

In the AMCP testing strategy as described in the AMCP BRD (**Figure 2-1**), the first test method used depends on knowledge of the chemical properties of the test substance. If the test substance is an oxidizer, which suggests that it will be an ocular corrosive or severe irritant, it is first tested in the BCOP test method. As noted above, test substances that produce an IVIS ≥ 75 would be classified as EPA Category I. If a test substance produces an IVIS < 75 , further assessment using histopathology evaluation can determine whether it meets the criteria for classification as EPA Category I, II, or III.

To determine whether the test substance is EPA Category III or IV, the test substance is subsequently tested in either the CM or EO test method to determine the final hazard category. Selection of the CM or EO test method depends on the water solubility of the test substance; water-soluble substances could be tested in either the CM test method or EO test method, but water-insoluble substances must be tested in the EO test method to determine their final hazard classification.

³ The *in vitro* irritancy score (IVIS) is calculated as the sum of the mean corrected opacity value (\pm standard deviation [SD]) and 15 times the mean corrected permeability value (OD₄₉₀ units \pm SD).

2.2 Alternate AMCP Testing Strategy

Because none of the 228 substances has been tested in all three of the *in vitro* test methods included in the AMCP testing strategy, as well as concerns regarding the validation status of the LVET (ICCVAM 2009), which was used as the *in vivo* reference test method for all of the CM data, an alternate AMCP testing strategy (**Figure 2-2**) that includes only the BCOP and EO test methods was evaluated. In the alternate AMCP testing strategy, the BCOP test method would be used to identify EPA Category I or II substances and the EO test method would be used to identify EPA Category III or IV substances.

Testing in the alternate AMCP testing strategy (**Figure 2-2**) could proceed in one of two approaches: (1) test in the BCOP test method first and then in the EO test method or (2) test in the EO test method first and then in the BCOP test method. Using the first approach, the BCOP test method would classify all EPA Category I and II substances. All other substances would then be tested in the EO test method and classified as either EPA Category III or IV. Using the second approach, substances would first be tested in the EO test method, which would classify all EPA Category III and IV substances. All other substances would then be tested in the BCOP test method and classified as either EPA Category I or II.

Figure 2-1 Combining the BCOP, CM, and EO Test Methods into a Testing Strategy: AMCP Testing Strategy

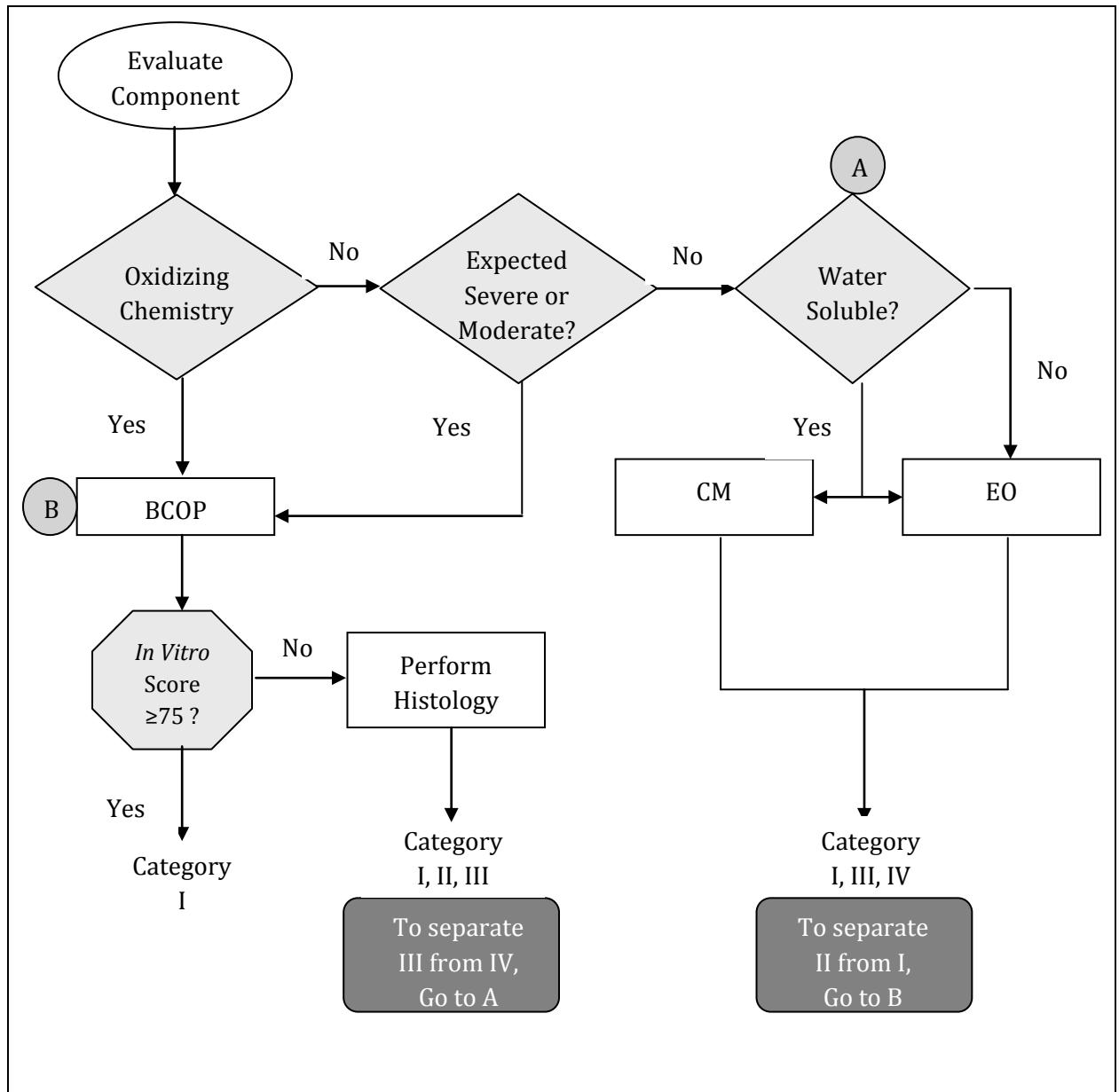
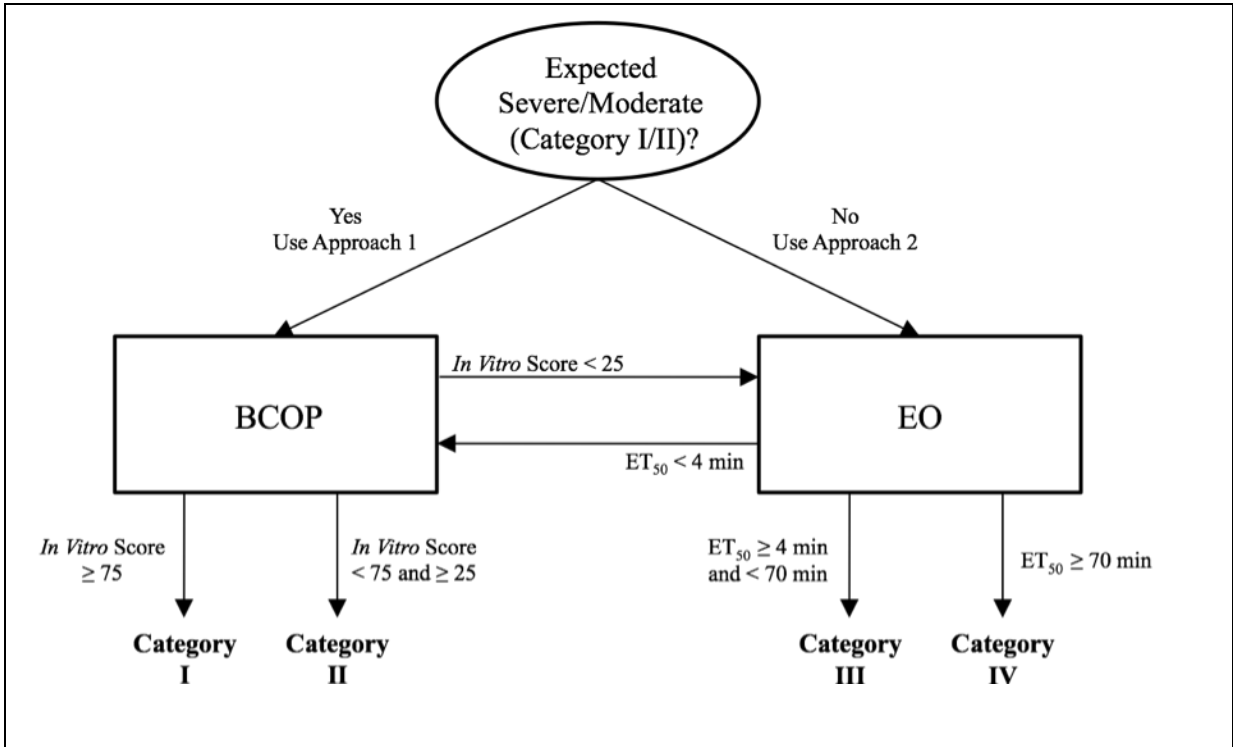


Figure 2-2 Combining the BCOP and EO Test Methods into a Testing Strategy: Alternate AMCP Testing Strategy



3.0 Substances Used for Validation of the Testing Strategies for EPA Classification of Antimicrobial Cleaning Products

3.1 Rationale for the Substances or Products Included in the AMCP Testing Strategy

A total of 228 substances were included in the validation database of the AMCP BRD (**Annex I**). It should be noted that, according to the submitter, “a minimum 28 of the materials are EPA registered anti-microbial cleaning products, with eight additional materials being in-use dilutions of concentrates which are EPA registered” (Rodger Curren, personal communication). Of these 228 substances, 68 substances were tested in the BCOP test method, 105 substances were tested in the CM test method, and 55 substances were tested in the EO test method. None of the 228 substances has been tested in all three of the *in vitro* test methods.

In the AMCP BRD, test substances were divided into “buckets” (i.e., chemical classes). The distribution of these chemical classes (solvents, oxidizers, surfactants, acids, bases, and others) by test method is presented in **Table 3-1**. Among the 68 substances tested in the BCOP test method, 18% (12/68) were solvents, 24% (16/68) were oxidizers, 33% (18/55) were surfactants, and 21% (14/68) were bases. Among the 105 substances tested in the CM test method, 17% (18/105) were solvents and 78% (82/105) were surfactants. Of 55 substances tested in the EO test method, 18% (10/55) were solvents, 24% (13/55) were oxidizers, 31% (17/55) were surfactants, and 20% (11/55) were bases.

Table 3-1 Distribution of Product Categories Evaluated in the AMCP Testing Strategy

Product Categories	Number of Substances Tested Per Test Method			
	BCOP	Cytosensor Microphysiometer	EpiOcular	Total
Solvents	12	18	10	39
Oxidizers	16	0	13	33
Surfactants	18	82	17	114
Acids	7	1	2	10
Bases	14	4	11	29
Others	1	0	2	3
Total	68	105	55	228

Abbreviations: AMCP = antimicrobial cleaning product; BCOP = bovine corneal opacity and permeability.

As reported in the AMCP BRD (**Annex I**), all 105 substances tested in the CM test method were tested in the LVET. No Draize rabbit eye test data were available for any of the substances tested in the CM test method. Of the 55 substances tested in the EO test method, 30 were tested in the Draize rabbit eye test and 25 were tested in the LVET. For the 68 substances tested in the BCOP test method, 58 were tested in the Draize rabbit eye test, 8 were tested in a nontraditional Draize rabbit eye test,⁴ and 2 were tested in the LVET.

⁴ The nontraditional Draize test data included seven substances tested with 30 µL rather than the traditional 100 µL instilled in the conjunctival sac of the rabbit and one substance that was tested as an aerosol sprayed directly on the cornea.

3.2 Rationale for the Substances or Products Included in the Alternate AMCP Testing Strategy

NICEATM requested additional ocular data on substances tested in either the BCOP or EO test methods. MatTek Corporation (Ashland, MA) provided additional EO data (for which BCOP and Draize rabbit eye test data were available). However, NICEATM determined that these data were generated using a different protocol or prediction model than described in the AMCP BRD (**Annex I**). No other data were found.

Of 29 substances tested in both the BCOP and EO test methods that were also tested in the Draize rabbit eye test, 28 substances met the criteria to assign an EPA hazard classification. The chemical categories for these 28 substances included five surfactants, two acids, ten alkalis, four oxidizers, six solvents, and one “other” (or nonspecified) as shown in **Table 3-2**. The composition of the 28 substances evaluated in the alternate AMCP testing strategy is provided in **Annex III**.

Table 3-2 Distribution of Product Categories Evaluated in the Alternate AMCP Testing Strategy

Product Category	Number of Products Tested	<i>In Vivo</i> Draize Classification - EPA			
		I	II	III	IV
Surfactant	5	0	0	2	3
Acid	2	0	0	1	1
Alkali	10	9	1	0	0
Oxidizer	4	3	0	0	1
Solvent	6	2	0	1	3
Other	1	0	0	0	1
Total	28	14	1	4	9

Abbreviations: AMCP = antimicrobial cleaning product; EPA = U.S. Environmental Protection Agency.

4.0 *In Vivo* Reference Data

As reported in the AMCP BRD (**Annex I**), all 105 substances tested in the CM test method were tested in the LVET. No Draize rabbit eye test data were available for these substances. For the 55 substances tested in the EO test method, 25 were tested in the LVET and 30 were tested in the Draize rabbit eye test. Of those tested in the BCOP, 85% (58/68) were tested in the Draize rabbit eye test, 12% (8/68) were tested in a nontraditional Draize rabbit eye test,⁵ and the remaining 3% (2/68) were tested in the LVET. The alternate AMCP testing strategy is based on the results for the 28 substances that were tested in both the BCOP and EO test methods, were also tested in the Draize rabbit eye test, and qualified for EPA hazard classification.

The Draize rabbit eye test (Draize et al. 1944) is the standard test method accepted by U.S. regulatory agencies such as the EPA for ocular irritation testing and for the classification and labeling of chemicals and products. The EPA (OPPTS 870.2400, EPA 1998) and the Organisation for Economic Co-operation and Development (OECD Test Guideline 405, OECD 2002) have published protocols describing the Draize rabbit eye test. The *in vivo* reference data are summarized in Section 4.2 of the AMCP BRD (**Annex I**), and the individual animal data are appended to that document.

The LVET is an *in vivo* rabbit eye test developed by Griffith et al. (1980) that differs from the Draize rabbit eye test by applying 10 μL (instead of 100 μL) of a test substance directly on the cornea (instead of the conjunctival sac). Scoring of corneal, iridal, and conjunctival lesions in the LVET is identical to that of the Draize rabbit eye test. Background information on the LVET and comparison of the LVET to the Draize rabbit eye test is available in the ICCVAM test method evaluation report (ICCVAM 2010).

⁵ The nontraditional Draize test data included seven substances tested with 30 μL rather than the traditional 100 μL instilled in the conjunctival sac of the rabbit and one aerosol test substance that was sprayed directly on the cornea.

5.0 Test Method Data and Results

5.1 AMCP Testing Strategy

The AMCP BRD (**Annex I**) includes, where available, the following specific information for each test substance: name, Chemical Abstracts Service Registry Number, physicochemical properties (e.g., purity, form tested), study reference, formulation ingredients, and chemical class. Test concentrations, individual and mean opacity scores, individual and mean permeability scores, ET₅₀ or MRD₅₀ values, and hazard classification information are also provided. If the source or purity of the test substance was missing, no attempt was made to identify it.

5.1.1 The Bovine Corneal Opacity and Permeability Test Method

Participating companies submitted BCOP data on 68 AMCPs generated using the ICCVAM-recommended BCOP protocol (ICCVAM 2006b). Of these substances, 66 had paired Draize rabbit eye test data (58 generated from the traditional Draize rabbit eye test protocol and 8 generated from a nontraditional Draize protocol, see **Section 3.1**). Two substances were tested in the LVET.

Supplemental BCOP data were included in the AMCP BRD (**Annex I**). These data were extracted from the BCOP BRD (ICCVAM 2006a).

5.1.2 The Cytosensor Microphysiometer Test Method

Participating companies submitted CM data on 105 unique AMCPs (with paired LVET data) generated using at least two different protocols. One protocol was based on the silicon microphysiometer (SM) test method, the predecessor of the CM test method, that used a 500-second exposure to L929 cells grown on a coverslip, compared to the CM protocol that used a 810-second exposure to cells grown on a Transwell™ membrane. An algorithm was derived and used to convert SM data to CM data. It should be noted that data analyses in the CM test method were based on 108 substances because three substances were tested twice, each with a different result.

Supplemental CM data were included in the AMCP BRD. The CTFA Phase III validation study provided data on surfactants and surfactant-based substances (n=25) with paired data from both the Draize rabbit eye test and the LVET (Gettings et al. 1996). CM data were also included from the EC/HO and COLIPA validation studies (Balls et al. 1995; Brantom et al. 1997).

5.1.3 The EpiOcular Test Method

Participating companies submitted EO data on 61 substances with formulations similar to those found in typical cleaning product formulations (**Annex I**). However, sufficient *in vivo* data to determine the EPA hazard classification were available for only 55 of these substances. Of these substances, 30 were tested in the Draize rabbit eye test data and 25 were tested in the LVET. Of the 30 substances tested in the Draize rabbit eye test, 29 qualified for EPA hazard classification (i.e., one substance producing a Draize score greater than 1 was not evaluated through day 21 as required by EPA).

Supplemental EO data were included in the AMCP BRD (**Annex I**). However, the EO protocol used in these studies differs significantly from the protocol being proposed in the AMCP BRD in that the test substance was diluted before testing; therefore, these studies were presented only as supporting information.

5.1.4 Combining the BCOP, CM, and EO Test Methods into a Testing Strategy: AMCP Testing Strategy

None of the 228 substances included in the AMCP BRD (**Annex I**) was tested in all three of the *in vitro* test methods in the AMCP testing strategy. Therefore, there are no data with which to characterize the actual performance of the AMCP testing strategy that includes the BCOP, CM, and EO test methods.

