ICCVAM Test Method Evaluation Report: Current Validation Status of *In Vitro* Test Methods Proposed for Identifying Eye Injury Hazard Potential of Chemicals and Products

Interagency Coordinating Committee on the Validation of Alternative Methods

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

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List of Abbreviations and Acronyms

AMCP	Antimicrobial cleaning product
BCOP	Bovine corneal opacity and permeability
BRD	Background review document
CAM	Chorioallantoic membrane
CEC	Commission of European Communities
CM	Cytosensor® Microphysiometer
CV	Coefficient of variation
ºC	Degrees centigrade
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
EU	European Union
FHSA	Federal Hazardous Substances Act
FR	*Federal Register*
g	Gram
GHS	United Nations Globally Harmonized System of Classification and Labelling of Chemicals
HET-CAM	Hen's egg test–chorioallantoic membrane
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICE	Isolated chicken eye
IRE	Isolated rabbit eye
IS	Irritation score
MeSH	Medical Subject Headings
mL	Milliliter
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NTP	U.S. National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OTWG	ICCVAM Ocular Toxicity Working Group
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
UN	United Nations
UV/VIS	Ultraviolet/Visible

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Preface

Eye injury is a leading cause of visual impairment in the United States 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 cause 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]

To prevent eye injuries, regulatory agencies require testing to determine if chemicals and products may cause eye damage. This testing information is used to classify the ocular hazard and determine appropriate labeling to warn consumers and workers of the potential hazard. Appropriate labeling tells users how to avoid exposure that could damage 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 (Draize et al. 1944), although *in vitro* methods can now be used to identify whether substances cause severe irritation or permanent eye damage. The Draize rabbit eye test involves instillation of 0.1 mL of the test substance into the conjunctival sac of one eye. 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 the iris (tissues inside the eye) are scored, and the duration that the injuries persist is recorded.

In 2006, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluated the validation status of the bovine corneal opacity and permeability (BCOP), hen's egg test–chorioallantoic membrane (HET-CAM), isolated chicken eye (ICE), and isolated rabbit eye (IRE) test methods for their ability to identify ocular corrosives and severe irritants. Based on the validation database and performance, ICCVAM recommended that positive results in the BCOP and ICE test methods could be used to identify ocular corrosives and severe irritants without the need for animal testing. These test methods should always be considered before using animals and should be used where determined appropriate. Following their acceptance by U.S. Federal regulatory agencies in 2008, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and ICCVAM developed Organisation for Economic Co-operation and Development (OECD) international test guidelines for the BCOP and ICE test methods. The OECD adopted the guidelines in 2009.[5] As a result, substances that may cause severe irritation or permanent damage to eyes can now be identified using these methods without the use of live animals in the 31 member countries of the OECD.

This test method evaluation report provides ICCVAM's recommendations regarding the BCOP, HET-CAM, ICE, and IRE test methods for identifying nonsevere ocular irritants and substances not labeled as irritants. The report also includes recommendations on the Cytosensor® Microphysiometer (CM) test method, which was not part of the 2006 evaluation. The report summarizes the validation status of each test method and provides the ICCVAM-recommended BCOP, CM, HET-CAM, ICE, and IRE test method protocols.

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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 Test Guideline 437. Bovine corneal opacity and permeability test method for identifying ocular corrosives and severe irritants; Test Guideline 438. Isolated chicken eye test method for identifying ocular corrosives and severe irritants. Both In: OECD Guidelines for Testing of Chemicals. Paris:Organisation for Economic Co-operation and Development

As part of ICCVAM's ongoing international collaborations, 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 ICCVAM Ocular Toxicity Working Group (OTWG). ICCVAM, NICEATM, and the OTWG prepared (1) draft background review documents (BRDs) describing the validation status of each test method, including reliability and accuracy, and (2) draft test method recommendations for their usefulness and limitations.

ICCVAM released these documents to the public for comment prior to a meeting of an independent international scientific peer review panel (Panel). The Panel met in public session on May 19–21, 2009, and prepared a report summarizing its conclusions and recommendations. The Panel report was provided to the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) along with the draft BRDs, draft test method recommendations, and all public comments. 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 BRDs, which are provided as appendices, 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 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, 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, until 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 the ECVAM liaisons Drs. João Barroso, Thomas Cole, and Valerie Zuang and the JaCVAM liaison Dr. Hajime Kojima for their participation and contributions.