5.1.5 Combining the BCOP and EO Test Methods into a Testing Strategy: Alternate AMCP Testing Strategy

The evaluation of the alternate AMCP testing strategy was limited to the 28 substances that were tested in both the BCOP and EO test methods and also tested in the Draize rabbit eye test.

6.0 Test Method Accuracy

6.1 AMCP Testing Strategy

The AMCP BRD (**Annex I**) details the performance of each test method (i.e., BCOP, CM, and EO) included in the AMCP testing strategy. Performance is discussed according to the EPA (EPA 2003a) and GHS (UN 2007) classification systems. Therefore, we only briefly summarize the performance of each test method. Additionally, because the results for the EPA and GHS classification systems are similar, only the EPA results are discussed. The data from the AMCP BRD are summarized in **Table 6-1**.

6.1.1 The Bovine Corneal Opacity and Permeability Test Method

Based on the validation database of 66 substances tested in both the BCOP test method and the Draize rabbit eye test, accuracy of the overall EPA classification (i.e., EPA Category I, II, III, and IV) was 55% (36/66) (**Table 6-1**). The BCOP test method correctly identified only 60% (3/5) of the EPA Category II and 50% (6/12) of the EPA Category III substances. However, the BCOP test method correctly identified 90% (27/30) of the EPA Category I substances. Among the three EPA Category I substances that the BCOP test method underpredicted as EPA Category II, two were oxidizers and one was a base. It should be noted that the base would have been correctly identified if the decision criteria were $IVIS \geq 55.1$, as recommended in the BCOP BRD (ICCVAM 2006a), instead of $IVIS \geq 75$ as proposed in the AMCP BRD (**Annex I**). However, such a change in decision criteria would also result in two EPA Category II substances (one oxidizer and one acid) and one EPA Category III substance (a base) being overpredicted as EPA Category I.

Among the EPA Category II substances that were incorrectly identified by the BCOP test method, one (a base) was underclassified as EPA Category III, and one (an oxidizer) was overclassified as EPA Category I. All six EPA Category III substances that were incorrectly identified by the BCOP test method were overclassified as either EPA Category I (two oxidizers and one base) or EPA Category II (one solvent, one base, and one surfactant). Because decision criteria for the BCOP test method are not proposed in the AMCP BRD for EPA Category IV, all 19 substances were overpredicted: two as EPA Category II (both solvents) and 17 as EPA Category III (8 surfactants, 3 solvents, 3 acids, one base, one oxidizer, and one “other”).

To assess the use of histopathology evaluation, BCOP test method data with histopathology evaluation were compared to BCOP test method data only. Data were available for 17 substances that had BCOP data with histopathology evaluation. As noted in **Table 6-2**, the overall accuracy for EPA hazard classifications (i.e., EPA Category I, II, III, and IV) was reduced from 41% (7/17) to 35% (6/17) with histopathology evaluation. Using histopathology evaluation with the BCOP test method removed one of the EPA Category I false negatives, but added three EPA Category II false positives.

6.1.2 The Cytosensor Microphysiometer Test Method

Based on the database of 108 substances tested in both the CM test method and the LVET (**Table 6-1**), accuracy of the overall EPA classification was 30% (32/108). It should be noted that the database consisted of 105 unique substances because three substances were tested twice. The CM test method overclassified the majority of EPA Category II, III, and IV substances included in the database (100% [11/11] of the EPA Category II substances, 67% [40/60] of the EPA Category III substances, and 89% [25/28] of the EPA Category IV substances). Among the 25 EPA Category IV substances that were overclassified, the CM test method classified 16% (4/25, all surfactants) as EPA Category I and 84% (21/25, 6 solvents, 2 bases, and 13 surfactants) as EPA Category III. Because decision criteria for the CM test method are not proposed in the AMCP BRD for EPA Category II, all

EPA Category II or III substances that were overclassified by the CM test method were classified as EPA Category I. All but one of the 40 EPA Category III substances that were overclassified by the CM test method were surfactants. The remaining one was a solvent. All 11 EPA Category II substances that were overclassified by the CM test method were surfactants. All nine of the EPA Category I substances (all surfactants) were correctly identified. None of the irritant categories (i.e., EPA Category I, II, or III) were underpredicted by the CM test method.

Table 6-1 Performance of AMCPs in the Bovine Corneal Opacity and Permeability, Cytosensor Microphysiometer, and EpiOcular Test Methods Compared to the Draize Rabbit Eye Test or the Low Volume Eye Test as Reported in the AMCP BRD Using the EPA Classification System

<i>In Vitro</i> Test Method	<i>In Vivo</i> Test Method	Overall Classification	Performance of the <i>In Vitro</i> Test Method Compared to the <i>In Vivo</i> Reference Test Method Using the EPA Classification System									
			I		II		III			IV		
			Actual	Under	Over	Actual	Under	Over	Actual	Under	Over	Actual
BCOP ¹	Draize	55% (36/66)	90% (27/30)	10% (3/30)	20% (1/5)	60% (3/5)	20% (1/5)	50% (6/12)	50% (6/12)	0% (0/12)	100% (19/19)	0% (0/19)
CM ²	LVET	30% (32/108)	100% (9/9)	0% (0/9)	100% (11/11)	0% (0/11)	0% (0/11)	67% (40/60)	33% (20/60)	0% (0/60)	89% (25/28)	11% (3/28)
EO ³	Draize	76% (22/29)	100% (15/15)	0% (0/15)	0% (0/1)	0% (0/1)	100% (1/1)	25% (1/4)	75% (3/4)	0% (0/4)	56% (5/9)	44% (4/9)
EO ⁴	LVET	44% (11/25)	100% (3/3)	0% (0/3)	100% (1/1)	0% (0/1)	0% (0/1)	33% (4/12)	67% (8/12)	0% (0/12)	100% (9/9)	0% (0/9)

Abbreviations: AMCP = antimicrobial cleaning product; BCOP = bovine corneal opacity and permeability; CM = Cytosensor Microphysiometer; EO = EpiOcular; EPA = U.S. Environmental Protection Agency; ET50 = estimated time to decrease keratinocyte viability in the EO test method by 50%; IVIS = in vitro irritancy score; LVET = low volume eye test; MRD50 = concentration of test substance that decreases the metabolic rate by 50% determined by a plot of the concentration-response curve.

- ¹ Classification of the BCOP data was based on IVIS ≥ 75 = EPA Category I; IVIS ≥ 25 and < 75 = EPA Category II; IVIS < 25 = EPA Category III. The BCOP test method was not proposed to identify EPA Category IV. All BCOP classifications, including high-solvent substances, used a 10-minute exposure time. The database comprised 66 substances tested in both the BCOP test method and the Draize rabbit eye test.
- ² Classification of the CM data was based on MRD50 < 2 mg/mL = EPA Category I; MRD50 ≥ 2 mg/mL and < 80 mg/mL = EPA Category III; MRD50 ≥ 80 mg/mL = EPA Category IV. The CM test method was not proposed to identify EPA Category II. The database consisted of 108 substances tested in both the CM test method and in the LVET (105 different substances because three substances were tested twice).
- ³ Classification of the EO data was based on ET50 < 4 min = EPA Category I; ET50 ≥ 4 min and < 70 min = EPA Category III; ET50 ≥ 70 min = EPA Category IV. The EO test method was not proposed to identify EPA Category II. The database consisted of 29 substances tested in both the EO test method and the Draize rabbit eye test that qualified for EPA hazard classification (i.e., one substance producing a Draize score greater than 1 was not evaluated through day 21 as required by EPA).
- ⁴ Classification of the EO data was based on ET50 < 4 min = EPA Category I; ET50 ≥ 4 min and < 70 min = EPA Category III; ET50 ≥ 70 min = EPA Category IV. The EO test method was not proposed to identify Category II. The database consisted of 25 substances tested in both the EO test method and the LVET.

Table 6-2 Comparison of the BCOP Test Method and the BCOP Test Method Using Histopathology Evaluation

EPA	Overall Classification	Draize									
		I		II			III			IV ¹	
		Actual	Under	Over	Actual	Under	Over	Actual	Under	Over	Actual
BCOP ² Only	41% (7/17)	50% (3/6)	50% (3/6)	0% (0/4)	75% (3/4)	25% (1/4)	75% (3/4)	25% (1/4)	0% (0/4)	100% (3/3)	0% (0/3)
BCOP ² with Histology	35% (6/17)	67% (4/6)	33% (2/6)	75% (3/4)	25% (1/4)	0% (0/4)	75% (3/4)	25% (1/4)	0% (0/4)	100% (3/3)	0% (0/3)

Abbreviations: BCOP = bovine corneal opacity and permeability.

¹ The BCOP test method decision criteria do not propose to identify EPA Category IV substances.

² The BCOP test method was based on the use of AMCP decision criteria with a cutoff for corrosives or severe irritants of ≥ 75 tested with a 10-minute exposure time.

6.1.3 The EpiOcular Test Method

Among the 55 substances tested in the EO test method (**Table 6-1**), 30 were also tested in the Draize rabbit eye test and 25 were tested in the LVET. Of the 30 substances tested in the Draize rabbit eye test, 29 qualified for EPA hazard classification (i.e., one substance producing a Draize score greater than 1 was not evaluated through day 21 as required by EPA). For these 29 substances, accuracy of the overall EPA classification was 76% (22/29). Among the four EPA Category III substances, the EO test method correctly identified 75% (3/4). The one substance incorrectly identified (a base) was overclassified as EPA Category I. Among the nine EPA Category IV substances, 44% (4/9) were correctly identified. Four of the five incorrectly identified substances were overclassified as EPA Category III (two solvents, one acid, and one surfactant). The remaining substance (a surfactant) was overclassified as EPA Category I. All of the EPA Category I substances (15/15, including nine bases, three oxidizers, two solvents, and one "other") were correctly identified.

The EO test method correctly classified 44% (11/25) of the 25 substances tested in both the EO test method and the LVET (**Table 6-1**). Among the 12 EPA Category III substances, (67% (8/12) were correctly identified by the EO test method. The four substances incorrectly identified (two surfactants and two oxidizers) were overclassified as EPA Category I. None of the nine EPA Category IV substances were correctly identified: 44% (4/9, including three surfactants and one solvent) were overclassified as EPA Category III, and 56% (5/9, including three oxidizers and two solvents) were overclassified as EPA Category I. The EO test method correctly identified all three of the EPA Category I substances (two oxidizers and one surfactant).

6.1.4 Combining the BCOP, CM, and EO Test Methods into a Testing Strategy: AMCP Testing Strategy

The performance of each test method included in the AMCP testing strategy is summarized in **Table 6-1**. None of the 228 substances included in the AMCP BRD was tested in all three of the *in vitro* test methods proposed for the AMCP testing strategy. Therefore, no data are available with which to characterize the actual performance of the AMCP testing strategy that includes the BCOP, CM, and EO test methods.

6.2 Combining the BCOP and EO Test Methods into a Testing Strategy: Alternate AMCP Testing Strategy

The performance of the alternate AMCP testing strategy was based on the 28 substances that were tested in both the BCOP and EO test methods with Draize rabbit eye test data (**Annex IV**). As noted in **Section 2.0**, these data were evaluated based on two approaches: (1) test in the BCOP test method first and then in the EO test method, or (2) test in the EO test method first and then in the BCOP test method. Using the first approach, the BCOP test method would first classify all EPA Category I or II results. All other substances would then be tested in the EO test method and classified as either EPA Category III or IV. Using the second approach, the EO test method would first classify all EPA Category III or IV results. All other substances would then be tested in the BCOP test method and classified as either EPA Category I or II.

Regardless of which approach was used, the performance of the alternate AMCP testing strategy was the same (see **Sections 6.2.1** and **6.2.2**). The overall correct classification of the BCOP data using either the decision criteria in the AMCP BRD (**Annex I**) (IIVS ≥ 75 to assign EPA Category I) or in the BCOP BRD (ICCVAM 2006a) (IIVS ≥ 55 to assign EPA Category I) yielded identical results. All BCOP classifications, including high-solvent substances, used a 10-minute exposure time. When using 3-minute data for high solvents, the overall classification is 74% (17/23). Five high-solvent substances did not have 3-minute data and, therefore, cannot be considered in this analysis.

6.2.1 Approach 1: Test in the BCOP Test Method First and then in the EO Test Method

Using Approach 1 and either the ≥ 55.1 or ≥ 75 cutoff value to identify EPA Category I substances, the overall correct classification was 79% (22/28) (**Table 6-3**). The boxes in **Table 6-3** represent the correct calls for the BCOP test method (bolded numbers) and for the EO test method (numbers in parentheses). All of the substances classified as EPA Category I by the Draize rabbit eye test were correctly identified by the alternate AMCP testing strategy using Approach 1 (100% [14/14]). The EO test method correctly predicted all (100%; 4/4) of the EPA Category III substances and 44% (4/9) of the EPA Category IV substances. Thus, the EO test method overpredicted 56% (5/9) as EPA Category III.

6.2.2 Approach 2: Test in the EO Test Method First and then in the BCOP Test Method

Using Approach 2 and either the ≥ 55.1 or ≥ 75 cutoff value to identify EPA Category I substances, the overall correct classification was 79% (22/28) (**Table 6-4**). The boxes in **Table 6-4** represent the correct calls for the BCOP test method (bolded numbers) and for the EO test method (numbers in parentheses). The EO test method correctly identified all (100%; 4/4) of the EPA Category III substances and 44% (4/9) of the EPA Category IV substances. Five EPA Category IV substances (56% [5/9]) were overclassified by the EO test method as EPA Category III. All of the substances classified as EPA Category I by the Draize rabbit eye test were correctly identified by the alternate AMCP testing strategy using Approach 2 (100% [14/14]). The BCOP test method overpredicted one EPA Category IV substance as EPA Category II.

Table 6-3 Performance of AMCPs Tested in Both the BCOP and EO Test Methods Using Approach 1¹

EPA		Classification (BCOP→EO) ² Using Approach 1										
		I		II		III		IV		Totals		
Draize Classification	I	14 (0)		0 (0)		0 (0)		0 (0)		14		
	II	0 (0)		0 (0)		0 (1)		0 (0)		1		
	III	0 (0)		0 (0)		0 (4)		0 (0)		4		
	IV	0 (1)		1 (0)		0 (3)		0 (4)		9		
	Total	14 (1)		1 (0)		0 (8)		0 (4)		28		
EPA	Overall Classification	Draize										
		I			II			III			IV	
		Actual	Under	Over	Actual	Under	Over	Actual	Under	Over	Actual	
Approach to Identify Ocular Corrosives and Severe Irritants	79% (22/28)	100% (14/14)	0% (0/14)	0% (0/1)	0% (0/1)	100% (1/1)	0% (0/4)	100% (4/4)	0% (0/4)	56% (5/9)	44% (4/9)	

Abbreviations: BCOP = bovine corneal opacity and permeability; EO = EpiOcular; EPA = U.S. Environmental Protection Agency.

¹ Boldface numbers represent the classification by the BCOP test method, and numbers in parentheses represent the classification by the EO test method when using the alternate AMCP testing strategy.

² In the alternate AMCP testing strategy, the BCOP test method is only intended to identify EPA Category I or II substances, and the EO test method is intended to identify only EPA Category III or IV substances.

Table 6-4 Performance of AMCPs Tested in Both the BCOP and EO Test Methods Using Approach 2¹

EPA		Classification (EO→BCOP) ² Approach 2									
		I		II		III		IV		Totals	
Draize Classification	I	14 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	14
	II	0 (0)	0 (0)	0 (0)	0 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1
	III	0 (0)	0 (0)	0 (0)	0 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4
	IV	0 (0)	0 (0)	0 (0)	1 (4)	0 (4)	1 (4)	0 (4)	0 (4)	0 (4)	9
	Totals	14 (1)	0 (0)	0 (0)	1 (9)	0 (9)	1 (9)	0 (9)	0 (4)	0 (4)	28
EPA	Overall Classification	Draize									
		I		II			III			IV	
		Actual	Under	Over	Actual	Under	Over	Actual	Under	Over	Actual
Approach to Identify Category IV	79% (22/28)	100% (14/14)	0% (0/14)	0% (0/1)	0% (0/1)	100% (1/1)	0% (0/4)	100% (4/4)	0% (0/4)	56% (5/9)	44% (4/9)

Abbreviations: AMCP = antimicrobial cleaning product; BCOP = bovine corneal opacity and permeability; EO = EpiOcular; EPA = U.S. Environmental Protection Agency.

¹ Boldface numbers represent the classification by the BCOP test method, and numbers in parentheses represent the classification by the EO test method when using the alternate AMCP testing strategy.

² In the alternate AMCP testing strategy, the BCOP test method is only intended to identify EPA Category I or II substances, and the EO test method is intended to identify only EPA Category III or IV substances.

7.0 Reliability of the Test Methods Used in the Antimicrobial Cleaning Product Testing Strategy

An assessment of test method reliability is essential to any evaluation of the performance of an alternative test method (ICCVAM 2003). NICEATM assessed test method reliability by analyzing the following:

- Intralaboratory repeatability: multiple runs of a substance in a test method conducted by a single laboratory over a short period of time
- Intralaboratory reproducibility: multiple runs of a substance in a test method conducted by a single laboratory over an extended period of time under similar conditions using identical protocols
- Interlaboratory reproducibility: multiple runs of a substance in a test method conducted among several laboratories over an extended period of time under similar conditions using identical protocols

While some measures of repeatability and reproducibility were conducted using data presented in the AMCP BRD (**Annex I**), insufficient data were available to accurately determine the reliability of the test methods. Additional data on the reliability of each test method were provided by the Institute for In Vitro Sciences, Inc. as an AMCP BRD Supplement (**Annex II**). Data from the BCOP BRD (ICCVAM 2006a) were also used to establish reliability of the BCOP test method.