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Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently evaluated the validation status of test methods to identify substances that cause reversible eye injuries or do not cause sufficient eye damage to require hazard labeling: the bovine corneal opacity and permeability (BCOP), Cytosensor® Microphysiometer (CM), hen's egg test–chorioallantoic membrane (HET-CAM), isolated chicken eye (ICE), and isolated rabbit eye (IRE) test methods. Nearly all ocular safety testing has been conducted using the *in vivo* Draize rabbit eye test (Draize et al. 1944) to evaluate the potential for substances to cause ocular irritation and other ocular injuries, an acute reaction that may involve corneal cloudiness and ulceration, swelling and redness of the conjunctiva, and/or visible damage to the inside of the eye (iritis). The BCOP, CM, HET-CAM, ICE, and IRE methods are *in vitro* test methods that predict the extent of ocular damage that might occur *in vivo* without requiring the use of live animals. This test method evaluation report provides ICCVAM's recommendations for each *in vitro* test method as an alternative to the Draize rabbit eye test, based on demonstrated validity (usefulness and limitations). This report includes (1) protocols recommended by ICCVAM for future data collection and evaluation for the BCOP, CM, HET-CAM, ICE, and IRE test methods, (2) final background review documents (BRDs) describing the validation status of these test methods, and (3) recommendations for future studies.

Following a nomination by the U.S. Environmental Protection Agency (EPA) requesting an evaluation of several alternative methods and approaches for reducing, replacing, and refining the use of rabbits in the current *in vivo* eye irritation test method, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), ICCVAM, and the ICCVAM Ocular Toxicology Working Group prepared draft BRDs and draft test method recommendations. The drafts were provided to an independent international scientific peer review panel (hereafter "Panel") and to the public for comment. The Panel met in public session on May 19-21, 2009, to discuss its peer review of the ICCVAM draft BRDs and to provide conclusions and recommendations regarding the validation status of the BCOP, CM, HET-CAM, ICE, and IRE test methods. The Panel also reviewed how well the information contained in the draft BRDs supported ICCVAM's draft test method recommendations.

In finalizing this test method evaluation report and the BRDs, which are included here as appendices, ICCVAM considered (1) the conclusions and recommendations of the Panel, (2) comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and (3) public comments.

The Bovine Corneal Opacity and Permeability (BCOP) Test Method

ICCVAM Recommendations: BCOP Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy and reliability of the BCOP test method does **not** support its use as a screening test to distinguish substances not labeled as irritants (EPA Category IV, European Union [EU] Not Labeled, Federal Hazardous Substances Act [FHSA] Not Labeled, United Nations Globally Harmonized System of Classification and Labelling of Chemicals [GHS] Not Classified) from all other hazard categories (EPA Category I, II, or III; EU R41 or R36; FHSA Irritant; GHS Category 1, 2A, or 2B) when results are to be used specifically to classify and label substances under the EPA, EU, FHSA, or GHS classification systems. For the BCOP validation database of 211 substances, false positive rates were high, ranging from 53% (24/45) to 70% (63/90), depending on the hazard classification system used. Therefore, all positive results from these tests would require additional testing in a valid test system that can accurately characterize whether such substances require hazard labeling. False negative rates were 0% for the EU (0/54) and GHS (0/97) classification systems, 5% (6/132) for the FHSA classification system, and 6% (8/142) for the EPA classification system.

Among the eight EPA false negatives were three substances (3/8 [38%]) that were classified as EPA eye irritants based on at least one rabbit with corneal injuries and opacity that did not resolve until day 3 of the study. A fourth substance was classified as an EPA eye irritant based on all six rabbits with a conjunctival redness score of 2 (n = 4; *diffuse, crimson color of the conjunctiva, individual blood vessels not easily discernable*) or 3 (n = 2; *diffuse beefy red*). The conjunctival redness scores for two of these animals did not recover to a score of 1 (*some blood vessels definitely hyperemic*) until day 6 of the study. The conjunctival redness scores for the remaining four rabbits recovered to a score of 1 on day 2 of the study. These four EPA false negative substances were also false negatives for the FHSA classification system. Given the significant lesions associated with these false negative substances, the BCOP test method cannot be recommended as a screening test to identify substances not labeled as irritants (i.e., EPA Category IV, FHSA Not Labeled) for the EPA or FHSA classification systems.

Furthermore, although the false negative rate was 0% (0/97) for the GHS classification scheme, the GHS does not classify substances as eye hazards that produce the corneal and conjunctival injuries described above, which are required to be labeled as eye hazards according to the EPA and FHSA classification systems. These findings led NICEATM-ICCVAM to look more closely at the GHS eye hazard classification criteria. NICEATM evaluated results from rabbit eye test studies from two independent databases: (1) 149 studies obtained from a publicly available database (ECETOC 1998) and (2) 144 studies included in the Organization for Economic Cooperation and Development (OECD) Detailed Review Document on Classification Systems for Eye Irritation/Corrosion in OECD Member Countries (OECD 1999). These data, which are included here as an appendix, confirmed that approximately 30% of the substances requiring labeling for eye irritation hazard based on current U.S. hazard classification requirements (EPA and FHSA) are not labeled as eye irritation hazards by the GHS system. This includes at least 70% of currently labeled EPA Category III irritants (those causing eye injuries persisting for 24 hours to 7 days) that would not require hazard labeling using the GHS system. The nature, severity, and duration of these eye injuries suggest the potential to cause human injury. The purpose of ocular toxicity labeling is to communicate potential hazards of chemicals and products to workers and consumers so that appropriate measures can be taken to avoid accidental or inadvertent contact with the eye. In addition, ocular safety labels provide the necessary first aid measures that should be taken in the event of accidental exposures.

The GHS was established based on principles agreed to by participants, which included assuring that "the level of protection offered to workers, consumers, the general public and the environment should not be reduced as a result of harmonizing the classification and labeling systems" (UN 2007). ICCVAM has conducted technical analyses to support the development of appropriate recommendations for GHS options that would continue to provide protection that is at least equivalent to current U.S. eye irritation hazard classification and labeling requirements. ICCVAM recommends that U.S. agencies consider the GHS eye irritation hazard classification criteria and hazard categories and the level of protection they provide compared to current U.S. hazard classification systems.

Federal law requires agencies to determine that new test methods recommended by ICCVAM generate data that are at least equivalent to data generated by current test methods required or recommended by each agency for hazard identification purposes. Until the issues associated with the GHS system as outlined above are further discussed, ICCVAM is deferring final recommendations on the usefulness and limitations of using the BCOP test method as a screening test to identify substances not labeled as irritants according to the GHS classification system.

ICCVAM Recommendations: BCOP Test Method Protocol

For use of the BCOP test method as a screening test to identify substances as ocular corrosives and severe irritants (EPA Category I, EU R41, GHS Category 1), ICCVAM recommends using the

updated ICCVAM BCOP test method protocol included as an appendix to this report. All future studies intended to further characterize the usefulness and limitations of the BCOP test method should be conducted using this protocol.

ICCVAM Recommendations: BCOP Future Studies
ICCVAM recommends additional studies to further characterize and potentially improve the usefulness and applicability of the BCOP test method to distinguish ocular irritants from all hazard categories:

- Additional optimization studies/evaluations should be conducted to improve the correct classification of mild and moderate ocular irritants and substances not labeled as irritants. After optimization, additional studies to further assess the reliability and accuracy of the test method are recommended.
- Histopathological evaluation of the corneal tissue, using standardized procedures, should be included when the BCOP test method is used. Such data will help develop 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.
- Users of the BCOP test method should provide all data that are generated from future studies, because they could help to further characterize the usefulness and limitations of the BCOP test method to identify all ocular hazard categories.

ICCVAM Recommendations: BCOP Performance Standards
Based on the available data and associated performance described above, ICCVAM recommends that the development of performance standards for the BCOP test method is not warranted at this time.

Validation Status of the BCOP Test Method
The BCOP test method is an *in vitro* method that provides short-term maintenance of physiological and biochemical function of the bovine cornea. Quantitative changes in opacity and fluorescein permeability are assessed as indicators of potential ocular irritation.

The accuracy of the BCOP test method was compared to hazard categories based on *in vivo* Draize rabbit eye test data according to the EPA, EU, FHSA, or GHS systems using the current BCOP validation database of 211 substances. When the BCOP test method was used to distinguish substances not labeled as irritants (EPA Category IV, EU Not Labeled, FHSA Not Labeled, GHS Not Classified) from all other categories, accuracy ranged from 64% (76/118) to 83% (161/194), depending on the hazard classification system used. While false positive rates were high (53% [24/45] to 70% [63/90], depending on the hazard classification system used), the false negative rates were low (5% [6/132] for the FHSA system, 6% [8/141] for EPA the system, and 0% [0/54 or 0/97] for the EU and GHS systems, respectively).

Qualitative analyses of interlaboratory reproducibility were conducted to evaluate how well the BCOP hazard classifications agreed among the participating laboratories from the three different interlaboratory validation studies (Balls et al. 1995; Gautheron et al. 1994; and Southee 1998). These evaluations were based on the use of the BCOP test method (1) to identify all ocular hazard categories according to the EPA, EU, or GHS systems, and (2) to distinguish substances not labeled as irritants from all other ocular hazard categories. For both approaches, there was 100% agreement among the multiple laboratories in each study for a majority of the correctly identified ocular irritant hazard categories. Because the performance of the BCOP test method was similar for the EPA and FHSA hazard classification systems, additional reliability analyses were not conducted for the FHSA hazard classification system.