7.1 The Bovine Corneal Opacity and Permeability Test Method

7.1.1 Intralaboratory Repeatability

Intralaboratory repeatability for the BCOP test method was quantitatively determined for 67 AMCPs (four substances have repeat tests) as the mean %CV for opacity, permeability, and IVIS in the AMCP BRD (**Annex I**). Because %CVs are significantly affected by scores in the very low range, the mean %CVs from materials with an IVIS ≤ 10 (arbitrarily set in the AMCP BRD) were not considered in the overall mean %CV calculations. The overall mean %CV for opacity, permeability, and IVIS was 21%, 25%, and 18%, respectively.

These test substances, tested in a total of 75 runs, were also qualitatively evaluated for their concordance using the EPA (EPA 2003a) and GHS (UN 2007) classification systems (**Annex II**). For the EPA and GHS classification systems, there was 100% agreement for 63 of the 75 runs (84%), 67% agreement for 11 of the 75 runs (15%), and 60% agreement for 1 of the 75 runs (1%). Of the 12 runs that did not have 100% agreement, seven had reactive chemistries, two were alkalis, two were surfactants, and one was an acid.

Intralaboratory repeatability for the BCOP test method was quantitatively determined for non-AMCPs predicted as severe or ocular corrosives in the BCOP test method in three studies (16–52 substances) (ICCVAM 2006a). The mean %CV for IVIS ranged from 39% to 71%.

7.1.2 Intralaboratory Reproducibility

Intralaboratory reproducibility for the BCOP test method was quantitatively determined for AMCPs (n=5) as the mean %CV for IVIS. For these five substances (2–6 experiments), the mean %CV for IVIS was 20% (see Section 7.3 of the AMCP BRD, **Annex I**).

These test substances were also qualitatively evaluated for their concordance using the EPA (EPA 2003a) and GHS (UN 2007) classification systems (see Section 3.2 of the AMCP BRD Supplement, **Annex II**). Using either the EPA or GHS classification systems, there was 100% agreement for the five test substances.

Intralaboratory reproducibility for the BCOP test method has been quantitatively determined for non-AMCPs predicted as severe or ocular corrosives in the BCOP test method in two studies (ICCVAM 2006a). In one study composed of 25 surfactant-based personal care cleaning formulations, the mean %CV for permeability values was 33%. In the second study (n=16), the mean %CV for IVIS ranged from 13% to 15%.

7.1.3 Interlaboratory Reproducibility

Interlaboratory reproducibility the BCOP test method cannot be determined specifically for the AMCPs presented in the AMCP BRD (**Annex I**) because only one laboratory conducted the testing.

Interlaboratory reproducibility for the BCOP test method has been quantitatively determined for non-AMCPs predicted as severe or ocular corrosives in the BCOP test method in three studies (3-12 laboratories each) (ICCVAM 2006a). The mean %CV for IVIS ranged from 25% to 36%. These test substances were also qualitatively evaluated (ICCVAM 2006a) for their concordance using the EPA (EPA 2003a), GHS (UN 2007), and EU (EU 2001) classification systems.

7.2 The Cytosensor Microphysiometer Test Method

7.2.1 Intralaboratory Repeatability

Reliability for the CM test method could not be evaluated specifically for AMCPs due to insufficient data. However, quantitative evaluations of reliability were conducted based on non-AMCPs tested in the CM test method (**Annexes I and II**).

Intralaboratory repeatability for the CM test method was quantitatively evaluated for non-AMCPs in seven studies (n=1–35 test substances per study) (**Annexes I and II**). The mean %CV for MRD₅₀ values for all materials tested, including surfactant and nonsurfactant materials, ranged from 6% to 25%.

7.2.2 Intralaboratory Reproducibility

Intralaboratory reproducibility for the CM test method was quantitatively determined for non-AMCPs in one laboratory (16 substances) (**Annex I**). The mean %CV for MRD₅₀ values for all materials tested, including surfactant and nonsurfactant materials, was 25%.

7.2.3 Interlaboratory Reproducibility

Interlaboratory reproducibility for the CM test method was quantitatively determined for non-AMCPs in two studies (2–4 laboratories each) (**Annexes I and II**). The mean %CV for MRD₅₀ values for all materials tested, including surfactant and nonsurfactant materials, ranged from 17% to 51%. Nonsurfactant materials had a higher mean %CV in each study.

7.3 The EpiOcular Test Method

7.3.1 Intralaboratory Repeatability

Intralaboratory repeatability for the EO test method was quantitatively determined specifically for a subset of AMCPs (n=15) presented in the AMCP BRD (**Annex I**). The mean %CV for ET₅₀ values ranged from 0% to 62%.

Qualitative analyses were conducted with three AMCPs that were tested more than once at the Institute for In Vitro Sciences, Inc. to evaluate the extent of agreement using the EPA (EPA 2003a) or GHS (UN 2007) hazard classification system (**Annex II**). There was 100% agreement for all three AMCPs for both EPA and GHS classification systems.

7.3.2 Intralaboratory Reproducibility

Intralaboratory reproducibility for the EO test method was also quantitatively determined from repeat testing of a single substance (0.3% Triton[®] X-100). Data were presented as combined data from MatTek Corporation and the Institute for In Vitro Sciences, Inc. (9-year period) and from the Institute for In Vitro Sciences, Inc., only (8-year period). The mean %CV for ET₅₀ values was 21% and 22%, respectively.

7.3.3 Interlaboratory Reproducibility

Interlaboratory reproducibility for the EO test method cannot be determined specifically for the AMCPs presented in the AMCP BRD (**Annex I**) because only one laboratory conducted the testing. However, interlaboratory reproducibility for the EO test method was quantitatively determined for non-AMCPs in a two-phase validation study for surfactants and surfactant-containing products, which is summarized in the AMCP BRD (**Annex I**). Based on the validation study, the mean %CVs ranged from 12% to 18%. However, it should be noted that this evaluation of reproducibility is based on an EO protocol that uses relative percent viability to assign an irritancy classification (i.e., irritant vs. nonirritant) and not on a calculated ET₅₀ value to predict the ocular hazard classification category (i.e., EPA Category I, II, III, and IV). The latter is the protocol included in the AMCP BRD.

These test substances were also qualitatively evaluated for their concordance using the EPA (EPA 2003a) and GHS (UN 2007) classification systems (**Annex II**). Using either the EPA or GHS classification systems in Phase II of the validation study, there was 100% agreement for 74% (14/19) of the substances, 75% agreement for 11% (2/19) of the substances, and 50% agreement for 16% (3/19) of the substances among four laboratories. In Phase III of the validation study using the EPA or GHS classification systems, there was 100% agreement for 94% (51/54) of the substances and 0% agreement for 6% (3/54) of the substances in two laboratories.

8.0 Data Quality: Antimicrobial Cleaning Product Background Review Document

8.1 Adherence to National and International Good Laboratory Practice Guidelines

The extent to which the studies included in the AMCP BRD (**Annex I**) complied with national and international Good Laboratory Practice (GLP) guidelines (OECD 1998; EPA 2003b, 2003c; FDA 2003) is based on the information provided in the AMCP BRD. While it could not be ascertained whether all of the *in vitro* data provided in the AMCP BRD were GLP compliant, the data determined to be GLP compliant were noted in the spreadsheets that contain the study data. All of the laboratories that contributed data for these studies have experience conducting GLP-compliant studies. All of the new data generated for the studies in the AMCP BRD were collected according to GLP guidelines.

8.2 Data Quality Audits

Formal assessments of data quality, such as quality assurance audits, generally involve a systematic and critical comparison of the data provided in the study report to the laboratory records generated during the study. No data quality audits were specifically conducted in the preparation of the AMCP BRD (**Annex I**). However, the studies conducted according to GLP guidelines would have included such an audit.

8.3 Impact of Deviations from GLP Guidelines

The impact of deviations from GLP guidelines cannot be evaluated because no information on data quality audits was obtained.

8.4 Availability of Laboratory Notebooks or Other Records

The original study notebooks, final reports, and other background information were available for the majority of the studies reported in the AMCP BRD (**Annex I**). The individual companies that contributed data to the AMCP BRD consider these materials confidential and requested that they not be associated with any particular product. Thus, the study materials are available for inspection, if requested by NICEATM or the EPA, with company identifiers removed to ensure compliance with this request.

9.0 Other Scientific Reports and Reviews

9.1 The Bovine Corneal Opacity and Permeability Test Method

For the BCOP test method, NICEATM identified four studies that had been published since the previous evaluation of the BCOP test method for the identification of ocular corrosives and severe irritants (ICCVAM 2006a): Debbasch et al. 2005; Van Goethem et al. 2006; Cater and Harbell 2006; and Cater and Harbell 2008. However, none of these publications included Draize rabbit eye test data; therefore, these studies were not added to the database.

9.1.1 Debbasch et al. (2005)

Twelve makeup removers were tested in both the BCOP test method and in a clinical in-use test under ophthalmological control. The undiluted test product (750 µL) was pipetted onto the corneas and exposure was conducted for 4 hours. Corneal opacity was determined using an adapted spectrophotometer and barrier disruption by fluorescein uptake using OD₄₉₀ mm. *In vitro* scores were classified according to Gautheron et al. (1994) and Harbell and Curren (1998).

9.1.2 Cater and Harbell (2006)

Surfactant-based “rinse-off” personal care formulations were tested in the BCOP test method using slight modifications of the BCOP test method protocol reported by Sina et al. (1995). Corneas were exposed to the test substances (750 µL) for 10, 30, or 60 minutes either undiluted or diluted in deionized water. Corneas were evaluated for opacity, fluorescein uptake, and histological alterations.

9.1.3 Van Goethem et al. (2006)

Van Goethem et al. tested 20 substances in the BCOP test method (7 compounds classified as GHS Not Classified and 13 GHS Category 1). Vanparys et al. (1993) and Gautheron et al. (1994) previously published these results, which were included in the BCOP BRD (ICCVAM 2006a).

9.1.4 Cater and Harbell (2008)

The BCOP test method was used on four commercial and one unregistered body wash. The purpose was to determine if the BCOP test method could be used as a prediction model for relative ranking of human eye responses to surfactant-based formulations under conditions of a standard human eye sting test. Test articles were prepared as 25% solutions in deionized water; 750 µL was applied to the corneas for a 30-minute exposure. Following exposure, opacity and fluorescein uptake were determined.

9.2 The Cytosensor Microphysiometer Test Method

A BRD for the CM test method,⁶ which includes a comprehensive review of all available data, was submitted to the European Centre for the Validation of Alternative Methods (ECVAM) for review of its validation status in Europe.

⁶ A redacted version of the ECVAM CM BRD is available on the NICEATM-ICCVAM website. The main body of the document is available at <http://iccvam.niehs.nih.gov/methods/ocutox/CM/ECVAM-CMBRD-Aug08redact.pdf> and the annexes to the document are available at <http://iccvam.niehs.nih.gov/methods/ocutox/CM/CMBRD-AnnexesAug08redact.pdf>.

9.3 The EpiOcular Test Method

A BRD for the EO test method, which includes a comprehensive review of all available data, was submitted to ECVAM for review of its validation status in Europe. To date, this document has not been made available to the public.

10.0 Animal Welfare Considerations

10.1 How the AMCP Testing Strategy and *In Vitro* Methods will Refine, Reduce, or Replace Animal Use

Draize rabbit eye test data are currently used to classify and label AMCPs. The AMCP testing strategy described in the AMCP BRD (**Annex I**) or the alternate AMCP testing strategy would provide a non-animal approach to EPA classification and labeling of AMCPs and could thereby eliminate the use of rabbits for this type of testing.

10.2 Requirements for the Use of Animals

The EPA currently requires a Draize rabbit eye test for classification and labeling of AMCPs. The Draize rabbit eye test protocol is provided in the EPA Health Effects Test Guideline (OPPTS 87.2440; EPA 1998) and in OECD Test Guideline 405 (OECD 2002). The Draize rabbit eye test requires only one animal if the test substance is shown to be corrosive or a severe (irreversible) eye irritant. It requires three animals per test substance for nonsevere irritants or nonirritants. These animals are in addition to similar sets of animals for both the positive and negative control groups within a study of multiple test substances. More animals may be required if the EPA classification results are equivocal.

The BCOP test method uses ocular tissue obtained from animals that are being procured for food. Cattle are not subject to pain and suffering during the harvest of corneal tissue, because it is obtained post mortem and would otherwise be discarded by the meatpacker.

No animals are used for the CM test method, except for the mice used to establish the original mouse fibroblast cell line.

The EO test method uses a three-dimensional corneal construct generated with primary human keratinocytes. These cells are obtained during routine surgical procedures, and their procurement to initiate a cell culture does not subject the donor to any pain or suffering.

11.0 Practical Considerations

Several issues in addition to performance evaluations must be considered when assessing the practicality of an alternative test method in comparison to the existing test method:

- Laboratory equipment and supplies needed to conduct the alternative test method
- Level of personnel training
- Labor costs
- Time required to complete the test method

The time, personnel cost, and effort required to conduct the proposed test method must be considered reasonable in comparison to those of the test method it is intended to replace.

11.1 Transferability of the Test Methods Included in the AMCP Testing Strategy

Test method transferability addresses the ability of a test method to be performed accurately and reliably by multiple laboratories (ICCVAM 2003), including those experienced in the particular type of procedure and those with less or no experience in the particular procedure. The degree of transferability of a test method can be evaluated based on interlaboratory reproducibility (see **Section 7.0**).

One important consideration regarding the transferability of the CM test method is that the instrument has been discontinued. Therefore, a user would have to obtain a used instrument or have one manufactured before testing.

11.2 Training Considerations

The AMCP BRD (**Annex I**) details the level of training and expertise needed to conduct the test methods used in the AMCP testing strategy and the training requirements needed to demonstrate proficiency based on the ICCVAM test method submission guidelines (ICCVAM 2003).

11.3 Cost Considerations

At the present time, the cost of running a GLP-compliant Draize rabbit eye test ranges from \$1200 to \$14,500 depending on the number of days the animals have to remain on the study (i.e., 21 days or less). A GLP-compliant BCOP test method will cost approximately \$1500 for a single test substance. The cost of performing the BCOP test method is approximately doubled when histopathology evaluation is included. A GLP-compliant CM test method will cost approximately \$2000 for each of a minimum of two test substances. A GLP-compliant EO test method will cost approximately \$3000 for a single test substance. For each of these *in vitro* test methods, the cost per sample is significantly reduced when multiple substances are run concurrently.

11.4 Time Considerations

The Draize rabbit eye test or the LVET could require up to 21 days, in addition to several pretest days to acclimatize the animals. The BCOP test method can be completed in one day, but histopathology evaluation may require an additional four weeks. The CM test method, including multiple runs of the test substance, can be completed in a one day. The EO test method can be performed in two days, although it may take several weeks to acquire the tissue.

12.0 References

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13.0 Glossary⁷

Accuracy:⁸ (a) The closeness of agreement between a test method result and an accepted reference value. (b) The proportion of correct outcomes of a test method. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *concordance* (see also *two-by-two table*). Accuracy is highly dependent on the prevalence of positives in the population being examined.

Antimicrobial cleaning product (AMCP): Commercially available household cleaning products are regulated by the CPSC. However, when an antimicrobial claim is made, these products must be registered as pesticides with the EPA.

Blepharitis: Inflammation of the eyelid.

Chemosis: A form of eye irritation in which the membranes that line the eyelids and surface of the eye (*conjunctivae*) become swollen.

Classification system: An arrangement of quantified results or data into groups or categories according to previously established criteria.

Coded substances: Substances labeled by code rather than name so that they can be tested and evaluated without knowledge of their identity or anticipation of test results. Coded substances are used to avoid intentional or unintentional bias when evaluating laboratory or test method performance.

Coefficient of variation: A statistical representation of the precision of a test. It is expressed as a percentage and is calculated as follows:

$$\left(\frac{\text{standard deviation}}{\text{mean}} \right) \times 100\%$$

Concordance:⁸ The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *accuracy* (see also *two-by-two table*). Concordance is highly dependent on the prevalence of positives in the population being examined.

Conjunctiva: The mucous membrane that lines the inner surfaces of the eyelids and folds back to cover the front surface of the eyeball, except for the central clear portion of the outer eye (the cornea). The conjunctiva is composed of three sections: palpebral conjunctiva, bulbar conjunctiva, and fornix.

Conjunctival sac: The space located between the eyelid and the conjunctiva-covered eyeball. Substances are instilled into the sac to conduct an *in vivo* eye test.

Cornea: The transparent part of the coat of the eyeball that covers the iris and pupil and admits light to the interior.

Corneal opacity: Measurement of the extent of opaqueness of the cornea following exposure to a test substance. Increased corneal opacity is indicative of damage to the cornea. Opacity can be evaluated

⁷ The definitions in this glossary are restricted to their uses with respect to the AMCP test methods and testing strategy.

⁸ Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM 2003).

subjectively as done in the Draize rabbit eye test, or objectively with an instrument such as an opacitometer.

Corneal permeability: Quantitative measurement of damage to the corneal epithelium by a determination of the amount of sodium fluorescein dye that passes through all corneal cell layers.

Corrosion: Destruction of tissue at the site of contact with a substance.

Corrosive: A substance that causes irreversible tissue damage at the site of contact.

Endpoint:⁸ The biological process, response, or effect assessed by a test method.

Essential test method components:⁸ Structural, functional, and procedural elements of a test method that are used to develop the test method protocol. These components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to essential test method components is necessary when the acceptability of a proposed test method is being evaluated based on performance standards derived from mechanistically and functionally similar validated test method. [Note: Previously referred to as *minimum procedural standards*]

False negative:⁸ A substance incorrectly identified as negative by a test method.

False negative rate:⁸ The proportion of all positive substances falsely identified by a test method as negative (see *two-by-two table*). It is one indicator of test method accuracy.

False positive:⁸ A substance incorrectly identified as positive by a test method.

False positive rate:⁸ The proportion of all negative substances that are falsely identified by a test method as positive (see *two-by-two table*). It is one indicator of test method accuracy.

Globally Harmonized System (GHS): A classification system presented by the United Nations that provides (a) a harmonized criteria for classifying substances and mixtures according to their health, environmental and physical hazards and (b) harmonized hazard communication elements, including requirements for labeling and safety data sheets.

Good Laboratory Practice (GLP):⁸ Regulations promulgated by the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency, and principles and procedures adopted by the OECD and Japanese authorities, which describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submissions to national regulatory agencies.

Hazard:⁸ The potential for an adverse health or ecological effect. Hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

Interlaboratory reproducibility:⁸ A measure of whether different qualified laboratories using the same protocol and test substances can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the prevalidation and validation processes and indicates the extent to which a test method can be transferred successfully among laboratories.

Intralaboratory repeatability:⁸ The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period.

Intralaboratory reproducibility:⁸ The first stage of validation; a determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times.

In vitro: In glass; Refers to test methods that are carried out in an artificial system (e.g., in a test tube or petri dish) and typically use single-cell organisms, cultured cells, cell-free extracts, or purified cellular components.

In vitro irritancy score (IVIS): An empirically derived formula used in the BCOP test method whereby the mean opacity and mean permeability values for each treatment group are combined into a single *in vitro* score for each treatment group. The *in vitro* irritancy score = mean opacity value + (15 x mean permeability value).

In vivo: In the living organism. Refers to test methods performed in multicellular organisms.

Iris: The contractile diaphragm perforated by the pupil and forming the colored portion of the eye.

Negative predictivity:⁸ The proportion of correct negative responses among substances testing negative by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the substances tested.

Nonirritant: (a) A substance that produces no changes in the eye following its application to the anterior surface of the eye. (b) Substances that are not classified as GHS Category 1, 2A, or 2B; or EU R41 or R36 ocular irritants.

Nonsevere irritant: (a) A substance that causes tissue damage in the eye following application to the anterior surface of the eye; the tissue damage is reversible within 21 days of application and the observed adverse effects in the eye are less severe than observed for a severe irritant. (b) Substances that are classified as GHS Category 2A or 2B; EPA Category II, III, or IV; or EU R36 ocular irritants.

Ocular: Relating to the eye.

Ocular corrosive: A substance that causes irreversible tissue damage in the eye following application to the anterior surface of the eye.

Ocular irritant: A substance that produces a reversible change in the eye following application to the anterior surface of the eye.

Opacitometer: An instrument used to measure “corneal opacity” by quantitatively evaluating light transmission through the cornea. The instrument has two compartments, each with its own light source and photocell. One compartment is used for the treated cornea, while the other is used to calibrate and zero the instrument. The difference between photocell signals in the two compartments is measured electronically as a change in voltage, and is displayed digitally, generating numerical opacity values with arbitrary units.

Pannus: A specific type of corneal inflammation that begins within the conjunctiva, and with time spreads to the cornea. Also referred to as “chronic superficial keratitis.”

Performance:⁸ The accuracy and reliability characteristics of a test method (see *accuracy, reliability*).

pH: A measure of the acidity or alkalinity of a solution. pH 7.0 is neutral; higher pHs are alkaline, lower pHs are acidic.

Positive control: A substance known to induce a positive response used to demonstrate the sensitivity of the test method and to allow for an assessment of variability in the conduct of the test method over time. For most test methods, the positive-control substance is tested concurrently with the test substance and the vehicle/solvent control. However, for some *in vivo* test methods, periodic studies using a positive-control substance is considered adequate by the OECD.

Positive predictivity:⁸ The proportion of correct positive responses among substances testing positive by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Positive predictivity is a function of the sensitivity of the test method and the prevalence of positives among the substances tested.

Prevalence:⁸ The proportion of positives in the population of substances tested (see *two-by-two table*).

Protocol:⁸ The precise, step-by-step description of a test, including the listing of all necessary reagents, criteria, and procedures for the evaluation of the test data.

Quality assurance:⁸ A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures is assessed independently by individuals other than those performing the testing.

Reduction alternative:⁸ A new or modified test method that reduces the number of animals required.

Reference test method:⁸ The accepted *in vivo* test method used for regulatory purposes to evaluate the potential of a test substance to be hazardous to the species of interest.

Refinement alternative:⁸ A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal wellbeing.

Relevance:⁸ The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the *accuracy* or *concordance* of a test method.

Reliability:⁸ A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and interlaboratory reproducibility and intralaboratory repeatability.

Replacement alternative:⁸ A new or modified test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate).

Reproducibility:⁸ The consistency of individual test results obtained in a single laboratory (*intralaboratory reproducibility*) or in different laboratories (*interlaboratory reproducibility*) using the same protocol and test substances (see intra- and *interlaboratory reproducibility*).

Sclera: The tough, fibrous tissue that extends from the cornea to the optic nerve at the back of the eye.

Secondary bacterial keratitis: Inflammation of the cornea that occurs secondary to another insult that compromised the integrity of the eye.

Sensitivity:⁸ The proportion of all positive substances that are classified correctly as positive in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Severe irritant: (a) A substance that causes tissue damage in the eye following application to the anterior surface of the eye that is not reversible within 21 days of application or causes serious physical decay of vision. (b) Substances that are classified as GHS Category 1, EPA Category I, or EU R41 ocular irritants.

Solvent control: An untreated sample containing all components of a test system, including the solvent that is processed with the test substance-treated and other control samples to establish the baseline response for the samples treated with the test substance dissolved in the same solvent. When tested with a concurrent negative control, this sample also demonstrates whether the solvent interacts with the test system.

Specificity:⁸ The proportion of all negative substances that are classified correctly as negative in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Test:⁸ The experimental system used; used interchangeably with *test method* and *test method*.

Test method:⁸ A process or procedure used to obtain information on the characteristics of a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified conditions. Used interchangeably with *test* and *test method*. See also *validated test method* and *reference test*.

Tiered testing: A testing strategy where all existing information on a test substance is reviewed, in a specified order, prior to *in vivo* testing. If the irritancy potential of a test substance can be assigned, based on the existing information, no additional testing is required. If the irritancy potential of a test substance cannot be assigned, based on the existing information, a step-wise animal testing procedure is performed until an unequivocal classification can be made.

Toxic keratoconjunctivitis: Inflammation of the cornea and conjunctiva due to contact with an exogenous agent. Used interchangeably with *contact keratoconjunctivitis*, *irritative keratoconjunctivitis*, and *chemical keratoconjunctivitis*.

Transferability:⁸ The ability of a test method or procedure to be accurately and reliably performed in different, competent laboratories.

Two-by-two table:⁸ The two-by-two table can be used for calculating accuracy (concordance) ($(a+d)/(a+b+c+d)$), negative predictivity ($d/(c+d)$), positive predictivity ($a/(a+b)$), prevalence ($(a+c)/(a+b+c+d)$), sensitivity ($a/(a+c)$), specificity ($d/(b+d)$), false positive rate ($b/(b+d)$), and false negative rate ($c/(a+c)$).

		New Test Outcome		
		Positive	Negative	Total
Reference Test Outcome	Positive	a	c	a + c
	Negative	b	d	b + d
	Total	a + b	c + d	a + b + c + d

Validated test method:⁸ An accepted test method for which validation studies have been completed to determine the relevance and reliability of this method for a specific proposed use.

Validation:⁸ The process by which the reliability and relevance of a procedure are established for a specific purpose.

Vehicle control: An untreated sample containing all components of a test system, including the vehicle that is processed with the test substance-treated and other control samples to establish the baseline response for the samples treated with the test substance dissolved in the same vehicle.

Weight of evidence (process): The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

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Annex I

Background Review Document of an *In Vitro* Approach for EPA Toxicity Labeling of Antimicrobial Cleaning Products

The Background Review Document is available on request from NICEATM.

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Annex II

Supplement to a Background Review Document of an *In Vitro* Approach for EPA Toxicity Labeling of Antimicrobial Cleaning Products

The BRD supplement is available on request from NICEATM.

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Annex III

Composition of Substances Evaluated in the Alternate AMCP Testing Strategy

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Composition of Substances Evaluated in the Alternate AMCP Testing Strategy

Formulation	Ingredients	% Conc. (w/w)
H, Disinfectant / Cleaner Concentrate	Water	90–95
	Solvent	1–5
	Inorganic phosphates	1–5
	Nonionic surfactant	<1
	Inorganic alkalis	<1
	Quaternary	<1
	Inorganic salt	<1
	Fragrance	<1
	Nonionic surfactant	<1
	Thickeners	<1
I, Disinfectant/ Cleaner RTU	Water	95–100
	Inorganic salt	<1
	Inorganic phosphate	<1
	Quaternary	<1
	Nonionic surfactant	<1
	pH adjuster	<1
	Chelator	<1
	Fragrance	<1
	Dyes	<1
J, Disinfectant/ Cleaner RTU	Water	90–95
	Solvent	1–5
	Nonionic surfactant	1–5
	pH adjuster	<1
	Fragrance	<1
	Quaternary	<1
	Amine	<1
	Thickener	<1
	Dye	<1
K, Disinfectant	Water	95–100
	Inorganic salt	<1
	Anionic surfactant	<1
	TCM	<1
	pH adjuster	<1

Composition of Substances Evaluated in the Alternate AMCP Testing Strategy

Formulation	Ingredients	% Conc. (w/w)
P, Disinfectant / Cleaner RTU	Water	95–100
	Phenolics	<1
	Anionic surfactant	<1
	Solvent	<1
	Chelator	<1
	pH adjuster	<1
	Solvent	<1
	Anionic surfactant	<1
	Antioxidants	<1
	Inorganic salts	<1
	Fragrance	<1
Dye	<1	
R, Disinfectant	Water	95–100
	Quaternary	<1
	Dye	<1
T, Descaler	Water	75–80
	Inorganic acid	10–15
	pH adjuster	1–5
W, Disinfectant RTU	Water	95–100
	Quaternary	<1
	Fragrance	<1
AG, Metal Cleaner	Water	65–70
	Chelator	5–10
	NaOH	5–10
	Nonionic surfactant	5–10
	KOH	<1
AH, Degreaser	Water	65–70
	Anionic surfactant	5–10
	Chelator	5–10
	Nonionic surfactant	5–10
	KOH	1–5
	Inorganic salt	1–5
	Glycol ether	1–5

Composition of Substances Evaluated in the Alternate AMCP Testing Strategy

Formulation	Ingredients	% Conc. (w/w)
AI, Heavy Duty Cleaner/ Degreaser	Water	75–80
	Inorganic salts	10–15
	Chelator	1–5
	NaOH	1–5
	Nonionic surfactant	1–5
	Amphoteric surfactant	1–5
	Dye	<1
AJ, Heavy Duty Cleaner	Water	65–70
	Inorganic salts	10–15
	Anionic surfactant	5–10
	Chelator	1–5
	NaOH	1–5
	Nonionic surfactant	1–5
	Amphoteric surfactant	1–5
AK, Floor Stripper	Water	55–60
	Glycol ether	10–15
	Inorganic salt	5–10
	Amphoteric surfactant	5–10
	MEA	1–5
	NaOH	1–5
	Chelator	1–5
	Nonionic surfactants	<1
	Fragrance	<1
AL, Cleaner/ Degreaser	Water	65–70
	Glycol ether	10–15
	Anionic surfactant	1–5
	Inorganic salt	1–5
	Chelator	1–5
	Nonionic surfactant	5–10
	NaOH	1–5
	Dye	<1

Composition of Substances Evaluated in the Alternate AMCP Testing Strategy

Formulation	Ingredients	% Conc. (w/w)
AM, Glass Cleaner	Water	65–70
	Glycol ether	20–25
	Ammonium Hydroxide	1–5
	Anionic surfactant	1–5
	Chelator	1–5
	Dye	<1
AN, General Cleaner	Water	75–80
	Anionic surfactant	10–15
	Nonionic surfactant	5–10
	Chelator	1–5
	Inorganic salt	1–5
AO, Floor Stripper	Water	60–65
	Glycol ether	10–15
	Anionic surfactant	10–15
	MEA	5–10
	Organic solvent	1–5
	Inorganic salt	1–5
	NaOH	1–5
	Chelator	<1
	Fluorochemical	<1
	Fragrance	<1
AP, All Purpose Cleaner	Water	80–85
	Inorganic salt	5–10
	NaOH	1–5
	Nonionic surfactant	1–5
	Anionic surfactant	1–5
	KOH	<1
	Chelator	<1
	Amphoteric surfactant	<1
	Fragrance	<1
	Dye	<1

Composition of Substances Evaluated in the Alternate AMCP Testing Strategy

Formulation	Ingredients	% Conc. (w/w)
AT, Drain Cleaner	Water	55–60
	Bleach	5–10
	Inorganic base	<1
	Inorganic salt	<1
	Anionic surfactant	<1
AU, Drain Cleaner	Water	55–60
	Bleach	5–10
	Inorganic base	1–5
	Inorganic salt	<1
	Anionic surfactant	<1
AV, Drain Cleaner	Water	55–60
	Bleach	5–10
	Inorganic base	1–5
	Inorganic salt	<1
	Anionic surfactant	<1
AX, Floor Stripper	Water	50–55
	Glycol ether	30–35
	MEA	10–15
	Organic solvent	1–5
	Ammonium Hydroxide	1–5
	Anionic surfactant	1–5
BB, Glass Cleaner	Water	90–95
	Dye	<1
	Inorganic base	<1
	Chelator	<1
	Solvent	5–10
BE, Toilet Bowl Cleaner	Water	90–95
	Organic acid	1–5
	Anionic surfactant	1–5
	Thickener	<1
	Dye	<1
	Fragrance	<1

Composition of Substances Evaluated in the Alternate AMCP Testing Strategy

Formulation	Ingredients	% Conc. (w/w)
BK, Glass Cleaner	Water	90–95
	Dye	<1
	Solvent	1–5
	Anionic surfactant	<1
	Fragrance	<1
	Chelator	<1
	Inorganic base	<1
BM, Glass Cleaner	Water	90–95
	Solvent	5–10
	Inorganic base	<1
	pH adjuster	<1
	Anionic surfactant	<1
BL, Glass Cleaner	Water	90–95
	Chelator	<1
	Solvent	5–10
	Anionic surfactant	<1
	Dye	<1
	Inorganic base	<1
BN, Toilet Bowl Cleaner	Water	90–95
	Nonionic surfactant	1–5
	Anionic surfactant	1–5
	Preservative	<1
	Thickener	<1
	Dye	<1
	Fragrance	<1
	Phosphate	<1

Annex IV

Data Used for the Performance Analysis of the Alternate AMCP Testing Strategy

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Code	High Solvent ¹	<i>In Vivo</i> Classification		EpiOcular		BCOP ³					
		GHS (Draize)	EPA (Draize)	ET ₅₀ (min) ²	EPA Classification	IVIS (10 min)	EPA Classification (Cat 1 ≥75; 10 min)	EPA Classification (Cat 1 ≥55.1; 10 min)	IVIS (3 min) ⁴	EPA Classification (Cat 1 ≥75; 3 min) ⁴	EPA Classification (Cat 1 ≥55.1; 3 min) ⁴
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 ¹	<i>In Vivo</i> Classification		EpiOcular		BCOP ³					
		GHS (Draize)	EPA (Draize)	ET ₅₀ (min) ²	EPA Classification	IVIS (10 min)	EPA Classification (Cat 1 ≥75; 10 min)	EPA Classification (Cat 1 ≥55.1; 10 min)	IVIS (3 min) ⁴	EPA Classification (Cat 1 ≥75; 3 min) ⁴	EPA Classification (Cat 1 ≥55.1; 3 min) ⁴
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.

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

² ET₅₀ values represent the time needed to reduce cell viability by 50%.

³ 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).

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

Appendix D1

Summary Minutes from the Peer Review Panel Meeting on May 19-21, 2009

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Summary Minutes

Independent Scientific Peer Review Panel Meeting

Evaluation of the Validation Status of Alternative Ocular Safety Testing Methods and Approaches

Consumer Product Safety Commission Headquarters

Fourth Floor Hearing Room

Bethesda Towers Building

Bethesda, MD

May 19 - 21, 2009

Peer Review Panel Members:

A. Wallace Hayes, Ph.D., DABT, FATS, ERT (Peer Review Panel Chair)	Visiting Scientist (Harvard), Harvard School of Public Health, Andover, MA; Principal Advisor, Spherix Incorporated, Bethesda, MD
Hongshik Ahn, Ph.D.	Professor, Stony Brook University, Stony Brook, NY
Paul Bailey, Ph.D.	Bailey & Associates Consulting, Neshanic Station, NJ
Richard Dubielzig, D.V.M.	Professor, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI
Henry Edelhauser, Ph.D. ¹	Professor of Ophthalmology and Director of Ophthalmic Research, Emory University School of Medicine, Atlanta, GA
Mark Evans, D.V.M., Ph.D., DACVP	Pathology Lead for Ophthalmology Therapeutic Area, Pfizer Global Research and Development at La Jolla Drug Safety Research and Development, San Diego, CA
James Jester, Ph.D.	Professor of Ophthalmology and Biomedical Engineering, Endowed Chair, University of California-Irving, Orange, CA

¹ Unable to attend the Panel meeting, but participated in the review of all materials.