The Cytosensor Microphysiometer (CM) Test Method

ICCVAM Recommendations: CM Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy and reliability of the CM test method support its use as a screening test to identify water-soluble substances (water-soluble surfactants, surfactant-containing formulations, and nonsurfactants) as ocular corrosives and severe irritants (EPA Category I, EU R41, GHS Category 1) in a tiered-testing strategy, as part of a weight-of-evidence approach. False positive rates ranged from 0% (0/17 or 0/18) to 10% (3/29), and false negative rates ranged from 9% (2/23) to 50% (6/12), depending on the classification system used and the type of substance tested. A substance that tests negative with the CM test method would need to be tested in another test method that can identify possible *in vitro* false negative ocular corrosives and severe irritants and distinguish between moderate and mild ocular irritants. Currently, the Draize rabbit eye test is the only test method that can make such a distinction.

ICCVAM further concludes that the accuracy and reliability of the CM test method are sufficient to support its use as a screening test to distinguish water-soluble surfactant chemicals and certain types of surfactant-containing formulations (e.g., cosmetics and personal care product formulations, but not pesticide formulations) as substances not labeled as irritants (EPA Category IV, EU Not Labeled, FHSA Not Labeled) from all other hazard categories (EPA Category I, II, III; EU R41, R36; FHSA Irritant) when results are to be used specifically to classify and label substances under the EPA, EU, and FHSA classification systems. As noted above, until the issues associated with the GHS classification system are further discussed (see "BCOP Test Method Usefulness and Limitations"), ICCVAM is deferring final recommendations on the usefulness and limitations of using the CM test method as a screening test to identify substances not labeled as irritants according to the GHS classification system.

When the CM test method was used to distinguish substances not listed as irritants from all other hazard categories the validation database of 53 water-soluble surfactants and surfactant-containing formulations, false positive rates were high, ranging from 50% (3/6) to 69% (18/26), depending on the hazard classification system used. However, such positive results would require additional testing in a valid test system that can accurately characterize whether such substances require hazard labeling. Positive results would also need to be additionally tested with methods that can correctly identify moderate and mild ocular irritants. False negative rates ranged from 0% (0/27, 0/28, or 0/40) to 2% (1/42 or 1/47) compared to results from the Draize rabbit eye test. The one false negative substance was EPA Category III or FHSA Irritant based on *in vivo* data. For this substance, six test animals were included in the *in vivo* test. One test animal had no observable effects, three test animals had conjunctival redness (score = 1), and two test animals had corneal opacity (score = 1) that cleared after one day.

Because of the high false negative rates (24% [5/21] to 40% [8/20]for the CM test method when testing water-soluble nonsurfactant substances and formulations, the CM test method is **not** recommended as a screening test to identify substances not labeled as irritants among these types of substances.

Given that the CM test method (INVITTOX Protocol 102) is proposed for use as a screening test to identify ocular corrosives and severe irritants and substances not labeled as irritants, users may want to consider using the CM test method before using another *in vitro* ocular test method for testing these types of substances. However, water-soluble substances that are not identified as ocular corrosives and severe irritants or water-soluble surfactant chemicals and specific types of surfactant-containing formulations that are not identified as substances not labeled as irritants with the CM test method would need to be tested in another test method able to correctly classify substances into each of the four EPA or GHS hazard classification categories. Currently, the only test method accepted for these purposes is the Draize rabbit eye test. Because the CM test method has a high false positive rate for

substances not labeled as irritants (50% [3/6] to 69% [18/26], depending on the hazard classification system used), users may not want to use it if the intended use is to start with identifying substances not labeled as irritants.

ICCVAM Recommendations: CM Test Method Protocol

For use of the CM test method as a screening test to identify water-soluble substances as ocular corrosives and severe irritants (EPA Category I, EU R41, GHS Category 1) or to identify substances not labeled as irritants (EPA Category IV, EU Not Labeled, FHSA Not Labeled), ICCVAM recommends using the updated ICCVAM CM INVITTOX Protocol 102[6] that is included as an appendix to this report. All future studies intended to further characterize the usefulness and limitations of the CM test method should be conducted using this protocol.