Peer Review Panel Members:

Tadashi Kosaka, D.V.M., Ph.D.	Associate Director, Chief, Laboratory of Immunotoxicology and Acute Toxicology, Toxicology Division, The Institute of Environmental Toxicology, Ibaraki, Japan
Alison McLaughlin, M.Sc., DABT	Health Canada, Environmental Impact Initiative, Office of Science and Risk Management, Health Products and Food Branch, Ottawa, Ontario, Canada
J. Lynn Palmer, Ph.D.	Associate Professor, Department of Palliative Care and Rehabilitation Medicine, University of Texas, MD Anderson Cancer Center, Houston, TX
Robert Peiffer, Jr., D.V.M., Ph.D., DACVO	Senior Investigator, Merck Research Laboratories, Safety Assessment Toxicology, West Point, PA
Denise Rodeheaver, Ph.D., DABT	Assistant Director, Alcon Research Ltd., Department of Toxicology, Fort Worth, TX
Donald Sawyer, D.V.M., Ph.D., DACVA	Professor Emeritus, Retired, College of Veterinary Medicine, Michigan State University, East Lansing, MI
Kirk Tarlo, Ph.D., DABT	Scientific Director, Comparative Biology and Safety Sciences, Amgen, Inc., Thousand Oaks, CA
Daryl Thake, D.V.M., Dipl. ACVP ¹	Midwest ToxPath Sciences, Inc., Chesterfield, MO
Scheffer Tseng, M.D., Ph.D. ¹	Director, Ocular Surface (OS) Center, Medical Director OS Research & Education Foundation, Directory R&D Department, Tissue Tech, Inc., Ocular Surface Center, P.A., Miami, FL
Jan van der Valk, Ph.D.	Senior Scientist, Departments of Animals, Science and Society, Faculty of Veterinary Medicine, Utrecht University, Netherlands Centre Alternatives to Animal Use (NCA), Utrecht, Netherlands
Philippe Vanparrys, Ph.D., DABT	Managing Director, CARDAM (VITO), Mol, Belgium
Maria Pilar Vinardell, Ph.D.	Director, Department of Physiology, Professor of Physiology and Pathology, Department Fisologia, Facultat de Farmacia, Universitat de Barcelona, Barcelona, Spain
Sherry Ward, Ph.D., M.B.A.	In Vitro Toxicology Consultant, BioTred Solutions, Science Advisor, International Foundation for Ethical Research, New Market, MD

Peer Review Panel Members:

Daniel Wilson, Ph.D., DABT	Mammalian Toxicology Consultant, Toxicology and Environmental Research Consulting, The Dow Chemical Company, Midland, MI
Fu-Shin Yu, Ph.D.	Director of Research, Department of Ophthalmology & Anatomy, School of Medicine, Wayne State University, Detroit, MI

ICCVAM and ICCVAM Ocular Toxicity Working Group Members:

Meta Bonner, Ph.D.	EPA, OPP, Washington, DC
Robert Bronaugh, Ph.D.	FDA, CFSAN, College Park, MD
Pertti Hakkinen	NLM, Bethesda, MD
Masih Hashim, D.V.M., Ph.D.	EPA, OPP, Washington, DC
Jodie Kulpa-Eddy, D.V.M. (ICCVAM Vice-Chair)	USDA, Riverdale, MD
Donnie Lowther	FDA, CFSAN, College Park, MD
Deborah McCall	EPA, OPP, Washington, DC
Jill Merrill, Ph.D. (OTWG Chair)	FDA, CDER, Silver Spring, MD
John Redden	EPA, OPP, Crystal City, VA
RADM William Stokes, D.V.M., DACLAM (Director, NICEATM)	NIEHS, Research Triangle Park, NC
Marilyn Wind, Ph.D., (ICCVAM Chair)	CPSC, Bethesda, MD

Invited Experts:

Rodger Curren, Ph.D.	Institute for In Vitro Sciences (IIVS), Gaithersburg, MD
Arnhold Schrage, Ph.D.	Experimental Toxicology and Ecology, BASF SE, Ludwigshafen, Germany

European Centre for the Validation of Alternative Methods, ICCVAM OTWG Liaison:

João Barroso, Ph.D.

European Centre for the Validation of Alternative
Methods, Ispra, Italy

Public Attendees:

Attendee	Affiliation	Day Attended		
		1	2	3
Odelle Alexander	Syngenta Crop Protection, Greensboro, NC	√	√	√
Ian Blackwell	EPA, Antimicrobials Division, Arlington, VA	√	√	-
Krishna Deb	EPA, Antimicrobials Division, Arlington, VA	√	√	-
Noe Galvan	Clorox Services Co., Pleasanton, CA	√	√	√
Earl Goad	EPA, Antimicrobials Division, Arlington, VA	√	√	√
John Harbell	Mary Kay Inc., Addison, TX	√	√	√
Leon Johnson	EPA, Antimicrobials Division, Crystal City, VA	√	-	-
Eli Kumeckpor	Invitrogen, Frederick, MD	√	-	√
Pauline McNamee	The Procter & Gamble Co., Egham, Surrey, U.K.	√	√	√
Michelle Piehl	MB Research Laboratories, Spinnerstown, PA	√	-	-
Patrick Quinn	Accord Group, Washington, DC	-	-	√
Hans Raabe	Institute for In Vitro Sciences, Gaithersburg, MD	-	√	√
Mary Richardson	Bausch & Lomb, Rochester, NY	√	√	√
Michael Rohovsky	Johnson & Johnson, New Brunswick, NJ	√	√	√
Kristie Sullivan	Physicians Committee for Responsible Medicine, Oakland, CA	-	-	√
Neil Wilcox	Consultant/FDA, College Park, MD	√	√	-

NICEATM:

RADM William Stokes, D.V.M., Director
DACLAM

Debbie McCarley Special Assistant to the Director

Support Contract Staff— Integrated Laboratory Systems, Inc.:

David Allen, Ph.D. Elizabeth Lipscomb, Ph.D.

Jonathan Hamm, Ph.D. Linda Litchfield

Nelson Johnson Greg Moyer, M.B.A.

Brett Jones, Ph.D. James Truax, M.A.

Abbreviations used in participants' affiliations:

CDER = Center for Drug Evaluation and Research

CFSAN = Center for Food Safety and Applied Nutrition

CPSC = U.S. Consumer Product Safety Commission

ECVAM = European Centre for the Validation of Alternative Methods

EPA = U.S. Environmental Protection Agency

FDA = U.S. Food and Drug Administration

ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods

ILS = Integrated Laboratory Systems, Inc.

NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

NIEHS = National Institute of Environmental Health Sciences

NLM = National Library of Medicine

OPP = Office of Pesticide Programs

OTWG = Ocular Toxicity Working Group

USDA = U.S. Department of Agriculture

TUESDAY, MAY 19, 2009

Call to Order and Introductions

Dr. Hayes (Peer Review Panel Chair) called the meeting to order at 8:30 a.m. and introduced himself. He then asked all Peer Review Panel (Panel) members to introduce themselves and to state their name and affiliation for the record. He then asked all the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) members, the ICCVAM Ocular Toxicity Working Group (OTWG) members, the European Centre for the Validation of Alternative Methods (ECVAM) staff person, and members of the public to introduce themselves. Dr. Hayes stated that there would be opportunities for public comments during the discussions associated with each of the ten test method topics. He asked that those individuals interested in making a comment register at the registration table and provide a written copy of their comments, if available, to NICEATM staff. Dr. Hayes emphasized that the comments would be limited to seven minutes per individual per public comment session, and that, while an individual would be welcome to make comments during each commenting period, repeating the same comments at each comment period would be inappropriate. He further stated that the meeting was being recorded and that Panel members should speak directly into the microphone.

Welcome from the ICCVAM Chair

Dr. Wind, U.S. Consumer Product Safety Commission (CPSC) and Chair of ICCVAM, welcomed everyone to CPSC and to the Panel meeting. Dr. Wind stressed the importance of this Panel's efforts, especially considering the public health importance of ocular safety testing and hazard labeling. Dr. Wind noted that approximately 125,000 home eye injuries occur each year and over 2,000 workers suffer eye injuries each day, many of which are caused by accidental exposure to chemicals or chemical products. Dr. Wind also reviewed the statutes and regulations requiring ocular testing.

Dr. Wind thanked the Panel members for giving their expertise, time, and effort and acknowledged their important role in the ICCVAM test method evaluation process. Dr. Wind also emphasized the importance of public comments that are considered by the Panel in this process and the Panel's role in the development of ICCVAM final test method recommendations.

Welcome from the Director of NICEATM, and Conflict-of-Interest Statements

Dr. Stokes, Director of NICEATM, stated the Panel meeting was being convened as a National Institutes of Health (NIH) Special Emphasis Panel and was being held in accordance with applicable U.S. Federal Advisory Committee Act regulations. As such, Dr. Stokes indicated that he would serve as the Designated Federal Official for this public meeting. He reminded the Panelists that, when they were originally selected, they had signed conflict-of-interest statements in which they identified any potential conflicts of interest. He then read the conflict-of-interest statement and again asked members of the Panel to identify any potential conflicts for the record. Dr. Hayes asked the Panel members to declare any direct or indirect conflicts based on Dr. Stokes' statements and to recuse themselves from voting on any aspect of the meeting where these conflicts were relevant.

Dr. Sawyer declared a potential conflict-of-interest regarding his employment with Minrad Inc., a company that manufactures inhalation anesthetics. Dr. Ward declared a potential conflict-of-interest regarding her consulting relationship with a company that manufactures antimicrobial cleaning products. Dr. Rodeheaver indicated that she worked for Alcon, a manufacturer of the topical anesthetics proparacaine and tetracaine. Dr. Vanparys declared a potential conflict-of-interest regarding his company's involvement in the conduct of the Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) test method.

Overview of the ICCVAM Test Method Evaluation Process

Dr. Stokes opened his presentation by thanking the Panel members for their significant commitment of time and effort preparing for and attending the meeting. He noted that this is an international Panel, made up of 22 different scientists from six different countries (Belgium, Canada, The Netherlands, Japan, Spain, and the United States). He explained that the purpose of the Panel was to conduct an independent scientific peer review of the information provided on several proposed alternative ocular safety test methods, a testing strategy, and proposed refinements to the *in vivo* rabbit eye test method. This assessment is to include an evaluation of the extent that each of the established ICCVAM criteria for validation and regulatory acceptance has been appropriately addressed for each test method or testing strategy. The Panel is then asked to comment on the extent that the available information and test method performance in terms of accuracy and reliability supports the ICCVAM draft recommendations. Dr. Stokes noted that the first ICCVAM Ocular Peer Review Panel met in 2005 to evaluate the validation status of four alternative test methods (Bovine Corneal Opacity and Permeability [BCOP], Isolated Chicken Eye [ICE], Isolated Rabbit Eye [IRE], and the HET-CAM) for their ability to identify ocular corrosives or severe irritants. The Panel recommended two of these test methods (BCOP and ICE) on a case-by-case basis for use in a tiered-testing strategy with test method-specific applicability domain restrictions. ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) endorsed the Panel's recommended use for these test methods. The Panel also recommended that, while the IRE and HET-CAM test methods were potentially useful in a tiered-testing strategy with appropriate restrictions, additional data were needed to fully assess their usefulness and limitations for regulatory testing. ICCVAM prepared a test method evaluation report (TMER) and provided a transmittal package (i.e., Panel report, SACATM and public comments, TMER and associated materials) to the ICCVAM Federal agencies for their response as required by the ICCVAM Authorization Act of 2000 (ICCVAM 2000). All Federal agencies with ocular testing requirements endorsed the BCOP and ICE test method recommendations. Dr. Stokes noted that five Panel members from the 2005 review are on the current Panel (i.e., Drs. Henry Edelhauser, A. Wallace Hayes, Robert Peiffer, Scheffer Tseng, and Philippe Vanparys).

Dr. Stokes then provided a brief overview of ICCVAM and NICEATM, and identified the 15 Federal agencies that comprise ICCVAM. He summarized the purpose and duties of ICCVAM (as described in the ICCVAM Authorization Act of 2000²), noting that ICCVAM, as an interagency committee, does not carry out research and development or validation studies. Instead, ICCVAM, in conjunction with NICEATM, carries out critical scientific evaluations of the results of validation studies for proposed test methods to assess their usefulness and limitations for regulatory testing, and then makes formal recommendations to ICCVAM agencies.

Dr. Stokes then described the ICCVAM test method evaluation process, emphasizing the many opportunities for stakeholder input during numerous public comment periods.

As part of this process, a working group of Federal scientists designated for the relevant toxicity testing area (e.g., the OTWG) and NICEATM prepare a draft background review document (BRD) that provides a comprehensive review of all available data and information. ICCVAM considers all of this available data and information and then develops draft test method recommendations on the proposed usefulness and limitations of the test methods, test method protocol, performance standards, and future studies. The draft BRD and the ICCVAM draft test method recommendations are made available to the Panel and the public for review and comment. The Panel reviews the draft BRD and evaluates the extent to which the established ICCVAM validation and regulatory acceptance criteria have been adequately addressed and the extent that the demonstrated accuracy and reliability support the ICCVAM draft test method recommendations. A Panel report is published and then considered, along with public and SACATM comments, by ICCVAM in developing final recommendations.

² http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf

ICCVAM forwards these final recommendations to the ICCVAM member agencies for their consideration and possible incorporation into relevant testing guidelines.

He concluded by summarizing the timeline for 2009 for the ICCVAM evaluation and peer review of the ocular test methods and approaches, including a *Federal Register* notice in March announcing the Panel meeting, the projected publication of the Panel report in July, and transmittal of ICCVAM final recommendations to Federal agencies in November.

ICCVAM Charge to the Panel

Dr. Stokes reviewed the charge to the Panel:

- (1) Review the ICCVAM draft BRDs for completeness and identify any errors or omissions (e.g., other relevant publications or available data).
- (2) Evaluate the information in the draft BRDs to determine the extent to which each of the applicable ICCVAM criteria for validation and regulatory acceptance of toxicological test methods have been appropriately addressed.
- (3) Consider the ICCVAM draft test method recommendations for the following and comment on the extent to which they are supported by the information provided in the BRDs: proposed test method usefulness and limitations, proposed recommended standardized protocols, proposed test method performance standards, and proposed future studies.

Dr. Stokes thanked the OTWG and ICCVAM for their contributions to this project and acknowledged the contributions from the participating liaisons from ECVAM, the Japanese Center for the Validation of Alternative Methods (JaCVAM), and Health Canada. He also acknowledged the NICEATM staff for their support and assistance in organizing the Panel meeting and preparing the review materials.

Overview of the Agenda

Dr. Hayes outlined the process for reviewing each of the topics. First, the test method developer or other expert will describe the test method protocol and procedures, followed by a presentation summarizing the test method validation database and test method performance for each draft BRD or summary review document (SRD) given by a member of the NICEATM staff. An ICCVAM OTWG member will then present the ICCVAM draft test method recommendations. Following presentations, the Evaluation Group Chair responsible for the topic under consideration will present the Evaluation Group's draft recommendations and conclusions followed by Panel discussion. Public comments will then be presented followed by the opportunity for questions to the public commenters and additional Panel discussion. After consideration of the public comments, the Panel will then vote to accept the Panel consensus, with any minority opinions being so noted with a rationale for the minority opinion provided.

Draize Rabbit Eye Test and Current Ocular Regulatory Testing Requirements and Hazard Classification Schemes

Ms. McCall of the U.S. Environmental Protection Agency (EPA) presented the relevant U.S. and international statutes and regulations for ocular safety testing (e.g., EPA, CPSC, Food and Drug Administration [FDA], Occupational Safety and Health Administration [OSHA], European Union [EU], and Organisation for Economic Co-operation and Development [OECD]). She summarized the Draize scoring system for corneal, iridal, and conjunctival lesions in the rabbit, using representative photographs for reference. She also discussed optional but potentially useful assessments of ocular injury (e.g., fluorescein staining, corneal thickness, depth of corneal injury, photographic documentation, and histopathology) that are not routinely included in the Draize eye test. Ms. McCall then provided an overview of the various U.S. and international hazard classification schemes for ocular corrosivity and irritation (i.e., EPA, EU, Globally Harmonized System of Classification and

Labelling of Chemicals [GHS], and Federal Hazardous Substances Act [FHSA]). She noted that, based on the recently adopted European Union Regulation on the Classification, Labelling and Packaging of Substances and Mixtures (i.e., the CLP Regulation), the EU will move to the GHS system after December 1, 2010, for substances and after June 1, 2015, for mixtures. Ms. McCall also identified the required signal words for labeling based on each regulatory classification.

Use of Topical Anesthetics and Systemic Analgesics to Avoid or Minimize Pain and Distress in Ocular Toxicity Testing

On behalf of NICEATM, Dr. Allen reviewed the relevant sections of the draft BRD on the routine use of topical anesthetics and systemic analgesics in *in vivo* ocular irritation testing.

Dr. Merrill then presented the ICCVAM draft recommendations for the routine use of topical anesthetics and systemic analgesics in *in vivo* ocular irritation testing for the Panel to consider.

Panel Evaluation

Dr. Sawyer (Evaluation Group Chair) presented the Evaluation Group's responses to questions posed to the Panel on the routine use of topical anesthetics and systemic analgesics in *in vivo* ocular irritation testing and ICCVAM draft test method recommendations. Dr. Sawyer indicated that anesthetic requirements vary enormously among species. For instance, cats require approximately 40% more anesthetic than humans to achieve a similar level of anesthesia. Therefore, any protocol designed to minimize or eliminate pain needs to be individualized to the target species. The Evaluation Group proposed an alternative to the ICCVAM anesthetic/analgesic protocol to be used during all *in vivo* rabbit ocular irritation testing. Dr. Sawyer outlined the Evaluation Group's proposed protocol, which is divided into pretreatment and posttreatment regimens as follows:

Pretreatment Analgesia:

Buprenorphine 0.01 mg/kg subcutaneous (SC) (60 minutes before test substance application [TSA]). Dr. Sawyer noted that buprenorphine is classified as an opioid agonist-antagonist analgesic with a wide margin of safety in rabbits, minimal sedation, and relatively long duration. It has been found to be effective in managing pain in small animals, and is given before application of the test substance because the most effective method of managing pain and distress is to administer the analgesic preemptively to prevent establishment of central sensitization.

One or two drops of 0.5% proparacaine hydrochloride, applied to the eye three times at 5-minute intervals starting 15 minutes pre-TSA. Last application would be five minutes pre-TSA. Anticipated duration of action: 30 - 60 minutes. Dr. Sawyer stated that proparacaine is preferred because application to the eye would be less painful and the suggested application sequence is to assure effective penetration of the epithelial layer.

Eight hours post-TSA:

Buprenorphine 0.01 mg/kg SC and meloxicam 0.5 mg/kg SC. Dr. Sawyer noted that the timing is to reinforce the initial level of analgesia to carry over until the next morning (the duration of analgesia is expected to be at least 12 hours for buprenorphine and at least 24 hours for meloxicam). The combination of an opioid and a nonsteroidal anti-inflammatory drug (NSAID) such as meloxicam is a well-tested approach to balanced analgesia. Used for post-operative or chronic pain in dogs since 1997, meloxicam has been found to have effective application in rabbits.

Day two through day seven post-TSA:

Buprenorphine 0.01 mg/kg SC every 12 hours and meloxicam 0.5 mg/kg SC every 24 hours. Dr. Sawyer noted that buprenorphine and meloxicam should be continued for seven days post-TSA unless signs of ocular injury sufficient to cause pain and discomfort appear. If so, this systemic analgesic protocol would continue until the test is completed.

Rescue Analgesia:

Dr. Sawyer also outlined a procedure where, if a test subject shows signs of physical pain or discomfort during the test interval using the above protocol, a rescue dose of buprenorphine at 0.03 mg/kg SC could be given as needed every eight hours instead of 0.01 mg/kg SC every 12 hours. Meloxicam would continue with the same dose and interval.

Dr. Sawyer pointed out that buprenorphine and meloxicam were synergistic and have an excellent safety profile in clinical practice. A question was raised concerning the interval of dosing throughout the test period and the burden that it would impose on the testing laboratory. The Panel agreed that a \pm 30-minute interval is appropriate for the administration of the systemic analgesics.

Dr. Dubielzig indicated that the impact of the NSAID on inflammatory aspects of the Draize rabbit eye test is unknown, but the Panel did not consider such effects to be limited and therefore not likely to be a problem. Dr. Jester questioned the need to continue analgesic treatment through day seven when Category III or IV substances would have cleared by day three. He suggested an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approach where treatment is continued through day four. Dr. Peiffer suggested that the temporal aspect be removed and that treatment be continued only if there are signs of discomfort. **The Panel agreed that treatment should be stopped after day four (instead of day 7, as suggested above) if there are no signs of discomfort.** The Panel agreed that pain assessment should be made and recorded daily.

Dr. Jester raised a concern that the use of preservatives in the topical anesthetics may interfere with the irritation response. The Panel agreed that the use of preservative-free proparacaine should be required. Dr. Stokes asked how long after the administration of the systemic analgesics a rescue dose can be administered. Dr. Sawyer indicated that, due to the wide margin of safety, the rescue dose can be given immediately afterward if necessary.

Dr. Jester expressed concern that dilution of the test substance could occur if a significant amount of liquid anesthetic remained in the eye. Dr. Peiffer indicated that, in his experience, the 5-minute interval is reasonable and should not pose a problem for test substance dilution.

In response to the evaluation guidance question specific to testing situations where the use of topical anesthetics would be considered inappropriate, the Panel indicated that drugs to be used for ocular effects, such as eye drops, need to be tested by other means. However, the focus of this evaluation is eye irritation hazard classification; therefore, the proposal would be relevant to all such testing. The Panel did not know of additional systemic analgesics that might have greater efficacy in relieving ophthalmic pain associated with chemically-induced injuries. The Panel also agreed that there were no additional pain-related chemically-induced injuries to the eye that the proposed alternate analgesic proposal would not adequately address.

The Panel expressed general concern about the use of transdermal patches to deliver anesthetics due to the need for shaving prior to patch application and the possibility of skin irritation. In addition, with multiple applications, the availability of irritation-free skin sites may pose a problem. Most importantly, analgesic patches have proven to be unreliable in clinical practice with significant animal-to-animal variation as well as species-to-species variation when comparing effectiveness and duration of effect. The Panel also indicated a greater concern about self-mutilation due to severe pain during eye irritation testing than about the potential for the systemic analgesics to alter the ocular injury response. Dr. Jester indicated that there was insufficient information in the BRD to make this assessment.

The majority of the Panel agreed that the tetracaine information provided in the ICCVAM BRD could be applied to other topical anesthetics such as proparacaine. Dr. Ward indicated that additional studies on cell proliferation, migration, and cytotoxicity could be done with topical anesthetics to provide some assurance that they behave in a manner similar to tetracaine. Although it was previously noted

that anesthetic/analgesic use was for all *in vivo* eye irritation tests, the Panel indicated that administration of post-application analgesics is not a concern if a standard dosing regimen is used throughout and not adjusted for each animal to avoid overdosing side effects.

The Panel also agreed that the clinical signs of post-application pain and distress are adequately described and that no other clinical signs should be added. In the event of an eye infection, the Panel agreed that secondary treatment should be considered, the signs and symptoms of the eye infection should be documented, and the animal should be immediately removed from the study. Finally, the Panel agreed that all relevant data had been adequately considered in the BRD.

The Panel considered its proposal to be more appropriate than the ICCVAM-proposed recommendations in terms of the type and frequency of dosing for topical anesthetics and systemic analgesics. The Panel agreed with the ICCVAM draft recommendations for future studies. Therefore, it recommended refinement of the current *in vivo* test system to evaluate ocular irritation utilizing contemporary/novel technologies to address both concerns. The Panel recommended the following:

- New animal studies should only be considered when absolutely necessary in developing new strategies for testing.
- Products that are overpredicted when anesthetic and analgesic pretreatment is used should be identified.
- Animal responses should be collected in tests currently being conducted to determine whether refinements are warranted in the dosing and timing of anesthetic, analgesic, and antibiotic treatments.
- Rabbit ocular specimens should be submitted for histopathological evaluation to develop an archive of specimens.
- Digital photographs of lesions/observations should be collected.
- Analysis of the variability in rabbit wound-healing responses would help determine whether or not it is due to variability in the ocular defense linking to the neuroanatomic integration.
- Studies should be conducted to determine whether the timing and dosing of systemic analgesics with topical anesthetics might alter the ocular defense enough to change the classification of test substances.
- Cytology samples from the surface of the eye should be collected.
- Studies should be conducted to investigate the appropriateness of using proparacaine instead of tetracaine.
- Studies should be conducted to evaluate the impact of using the NSAID meloxicam with buprenorphine.
- New technologies (e.g., new imaging modalities and quantitative/mechanistic endpoints) should be incorporated into the Draize rabbit eye test, refining/changing it to make it a more humane test that is also more reliable.

Public Comments

No public comments were made.

Panel Conclusions and Recommendations

Dr. Hayes asked if the Panel was in agreement with its preliminary conclusions. The Panel voted unanimously to approve the recommendations as revised during the discussion with one abstention,

Dr. Rodeheaver, who cited a potential conflict-of-interest due to her employment by a manufacturer of anesthetic products.

Use of Humane Endpoints in *In Vivo* Ocular Irritation Testing

On behalf of NICEATM, Dr. Allen reviewed the relevant sections of the draft BRD on the use of humane endpoints in *in vivo* ocular irritation testing for the Panel.

Dr. Merrill then presented the ICCVAM draft recommendations for the use of humane endpoints in *in vivo* ocular irritation testing for the Panel to consider.

Panel Evaluation

Dr. Sawyer (Evaluation Group Chair) presented the Evaluation Group's responses to questions posed to the Panel on the use of humane endpoints in *in vivo* ocular irritation testing and ICCVAM draft test method recommendations. The Panel agreed that each of the current and proposed humane endpoints detailed in the BRD are sufficiently predictive of irreversible or severe effects (i.e., GHS Category 1, U.S. EPA Category I, EU R41) that they should be used routinely as humane endpoints to terminate a study as soon as they are observed. The Panel also agreed that animals should be observed at least once per day (at least twice daily for the first three days) to ensure that termination decisions are made in a timely manner. The Panel agreed that there was insufficient data in the BRD to determine the adequacy of pannus as a recommended humane endpoint. The Panel also agreed that the use of fluorescein staining was an appropriate technique for evaluating eye injury; however, the technique needs to be better described before a reasonable conclusion regarding its value can be made.

Dr. Jester suggested that the use of fluorescein staining had not been adequately discussed in this BRD.

The Panel emphasized that, in some cases, decisions to terminate a study should be based on more than one endpoint. Very severe endpoints (e.g., corneal perforation) would be adequate alone to terminate a study. Other biomarkers considered useful by the Panel as routine humane endpoints included extent of epithelial loss, limbal ischemia, and/or stromal loss, and depth of corneal damage.

In response to the question regarding other earlier biomarkers/criteria indicative that painful lesions can be expected to fully reverse, the Panel indicated eyes with conjunctival scores without corneal/iris scores would be expected to recover. The Panel indicated that the destruction of 50% of the limbus will result in pannus in rabbits and, therefore, the ICCVAM draft recommendation requiring 75% for early termination may be excessive. In addition, the Panel indicated that the humane endpoints described in the BRD were sufficient to ensure that the lesions would not reverse. The Panel did agree that the available data and information supported the ICCVAM draft recommendations on humane endpoints. The Panel recommended that studies be developed to identify better and earlier endpoints, such as those seen with fluorescein staining, and that these endpoints should be incorporated into current testing guidelines.

Public Comments

No public comments were made.

Panel Conclusions and Recommendations

Dr. Hayes asked if the Panel was in agreement with its preliminary conclusions. The Panel voted unanimously to approve the recommendations as revised during the discussion.

Adjournment

Dr. Hayes adjourned the Panel for the day at 5:45 p.m., to reconvene at 8:30 a.m. on Wednesday, May 20, 2009.

WEDNESDAY, MAY 20, 2009

Dr. Hayes called the meeting to order at 8:28 a.m. and asked Dr. Stokes to discuss the conflict-of-interest for the day's planned topics. Dr. Stokes read the conflict-of-interest statement and Dr. Hayes asked the Panel to declare any conflicts-of-interest. The conflicts-of-interest declared by Panel members on day one of the meeting were repeated.

Dr. Hayes then asked for introductions from the Panel, NICEATM staff, members of ICCVAM and the OTWG, and those in attendance for the public session.

HET-CAM Test Method

Dr. Schrage reviewed the various HET-CAM test method protocols (i.e., IS[A], IS[B], S-Score, Q-Score, and IT) and BASF experience with the test method. Dr. Schrage stressed the need for harmonization of HET-CAM protocols, endpoints, and scoring methods. BASF has conducted a retrospective review of 145 test substances, including a broad variety of chemicals and formulations, which revealed that overall accuracy, false positive rates, and false negative rates were not acceptable. The specificity and sensitivity were especially affected by solubility in both water and oil. These data were submitted to the journal *Alternatives to Laboratory Animals* in April 2009. Dr. Schrage said she would be willing to share the HET-CAM data on these 145 substances with NICEATM following publication.

Dr. Vanparys said that he would be willing to provide NICEATM with HET-CAM data using the IS(B) analysis method to determine if conversion to the IS(A) method was feasible. He added that, in his experience, the HET-CAM test method can be sensitive for the identification of substances not labeled as irritants.

On behalf of NICEATM, Dr. Allen reviewed the HET-CAM draft BRD.

Dr. Merrill then presented the ICCVAM draft recommendations for the HET-CAM test method for the Panel to consider.

Panel Evaluation

Dr. Wilson (Evaluation Group Chair) presented the Evaluation Group's responses to questions posed to the Panel on the validation status of the HET-CAM test method and ICCVAM draft test method recommendations. He noted that HET-CAM classified four EPA Category III substances incorrectly as Category IV (i.e., they were false negative in HET-CAM). However, he said that regulators would be more concerned if the false negative substances were EPA Category I or Category II. Some Panelists did not consider these substances likely to be a significant risk. Dr. Stokes suggested adding a statement defining an acceptable rate for false positives and false negatives. Dr. Wilson expressed concern that, while three of the four animals had an EPA Category III classification that cleared in seven days, one animal had a conjunctival redness score of two that cleared to one in seven days but required 14 days to completely resolve (i.e., return to a score of zero). Such lesions would not be considered inconsequential.

The Panel discussed the low number of mild and moderate substances used in the performance analyses, and that additional substances in these categories would be needed before a conclusion on the usefulness of HET-CAM could definitively be reached. The Panel also recognized that the validation database does not include substances currently regulated by EPA and that collection of additional data is needed. Therefore, given the limited data for mild and moderate substances, the Panel did not support the ICCVAM draft test method recommendation for use of the HET-CAM to identify substances not labeled as irritants from all other classes.

Dr. Peiffer said that he was concerned with the recommendation to test increasing concentrations of test substances. He stated that while dose-response curves are preferred for scientific studies, they are

not practical for regulatory testing. Dr. Sawyer agreed that increasing concentrations should not be a requirement. Ms. McLaughlin argued that use of different concentrations allows the investigator to see if increasing the concentration affects the outcome. She stated that poor predictivity might result from use of a concentration that produces an ineffectual or weak response, whereas the comparative effect of a higher concentration would provide useful information. The Panel agreed to remove the concentration requirement from the test method protocol but to include it as a general recommendation for additional research.

Ms. McLaughlin offered a minority opinion with respect to the Panel's recommendation on the use of the HET-CAM test method to identify substances not labeled as irritants from all other classes. Ms. McLaughlin stressed that personal care products are not regulated in the U.S. as they are in Europe and Canada. Ms. McLaughlin stated that the HET-CAM test method could be used as an alternative to the Draize rabbit eye test to evaluate personal care products in situations where they are regulated. Dr. Hayes asked Ms. McLaughlin to write a short paragraph to note the rationale for her opposition to the majority view for inclusion in the Panel report. Ms. McLaughlin drafted the following text:

Based on the demonstrated performance as outlined in the ICCVAM draft recommendations, HET-CAM can be used to screen not labeled as irritants from other irritant categories for the restricted applicability domain (surfactant-based formulations and oil/water emulsions). The rationale for this dissenting view is based on the fact that there were 60 substances in the overall database. The hazard category distribution was: 25 Category I; 2 Category II; 18 Category III; and 15 Category IV. The sensitivity of HET-CAM is 91% (41/45), resulting in a false negative rate of 9% (4/45). Among the four false negatives for the EPA system, 100% (4/4, all oil/water emulsion cosmetic formulations) were EPA Category III substances based on conjunctival redness score of two that required at least three days to resolve. The lesions noted *in vivo* indicated mild ocular irritation and are unlikely to represent a significant hazard. As such, the HET-CAM could be considered useful as a screening test for EPA Category IV substances not labeled as irritants from all other categories for the restricted applicability domain of surfactant-based formulations and oil/water emulsions. The sensitivity for GHS and EU was high enough for each system to warrant HET-CAM test method use (i.e., 100% sensitivity; 31/31 and 26/26, respectively for GHS and EU [from the ICCVAM draft BRD, Tables 6-2 and 6-12]) also with domain restriction. This performance demonstrates that HET-CAM could be used to screen EU or GHS hazard not labeled as irritant classifications from other irritant categories for the restricted applicability domain of surfactant-based formulations and oil/water emulsions. It should be noted that, for regulatory purposes, sensitivity (the proportion of all positive substances that are classified as positive) is most important from a public health perspective and the HET-CAM performed well in this regard.

The Panel discussed the ICCVAM draft recommended protocol for the HET-CAM test method. Dr. Vinardell said that she would like to see a statement added to the protocol to wash out any leftover solids after 30 seconds (as currently recommended in the EU Annex V). Dr. Hayes asked Dr. Vinardell to provide a statement for Dr. Wilson to include in the Panel report.

The Panel discussed the HET-CAM test method performance. One Panelist suggested that a Chi-square analysis should be included to ensure that differences in classification were statistically significant. Dr. Ahn was asked if a power analysis could be used to determine if the number of substances in the mild and moderate classification was adequate to differentiate the irritant classifications. Dr. Ahn said that there should be at least three substances in each classification category to conduct a power analysis.

The Panel discussed the need for Good Laboratory Practice (GLP) studies. Dr. Hayes emphasized that a study is either GLP compliant or it is not. He said that the phrase "spirit of GLP" should not be used in the Panel report. He also said that the term "original data" should be used rather than "raw data."

The Panel agreed that data from studies not conducted under GLP guidelines could be used to increase knowledge about the applicability domain of a test method but that laboratories should provide sufficient detail about the conduct of the study to understand any deviations from GLP guidelines.

The Panel discussed additional sources of HET-CAM data to expand the applicability domain and the number of mild and moderate substances tested. Dr. Allen noted that Dr. Debbasch, a principal contact for data acquisition, had left L’Oreal. Dr. Hayes said that *cosmeceuticals* represented a gray zone between cosmetics and personal-care formulations, and this class of products should be considered. Ms. McLaughlin said that the inclusion of a single ingredient (e.g., a UV-blocking material) could change the regulatory requirements for a formulation from an unregulated personal care product to a regulated material in Canada. She said that the applicability domain and database used in the ICCVAM draft BRD should be adequate to warrant use of the HET-CAM test method for personal care products that are not labeled as irritants. The Panel did not support the use of additional studies to identify the full range of irritation but supported additional studies to identify substances not labeled as irritants from all other classifications.

Public Comments

Dr. Barroso from ECVAM commented that the false negatives using the EPA classification system, which are substances not labeled as irritants using the GHS classification system, result because the EPA classification system categorizes substances based upon the most severe category observed among the test rabbits (i.e., not based on the majority classification among rabbits tested). Dr. Barroso also said that because the types of formulations regulated by EPA are not present in the database that the EPA classification system should not be given too much weight.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Panel Conclusions and Recommendations

Dr. Hayes asked if the Panel was in agreement with its preliminary conclusions. The Panel voted to approve the recommendations as revised during the discussion with one minority opinion, Ms. McLaughlin, and one abstention, Dr. Vanparys, who cited a potential conflict-of-interest with the HET-CAM test method, which he had worked on at Johnson & Johnson.

Isolated Chicken Eye Test Method

On behalf of NICEATM, Dr. Allen presented an overview of the ICE test method protocol and reviewed the ICE draft BRD. One Panelist asked why the test method was limited to three eyes. Dr. Allen explained that the incubation apparatus contained 10 chambers, sufficient for three groups of three eyes and a negative control. However, the ICCVAM ICE test method protocol, upon which the recently submitted OECD Test Guideline is based, includes both positive and negative controls.

Dr. Jester said that the term fluorescein *staining* should be used rather than *retention*. He also asked how the EPA classification categories were determined using the ICE test method. Dr. Allen replied that the four-tiered EPA classification system was considered equivalent to the four-tiered GHS system and used the same ICE test method decision criteria (e.g., EPA Category I – GHS Category 1, EPA Category II = GHS Category 2A, EPA Category III = GHS Category 2B, EPA Category IV = GHS Category Not labeled).

Dr. Yu asked if the evaluation of the eyes was subjective and whether photographs were taken. Dr. Allen said that the evaluation of the eyes for corneal lesions was subjective, except for the measurement of corneal swelling, which is measured quantitatively using a pachymeter. He said that photographs were not typically taken but were recommended by the previous ocular Panel.

Dr. Merrill then presented the ICCVAM draft recommendations for the ICE test method for the Panel to consider.

Panel Evaluation

Dr. Tarlo (Evaluation Group Chair) presented the Evaluation Group's responses to questions posed to the Panel on the validation status of the ICE test method and ICCVAM draft test method recommendations. The Panel agreed that the available data and test method performance supported the ICCVAM draft recommendations that the ICE test method is not recommended to identify substances from all hazard categories as defined by GHS, EPA, and EU classification systems. The Panel further agreed that the ICE test method is not recommended as a screening test to identify substances not labeled as irritants from all other hazard classifications defined by GHS, EPA, and EU, because one of the false negatives included a GHS Category 1 substance. The Panel agreed with the ICCVAM draft recommendation that the ICE test method should not be used as a screening test to identify GHS substances not labeled as irritants. Dr. van der Valk noted that the ICE test method is used by the Netherlands Organisation for Applied Scientific Research (TNO) to obtain good results, but the results obtained by other laboratories using the ICE test method in the validation study were variable. Dr. Vanparys recommended that the source of the variability be noted in the appropriate text.

The Panel agreed that the available data supported the ICCVAM draft recommendations that the proposed standardized protocol appeared acceptable. However, the Panel suggested that the protocol could be improved by adding objective endpoints for corneal opacity and fluorescein staining. The Panel also added that inclusion of a histopathological evaluation might improve ICE test method performance.

The Panel agreed with the ICCVAM draft recommendations for the ICE test method in terms of the proposed future studies that additional optimization studies would be required to validate the test method for the identification of all ocular irritancy hazard categories. The use of histopathology evaluation might add to the accuracy and determination of the test. The Panel also agreed with ICCVAM that the ICE test method performance standards are not warranted at this time.

Public Comments

Dr. Barroso said that variability of the ICE test method was similar to that of the Draize rabbit eye test because of the subjective assessments. He stated that the ICE test method should not be held to a higher standard than the Draize test. He also noted that the concordance among laboratories was reasonable.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Panel Conclusions and Recommendations

Dr. Hayes asked if the Panel was in agreement with its preliminary conclusions. The Panel voted unanimously to approve the recommendations as revised during the discussion.

Isolated Rabbit Eye (IRE) Test Method

On behalf of NICEATM, Dr. Allen presented an overview of the IRE test method and reviewed the IRE draft BRD. Dr. Hayes asked whether the rabbits used by GlaxoSmithKline (GSK) were from PelFreeze Biologicals or if fresh eyes were used for each test. Dr. Allen replied that at least some of the rabbits were obtained from other GSK laboratories and had been used as negative controls from other acute safety testing. Dr. Ward noted that PelFreeze ships rabbit eyes from its facility in Rogers, Arkansas, adding that their rabbits are used for multiple purposes. She was not aware of a formal study to determine the acceptability of eyes shipped from the U.S. to Europe. Dr. Peiffer suggested

that shipped eyes should be carefully examined prior to use. Dr. Jester said that his laboratory has compared eyes obtained from an abattoir to fresh eyes and found no significant differences.

Dr. Merrill then presented the ICCVAM draft recommendations for the IRE test method for the Panel to consider.

Panel Evaluation

Dr. Tarlo (Evaluation Group Chair) presented the Evaluation Group's responses to questions posed to the Panel on the validation status of the IRE test method and ICCVAM draft test method recommendations. The Panel agreed with ICCVAM that additional optimization and validation studies using a protocol that includes all four recommended endpoints are needed to further evaluate the relevance and reliability of the IRE test method and to develop more definitive recommendations.

The Panel recommended that the planned validation study with GSK/SafePharm include an evaluation of fresh versus shipped eyes. In general, the Panel felt there should be rigid criteria on the handling and storage of the eyes. Finally, the Panel recommended that criteria on test article administration/washout (e.g., viscous substances) were warranted.

Public Comments

No public comments were made.

Panel Conclusions and Recommendations

Dr. Hayes asked if the Panel was in agreement with its preliminary conclusions. The Panel voted unanimously to approve the recommendations as revised during the discussion.

Bovine Corneal Opacity and Permeability Test Method (BCOP)

Dr. Curren, Institute for In Vitro Sciences, provided an overview of the BCOP test method. He noted that Pierre Gautheron and his colleagues initially developed the test method for occupational safety. Dr. Curren said that as many as 30% of bovine eyes are rejected upon inspection because of scratches and other defects, and emphasized the importance of including concurrent positive and negative controls in each study. With respect to histopathology evaluation, he said that it was important to carefully choose a qualified laboratory because of the impact of quality on the evaluation.

Dr. Vanparys pointed out that the 15x OD₄₉₀ value in the *In Vitro* Score calculation was chosen to equate the data to *in vivo* data. One Panel member asked if there was an equilibration period, and Dr. Curren indicated that the bovine corneas were equilibrated for one hour before dosing.

Dr. Bailey asked if there was an example for when histopathology evaluation should be recommended based on effects associated with a particular chemical class. Dr. Curren cited as an example oxidizers, which may not produce opacity or permeability changes, but still produce substantive corneal damage that is observable only by histopathology. A Panel member asked why corneal thickness was not measured to provide a quantitative endpoint. Dr. Curren said that corneal thickness has been evaluated, but is less reliable than the opacity and permeability measurements and therefore is not measured in the current protocol.

Dr. Peiffer asked how the BCOP decision criteria for histopathology evaluation are applied to the EPA categorization scheme. Dr. Curren replied that a substance labeled as EPA Category IV would not penetrate further than the superficial corneal epithelium, whereas a Category III substance would penetrate to the basal layer, a Category II substance into the top third of the stroma, and a Category I substance into the bottom third of the stroma or to the endothelium. Minimal damage to the epithelium heals quickly, moderate damage heals more slowly, and significant damage (e.g., deep stromal or endothelial penetration) may be irreversible.

On behalf of NICEATM, Dr. Hamm reviewed the BCOP draft BRD.

Dr. Merrill then presented the ICCVAM draft recommendations for the BCOP test method for the Panel to consider.

Panel Evaluation

Dr. Tarlo (Evaluation Group Chair) presented the Evaluation Group's responses to questions posed to the Panel on the validation status of the BCOP test method and ICCVAM draft test method recommendations. With respect to the substances used in the validation studies, the Panel requested additional chemical classes be added as data becomes available to provide a more significant statistical inference. The Panel requested that Drs. Ahn and Palmer conduct a power analysis to determine the number of substances needed in each hazard classification to provide statistical significance.

The Panel discussed the performance of the BCOP test method to identify the intended range of classification categories. The Panel indicated that the available data and analyses were adequate for the intended purpose. The Panel indicated that all available and relevant data had been used in the ICCVAM BCOP test method analyses.

The Panel agreed with ICCVAM that the test method performance supported the ICCVAM draft recommendations. Accordingly, the BCOP test method was not recommended to identify substances from all hazard categories as defined by GHS, EPA, and EU classification systems. However, the BCOP test method can be used as a screening test to distinguish substances not labeled as irritants from all other hazard categories when results are to be used for EU or GHS hazard classifications. Because of the significant lesions associated with 50% (4/8) of the EPA Category III substances that tested as false negatives, the BCOP test method cannot be recommended as a screening test to identify EPA Category IV substances.

The Panel agreed with the ICCVAM draft recommendation that the BCOP test method could be used to distinguish substances not labeled as irritants from all other irritant classes, because the false negative rate for the EU and GHS systems was 0% (0/54 or 0/97, respectively). By comparison, the false negative rate was 6% (8/141) for the EPA system. Among the eight false negatives for the EPA system, 100% (8/8) were EPA Category III substances based on Draize rabbit eye test data.

The Panel said that, while the BCOP test method is unable to identify all irritant classifications, further test method development and refinement in future studies was encouraged.

The Panel recommended that performance standards should be developed, because the BCOP test method is now being considered as a screening test for both ocular corrosives/severe irritants and for the identification of substances not labeled as irritants.

Public Comments

Dr. Curren said that, based on his experience with the BCOP test method, performance of the BCOP for the four hazard classification systems was unlikely to improve based on the lack of Draize rabbit eye test reproducibility in the mild and moderate categories. He said that results from Weil and Scala (1971) show that the extremes are reproducible, but the mild and moderate levels of ocular irritation are highly variable. He referenced the antimicrobial cleaning products (AMCP) BRD that includes an analysis of the impact on the ocular hazard category when the results of a six-rabbit Draize test are randomly sampled for a three-rabbit test.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Dr. Harbell, Mary Kay Inc., said that his laboratories have used over 30,000 bovine eyes that were kept cold at 4°C. He added that damaged eyes are quickly removed and excluded from the test. He pointed out that Gautheron et al. (1992) used both fresh eyes and eyes maintained at 4°C and found no differences in their test method results. Dr. Harbell emphasized the utility of the BCOP in comparison to the other methods being considered given its focus on quantitative measurements.

Dr. Harbell also asked the Panel to consider how histopathology evaluation might contribute to the BCOP test method performance. He said that the experts at the 2005 ICCVAM workshop considered the depth of injury to be an important consideration in the assessment of ocular injury. The purpose of including histopathology evaluation is to evaluate the depth of injury that may not be visible to the naked eye. Dr. Harbell cited the example of oxidizing chemicals that may not affect the opacity or permeability of bovine eyes but do still damage the corneal tissue. Therefore, for these substances, depth-of-injury analysis may be important to differentiate corrosives or severe irritants from moderate irritants. Dr. Harbell said he would like to see histopathology evaluation reconsidered. Dr. Ward asked if he was recommending histopathology evaluation for all classes. Dr. Harbell said that he was but that it would be used primarily for EPA Categories I and II.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Dr. Barroso commented on what he referred to as the “top-down” (i.e., screening for corrosives/severe irritants) and “bottom-up” (i.e., screening for substances not labeled as irritants) approaches using the ICE and BCOP test methods. ECVAM is developing a paper to recommend the use of these proposed testing strategies for both ICE and BCOP, where substances could be tested in the BCOP or ICE test methods in order to identify corrosives/severe irritants or substances not labeled as irritants without using an animal test.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Panel Conclusions and Recommendations

Dr. Hayes asked if the Panel was in agreement with its preliminary conclusions. The Panel voted unanimously to approve the recommendations as revised during the discussion (pending the results of a power analysis by Dr. Ahn) with one abstention, Dr. Vanparys, who cited a potential conflict-of-interest with the BCOP test method, which he had worked on at Johnson & Johnson.

Adjournment

After the discussion, Dr. Hayes adjourned the Panel for the day at 7:25 p.m., to reconvene at 8:30 a.m. on Thursday, May 21, 2009.

THURSDAY, MAY 21, 2009

Dr. Hayes convened the Panel at 8:30 a.m. and asked Dr. Stokes to discuss the conflict-of-interest for the day's planned topics. Dr. Stokes read the conflict-of-interest statement and Dr. Hayes asked the Panel to declare any conflicts-of-interest. The conflicts-of-interest declared by Panel members on day one of the meeting were repeated.

Dr. Hayes then asked for introductions from the Panel, NICEATM staff, members of ICCVAM and the OTWG, and those in attendance for the public session.

The first order of business was to address issues from the preceding day.

BCOP Power Calculation

Dr. Ahn reported on the power calculation requested on Wednesday May 20, 2009, for the BCOP test method. He determined that, for each of the four hazard classification systems, a sample size of 13 substances in each chemical class represented (i.e., 13 x 4 for each chemical class for a four-category hazard classification system) is required to achieve 80% power using a two-group normal approximation test for proportions with a one-sided 0.05 significance level. This is necessary to reject the null hypothesis that the BCOP test is inferior to the Draize rabbit eye test (the accuracy of the BCOP test is more than 0.1 less than that of the Draize test) in favor of the alternative hypothesis that the accuracies in the two groups are equivalent. Dr. Ahn also noted that his analysis included the assumption that the expected accuracy of the BCOP test is 0.6 and the expected accuracy of the Draize rabbit eye test is 0.9.

The Panel voted unanimously to include the recommendation that a sample size of 13 be used for each chemical class in each of the four hazard classifications to achieve statistical significance.

ICE Test Method False Negative Substances

Dr. Vanparys commented on the ability of the ICE test method to identify GHS substances not labeled as irritants. Dr. Vanparys indicated that the false negative substances listed in the ICCVAM BRD were either paints that stick to the cornea or solids, which are known to give inaccurate results with the ICE test method. Dr. Vanparys suggested that the ICE test method is capable of identifying GHS substances not labeled as irritants with the exception of solids and substances that stick to the cornea. The overall Panel recommendations, as stated the previous day, remained unchanged.

Low Volume Eye Test (LVET) Test Method

On behalf of NICEATM, Dr. Allen provided a brief overview of the LVET test method and reviewed the LVET draft SRD.

Dr. Merrill then presented the ICCVAM draft recommendations for the LVET for the Panel to consider.

Panel Evaluation

Dr. Sawyer (Evaluation Group Chair) presented the Evaluation Group's responses to questions posed to the Panel on the validation status of the LVET and ICCVAM draft test method recommendations. The Panel noted that the LVET has been used on a wide range of substances and that it does detect the full range of ocular irritancy, but recognized that the majority of the LVET database was for surfactants and surfactant-containing products. The Panel identified several references that should be added to the SRD and noted the need to review the ECVAM BRD. If any additional historical data were obtained, there might be sufficient data to determine the performance of the LVET on several other chemical classes.

The Panel indicated that pain associated with direct application of the test substance to the cornea should not be an issue in light of the recommendations for topical anesthetic and systemic analgesic use.

When discussing the performance of the LVET compared to the Draize test, the Panel indicated that the evaluation was adequate, noting that the LVET appeared to overpredict the human response to a lesser degree than the Draize rabbit eye test. They also recommended that the full range of irritation categories are represented in the LVET validation database.

In considering whether all available data had been made available, the Panel indicated that all data had not been evaluated. Additional published sources should be considered as well as the ECVAM BRD, on which the Panel was unable to comment during this meeting. The Panel stated that in the absence of all existing data, including a background review document prepared by the European Centre for the Validation of Alternative Methods, it could not make definitive conclusions or recommendations on the validation status of the LVET. Nonetheless, the Panel did consider the limited data that are available for the LVET to support the use of historical LVET data as acceptable *in vivo* reference data on which to base comparisons to *in vitro* study results.

Public Comments

Dr. Harbell commented that eye irritation testing is done to protect the public and that accidental exposure data should be included in the evaluation. Dr. Harbell also commented on Dr. Merrill's presentation that outlined the ICCVAM draft recommendations. He stated that the suggestion in the ICCVAM draft recommendations that severe substances should be tested in humans is terrifying. (Note: This comment was in response to a misinterpretation by the commenter, which was clarified by Dr. Merrill who stated that the ICCVAM draft recommendations do not recommend human testing to be conducted [see below]).

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Dr. Curren commented that the LVET is being discussed because it was used as an *in vivo* reference test method for some of the data provided for the antimicrobial cleaning product (AMCP) testing strategy. He stated that only biologic or LVET data exist for many of the AMCPs, and these data were used to determine the prediction model to support registration of these AMCPs. The LVET test method is no longer used, but there is historical data that can and should be used. Dr. Curren stated that the question is whether we are putting people at risk based upon the cut-off points suggested in the AMCP BRD.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Dr. McNamee (Procter & Gamble) reiterated the comments by Dr. Curren regarding the LVET and noted that 30 years of human experience data with a chemical substance are sufficient for licensing in the United Kingdom.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Dr. Merrill responded to the comment by Dr. Harbell regarding human testing. Dr. Merrill clarified that the ICCVAM draft recommendation states that if an organization or sponsor desires to more adequately characterize the usefulness and limitations of the LVET, ICCVAM recommends that a comprehensive set of substances be tested and compared with the Draize rabbit eye test results. She stated that there was no recommendation for human testing to be conducted, but that existing accidental human injury data and ethical human study data should always be considered.

Panel Conclusions and Recommendations

Dr. Hayes asked if the Panel was in agreement with its preliminary conclusions. The Panel voted unanimously to approve the recommendations as revised during the discussion with one abstention,

Dr. Ward, who cited a potential conflict-of-interest because of her previous consulting work for a company that conducts the LVET.

Cytosensor[®] Microphysiometer Test Method

Dr. Curren provided an overview of the Cytosensor Microphysiometer (CM) test method protocol.

On behalf of NICEATM, Dr. Lipscomb reviewed the CM test method performance as detailed in the AMCP draft SRD.

Dr. Merrill then presented the ICCVAM draft recommendations for the CM test method for the Panel to consider.

Panel Evaluation

Dr. Bailey (Evaluation Group Chair) presented the Evaluation Group's responses to questions posed to the Panel on the validation status of the CM test method and ICCVAM draft test method recommendations. The Panel indicated that the test method protocol was sufficiently detailed; however, it was unlikely to be widely used because the CM instrument has been discontinued and a new instrument would require revalidation.

The Panel recommended the use of relevant positive controls in any future validation studies and, because surfactants form micelles that can influence response, surfactant concentrations should be included. The Panel recommended that an evaluation of the different classes of surfactants (i.e., nonionic, anionic, cationic, and zwitterionic) be conducted to determine if restrictions should be imposed on use of the CM test method.

The Panel agreed that, based on the database of surfactants and surfactant-based formulations, LVET data could be used to support the validity of the CM test method in the proposed AMCP testing strategy.

The Panel also agreed that the additional data on the surfactants and surfactant-containing formulations in the ECVAM BRD provided sufficient support for the use of the CM test method as a screening test to identify water-soluble surfactant chemicals and certain types of surfactant-containing formulations (e.g., cosmetics and personal care product formulations but not pesticide formulations) as either severe or corrosive irritants or substances not labeled as irritants in a tiered-testing strategy, as part of a weight-of-evidence approach. The Panel also agreed that the intra- and interlaboratory reproducibility of the CM test method had been adequately evaluated, although for a limited range of substances as previously discussed. The Panel again noted that the instrument has been discontinued and is currently not supported by the manufacturer, making its use difficult. However, if the CM instrument were redesigned, the remanufactured instrument would require "catch-up" validation (i.e., not a full validation study).

Based upon the lesions noted for one false negative substance in the EPA classification system, the Panel expressed concern with the ability of the CM test method to identify EPA Category IV substances. The Panel noted that the rabbit data indicated that this substance would be classified as a Category III and, therefore, may cause irritation in a human. The Panel noted that further CM studies are needed, in particular for EPA Categories III and IV substances.

The Panel also expressed concern with the high false positive rate of the CM test method when identifying all four hazard categories.

Public Comments

Dr. Curren noted a correction to his presentation where he did not specifically state that the CM test method is limited to water-soluble substances. He questioned the need for performance standards for the CM test method, given that the Panel did not recommend performance standards for the BCOP

and ICE test methods. Dr. Curren commented that the surfactants referred to as *personal care products* are really detergents.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Panel Conclusions and Recommendations

Dr. Hayes asked if the Panel was in agreement with its preliminary conclusions. The Panel voted unanimously to approve the recommendations as revised during the discussion.

EpiOcular Test Method

Dr. Curren provided an overview of the EpiOcular (EO) test method protocol.

On behalf of NICEATM, Dr. Lipscomb reviewed the EO test method performance as detailed in the AMCP draft SRD.

Dr. Merrill then presented the ICCVAM draft recommendations for the EO test method for the Panel to consider.

Panel Evaluation

Dr. Bailey (Evaluation Group Chair) presented the Evaluation Group's responses to questions posed to the Panel on the validation status of the EO test method and ICCVAM draft test method recommendations. The Panel agreed that the EO test method protocol is adequately detailed but emphasized that the manufacturer should provide a "certificate of quality" for each batch of EO. The Panel also agreed that the critical aspects of the protocol had been justified and described in the BRD; however, in order to use the EO test method in a testing strategy to identify mild irritants and substances not labeled as irritants, positive controls that represent these hazard categories should be included in any future validation studies. The Panel noted that the EO test method cannot distinguish Category III from Category IV substances.

The Panel commented that the performance of the EO test method had not been adequately evaluated and compared to the Draize test for the types of substances included in the AMCP database. The Panel noted that the total number of products and their distribution across hazard categories were not sufficient. The Panel commented that the intralaboratory variability was not adequately assessed, although interlaboratory variability was considered to be adequate.

Public Comments

Dr. Curren indicated that he felt that it was appropriate to include EO data that used a different protocol as a measure of test method reproducibility.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Panel Conclusions and Recommendations

Dr. Hayes asked if the Panel was in agreement with its preliminary conclusions. The Panel voted unanimously to approve the recommendations as revised during the discussion with one abstention, Dr. Ward, who cited a potential conflict-of-interest because of her previous consulting work for a company that conducts the EO test method.

Strategy for U.S. Environmental Protection Agency Ocular Hazard Classification and Labeling of Antimicrobial Cleaning Products (AMCPs) Using *In Vitro* Alternative Test Methods

Dr. Curren provided an overview of the AMCP testing strategy.

On behalf of NICEATM, Dr. Lipscomb reviewed the AMCP draft SRD.

Dr. Merrill then presented the ICCVAM draft recommendations for the AMCP testing strategies for the Panel to consider.

Panel Evaluation

Dr. Bailey (Evaluation Group Chair) presented the Evaluation Group's responses to questions posed to the Panel on the validation status of the AMCP testing strategies and ICCVAM draft test method recommendations. The Panel also suggested adding more discussion of the cells used in the CM and EO test methods.

Regarding the BCOP test method, the Panel reflected on its previous discussions of the BCOP test method for the total database. The Panel indicated that use of the BCOP test method in a testing strategy to identify severe irritants (Category I) and moderate irritants (Category II), should include positive controls that represent these hazard categories in any future validation studies. The Panel noted that histopathology evaluation, as it is proposed at this time as an additional endpoint for the BCOP test method, does not justify its use for hazard classification of AMCPs. However, histopathology evaluation may prove to be a useful endpoint and, as such, collection of histopathology data and further efforts to optimize its use are encouraged.

The Panel agreed with the ICCVAM draft recommendations that there is insufficient data to support the testing strategy in terms of the proposed test method usefulness and limitations (i.e., the classification of substances in all four ocular hazard categories). There were also insufficient available data on which to base definitive recommendations on the proposed alternate testing strategy for classifying substances in all four ocular hazard categories. In discussing the validity of retrospective evaluations, the Panel stated that a retrospective evaluation of results could be considered adequate if the studies were performed with GLP compliance, coded samples, and pre-established evaluation criteria. The Panel commented that any definitive recommendations on a testing strategy should be based on prospective testing of a list of reference substances in each of the proposed *in vitro* test methods.

The Panel concurred with the ICCVAM draft recommendations in terms of the proposed test method standardized protocols. The Panel stated that routine fixation of tissue from the BCOP test method for possible histopathology evaluation should be continued. The Panel emphasized that no single *in vitro* test method alone was applicable to all types of test materials, and therefore suggested several future studies that could potentially expand the usefulness of AMCP test strategies.

Finally, the Panel commented that the development of performance standards for the AMCP testing strategy was not currently warranted and that a new approach needed to be defined for comparing testing strategies.

Public Comments

Dr. Barroso commented that ECVAM is working on a guideline for the detection of severe irritants with the BCOP test method. He indicated that they see a small change in classification when the cut-off is changed from 55 to 75. ECVAM considers 55 the best cut-off for their intended purpose.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Dr. Curren commented that concern regarding the limited number of AMCPs is misplaced due to the intended narrow applicability domain. He stated that industrial-strength cleaners are mostly severe irritants and that household cleaners are mostly mild irritants. Very few, if any, substances are in the moderate range. Dr. Curren expressed concern with the recommendation by the Panel that substances need to be tested by each test method in the testing strategy. He noted that histopathology evaluation with the BCOP test method was included in the testing strategy to provide additional safety, and clarified that most of the histopathology evaluation was performed by a certified veterinary

pathologist. He also questioned the Panel's suggested use of a transformed ocular cell line rather than a normal epidermal cell line.

Dr. Hayes asked the Panel if they needed clarification from the commenter; none were requested.

Panel Conclusions and Recommendations

Dr. Hayes asked if the Panel was in agreement with its preliminary conclusions. The Panel voted unanimously to approve the recommendations as revised during the discussion with one abstention, Dr. Ward, who cited a potential conflict-of-interest because of her previous consulting work for a company that manufactures AMCPs.

Concluding Remarks

Dr. Hayes, on behalf of the Panel, thanked Dr. Stokes and the NICEATM staff for their continued assistance during the review process and Panel meeting. He also thanked Dr. Wind, ICCVAM Chair, and the members of ICCVAM and the OTWG for their contributions to the project. Finally, Dr. Hayes thanked the Panel and the Evaluation Group Chairs.

Drs. Wind and Stokes thanked the Panel again for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes further thanked public attendees for their participation and the invited test method developers for their excellent test method summaries. Dr. Stokes concluded by saying he looked forward to working further with Panel members to complete the Panel report.

Adjournment

Dr. Hayes adjourned the Panel at 7:40 p.m., concluding the meeting.

Appendix D2
Independent Scientific Peer Review Panel Report:
Evaluation of the Validation Status of
Alternative Ocular Safety Testing Methods and Approaches

The peer review panel report is available at:
https://ntp.niehs.nih.gov/iccvam/docs/ocutox_docs/ocularprprept2009.pdf

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Appendix E

Federal Register Notices and Public Comments

- E1 *Federal Register* Notices
- E2 Public Comments Received in Response to *Federal Register* Notices
- E3 Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)
Comments: SACATM Meeting on June 25-26, 2009

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Appendix E1

Federal Register Notices

Federal Register notices are available at <https://www.federalregister.gov/>

70 FR 13512 (March 21, 2005)

Request for Data on Non-Animal Methods and Approaches for Determining Skin and Eye Irritation Potential of Antimicrobial Cleaning Product Formulations; Request for Nominations for an Independent Expert Panel

72 FR 26396 (May 9, 2007)

Request for Data on the Use of Topical Anesthetics and Systemic Analgesics for *In Vivo* Eye Irritation Testing

72 FR 31582 (June 7, 2007)

Request for Ocular Irritancy Test Data from Human, Rabbit, and *In Vitro* Studies Using Standardized Testing Methods

73 FR 18535 (April 4, 2008)

Non-Animal Methods and Approach for Evaluating Eye Irritation Potential for Antimicrobial Cleaning Products (AMCPs): Request for Nominations for an Independent Expert Panel and Submission of Relevant Data

74 FR 14556 (March 31, 2009)

Announcement of an Independent Scientific Peer Review Panel on Alternative Ocular Safety Testing Methods; Availability of Draft Background Review Documents (BRDs); Request for Comments

74 FR 19562 (April 29, 2009)

Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

74 FR 33444 (July 13, 2009)

Independent Scientific Peer Review Panel Report: Evaluation of the Validation Status of Alternative Ocular Safety Testing Methods and Approaches: Notice of Availability and Request for Public Comments

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Appendix E2

Public Comments Received in Response to *Federal Register* Notices

Public comments are available on request from NICEATM

70 FR 13512 (March 21, 2005)

Request for Data on Non-Animal Methods and Approaches for Determining Skin and Eye Irritation Potential of Antimicrobial Cleaning Product Formulations; Request for Nominations for an Independent Expert Panel

- No responses received.

72 FR 26396 (May 9, 2007)

Request for Data on the Use of Topical Anesthetics and Systemic Analgesics for *In Vivo* Eye Irritation Testing

- Robert Guest (Safeparm Laboratories, Ltd.)

72 FR 31582 (June 7, 2007)

Request for Ocular Irritancy Test Data from Human, Rabbit, and *In Vitro* Studies Using Standardized Testing Methods

- No responses received.

73 FR 18535 (April 4, 2008)

Non-Animal Methods and Approach for Evaluating Eye Irritation Potential for Antimicrobial Cleaning Products (AMCPs): Request for Nominations for an Independent Expert Panel and Submission of Relevant Data

- No responses received.

74 FR 14556 (March 31, 2009)

Announcement of an Independent Scientific Peer Review Panel on Alternative Ocular Safety Testing Methods; Availability of Draft Background Review Documents (BRDs); Request for Comments

- Dr. Raymond David (BASF Corporation)
- Dr. John Harbell
- MatTek Corporation

- Dr. Wolfgang Pape (R&D Brands)
- Dr. Ruud Woutersen and Mr. Menk Prinsen (TNO)
- Dr. Robert Rapaport (The Procter & Gamble Company)
- Dr. Gerald Renner (Colipa, the European Cosmetics Association)
- Dr. Sherry Ward

74 FR 19562 (April 29, 2009)

Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

- Mr. Troy Seidle, Ms. Sara Amundson, and Dr. Martin Stephens (HSUS), Dr. Kate Willet (PETA), and Dr. Chad Sandusky (PCRM)
- Dr. Catherine Willet (PETA)

74 FR 33444 (July 13, 2009)

Independent Scientific Peer Review Panel Report: Evaluation of the Validation Status of Alternative Ocular Safety Testing Methods and Approaches: Notice of Availability and Request for Public Comments

- No responses received.

Appendix E3

Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments

SACATM Meeting on June 25-26, 2009

SACATM meeting minutes are available at
<https://ntp.niehs.nih.gov/events/past/index.html?type=SACATM>

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Appendix F

Relevant U.S. Federal and International Ocular Toxicity Regulations, Labeling, and Test Guidelines

F1	Table of Relevant U.S. Federal and International Ocular Testing Regulations for Hazard Classification and Labeling.....	F-3
F2	EPA OPPTS Guidance Document 870.2400 (August 1998).....	F-9
F3	EPA Office of Pesticide Programs Label Review Manual (August 2003).....	F-19
F4	Organisation for Economic Co-operation and Development (OECD) Test Guideline 405 (Adopted April 2002).....	F-21

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Appendix F1

Table of Relevant U.S. Federal and International Ocular Testing Regulations for Hazard Classification and Labeling

Note to the Reader:

Regulations may be updated in the future. It is recommended that users review the most current version of all regulations identified.

Electronic versions of United States Code (U.S.C.) can be obtained at:
<http://www.gpoaccess.gov/uscode/index.html>

Electronic versions of the Code of Federal Regulations (CFR) can be obtained at:
<http://www.gpoaccess.gov/cfr/index.html>

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Eye Irritation/Corrosion Testing: Relevant U.S. Federal Laws, Regulations, Guidelines, and Recommendations				
Agency, Center, or Office	Regulated Products	Statutory Requirements	Regulations (Applications)	Guidelines and Recommendations
CPSC	Consumer Products	Federal Hazardous Substances Act (U.S.C. Title 15, Chapter 47)	16 CFR 1500.3 (Definitions) 16 CFR 1500.42 (Test for Eye Irritants) 16 CFR 1500.121 (Labeling)	Animal Testing Policy (1984)
EPA/OPPTS	Chemicals as defined by the Toxic Substances Control Act Pesticides	Toxic Substances Control Act (U.S.C. Title 15, Chapter 53) Federal Insecticide, Fungicide, and Rodenticide Act (U.S.C. Title 7, Chapter 6)	40 CFR 716 (Safety Data) 40 CFR 717 (Adverse Reactions) 40 CFR 720 (Premanufacture Notification) 40 CFR 156 (Labeling) 40 CFR 158 (Pesticide Data)	OPPTS 870.2400 (1998) ¹ Label Review Manual (2003) ²

continued

¹ See **Appendix F2**.

² Available at: <http://www.epa.gov/oppfead1/labeling/lrm/>.

Eye Irritation/Corrosion Testing: Relevant U.S. Federal Laws, Regulations, Guidelines, and Recommendations (continued)				
Agency, Center, or Office	Regulated Products	Statutory Requirements	Regulations (Applications)	Guidelines and Recommendations
FDA/CFSAN FDA/CDER	Cosmetics ³ Pharmaceuticals	Federal Food, Drug, and Cosmetic Act (U.S.C. Title 21, Chapter 9) Public Health Service Act (U.S.C. Title 42, Chapter 6A)	21 CFR 70 (Color additives in food, medical devices, and cosmetics) 21 CFR 312 (IND Application) 21 CFR 314 (IND Approval) 21 CFR 701 (Cosmetic Labeling) 21 CFR 740 (Cosmetic Warning Statement)	No Specific Guidelines or Recommendations on Eye Irritation/Corrosion Testing Are Provided.
OSHA	Chemicals	Occupational Safety and Health Act of 1970 (U.S.C. Title 29, Chapter 15)	29 CFR 1910.1200 (Hazard Communication Standard) 16 CFR 1500.42 (Test for Eye Irritants)	No Specific Guidelines or Recommendations on Eye Irritation/Corrosion Testing Are Provided.

³ FDA does not have authority for pre-market approval of cosmetics or cosmetic ingredients with the exception of color additives. However, the FDA may enforce action against products or ingredients that are in violation of Federal labeling laws, including provision of adequate safety information.

Relevant Ocular Testing Regulations for Hazard Classification and Labeling: European Union	
Regulated Products	Regulations and Directives
Substances and Mixtures	<p>Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 (CLP, Classification Labelling and Packaging), amending and repealing Directives 67/548/EEC (DSD, Dangerous Substances Directive) and 1999/45/EC (DPD, Dangerous Preparations Directive), and amending Regulation (EC) No 1907/2006.</p> <p>Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 (REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals)</p>
Plant Protection Products	Council Directive 91/414/EEC of 15 July 1991 as amended
Cosmetics	Council Directive 76/768/EEC of 27 July 1976 as amended
Biocidal Products	Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 as amended

Relevant Ocular Testing Regulations for Hazard Classification and Labeling: United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS)	
Scope	Legal Instruments and Recommendations
Chemicals (Substances and Mixtures)	Globally Harmonized System of Classification and Labelling of Chemicals (UN 2007), Part 3, Chapter 3.2.4 (Serious eye damage/eye irritation)

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Appendix F2

EPA OPPTS Guidance Document 870.2400 (August 1998)

EPA Health Effects Test Guidelines are available at
<https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-870-health-effects-test-guidelines>

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Appendix F3

EPA Office of Pesticide Programs Label Review Manual (August 2003)

Electronic versions of the EPA LRM can be obtained at:
<http://www.epa.gov/oppfead1/labeling/lrm/>

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Appendix F4

Organisation for Economic Co-operation and Development (OECD) Test Guideline 405 (Adopted April 2002)

OECD Test Guideline 405 is available at

https://www.oecd-ilibrary.org/environment/test-no-405-acute-eye-irritation-corrosion_9789264185333-en

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