ICCVAM Recommendations: CM Future Studies

ICCVAM recommends that additional studies be conducted to further characterize the usefulness and limitations of the CM test method for use as a screening test to identify ocular corrosives and severe irritants (EPA Category I, GHS Category 1, EU R41) and substances not labeled as irritants (EPA Category IV, EU Not Labeled, FHSA Not Labeled, GHS Not Classified). This includes additional testing using a broader range of materials to expand the recommended types of substances appropriate for testing.

ICCVAM recommends that a subset of the ICCVAM-recommended reference substances for validation of *in vitro* ocular toxicity test methods for the evaluation of ocular corrosives and severe irritants[7] be tested in the CM test method in order to provide for more direct assessment of the CM test method's utility as a screening test for identifying ocular corrosives and severe irritants. Similarly, a reference set could also be selected from this list for the purposes of assessing the utility of the CM test method as a screening test for identifying substances not labeled as irritants.

Finally, ICCVAM recommends future optimization studies to increase the ability of the CM test method to identify all categories of ocular irritancy hazard classification according to the EPA, EU, or GHS hazard classification systems. This will require more substances in the moderate and mild ocular irritant categories (EPA Category II and III, EU Category R36, or GHS Category 2A and 2B, respectively) be identified and tested.

ICCVAM Recommendations: CM Performance Standards

Based on the available data and associated performance described above, ICCVAM recommends that the development of performance standards for the CM test method is not warranted at this time.

Validation Status of the CM Test Method

The CM test method exposes a population of cells to increasing concentrations of a test substance. The concentration that leads to a 50% decline in the metabolic rate of the cells (the MRD_{50}) is used as an indicator of ocular irritancy potential. An abbreviated version of the European Centre for the Validation of Alternative Methods (ECVAM) CM BRD that does not include confidential business information describes the current validation status of the CM test method, including what is known about its reliability and accuracy, the scope of substances tested, and standardized protocols for the validation study. The following is a synopsis of the information contained within three peer-reviewed publications (Balls et al. 1995; Gettings et al. 1996; Brantom et al. 1997) described in the ECVAM CM BRD and used in the ICCVAM review.

[6] Available at http://ecvam-dbalm.jrc.ec.europa.eu/

[7] http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_tmer.htm

The database of 53 water-soluble surfactants tested in the CM test method included 21 surfactant chemicals and 32 surfactant-containing formulations tested across seven different laboratories. Using INVITTOX Protocol 102 to identify ocular corrosives and severe irritants among the water-soluble surfactants and surfactant-containing formulations, the false positive rate ranged from 3% (1/30) to 10% (3/29), depending on the hazard classification system used, compared to *in vivo* results. The three false positives when using the EPA classification system are classified as Category II (n = 2) or III (n = 1) based on *in vivo* data. The one false positive when using the GHS and EU classification systems is classified as Not Classified and Not Labeled, respectively, based on *in vivo* data. The false negative rate ranged from 9% (2/23) to 22% (5/23), depending on the hazard classification system used, compared to *in vivo* results. In each case, these substances were classified as moderate or mild irritants *in vitro* based on the EPA, EU, and GHS classification systems (i.e., EPA Category II or III; EU R36; or GHS Category 2A or 2B).

The nonsurfactant substances database (n = 29) consisted of 27 water-soluble nonsurfactant chemicals, which included a range of chemical classes (e.g., acids, alcohols, alkalis, and ketones), and water-soluble nonsurfactant formulations (n = 2) tested in seven laboratories. Using INVITTOX Protocol 102 to identify ocular corrosives and severe irritants among the nonsurfactant substances, the false positive rate was 0% (0/17 or 0/18) for all hazard classification systems compared to *in vivo* results. The false negative rate ranged from 29% (2/7) to 50% (6/12), depending on the hazard classification system used, compared to *in vivo* results. Two substances were false negatives when using the EPA classification system and were classified *in vitro* as either Category II/III (n = 1) or IV (n = 1). Five substances were false negatives when using the GHS classification system and were classified *in vitro* as either Category 2A/2B (n = 4) or Not Labeled (n = 1). Six substances were false negatives when using the EU classification system and were classified *in vitro* as either R36 (n = 5) or Not Labeled (n = 1).

Using INVITTOX Protocol 102 to identify substances not labeled as irritants among the database of 53 water-soluble surfactants and surfactant-containing formulations, the false negative rate ranged from 0% (0/27 or 0/28, or 0/40) to 2% (1/46 or 1/47), depending on the hazard classification system used, compared to *in vivo* results. The one substance that was a false negative is classified as EPA Category III based on *in vivo* data from a six-rabbit *in vivo* test. One rabbit had no observable effects, three rabbits had conjunctival redness (score = 1), and two rabbits had corneal opacity (score = 1) that cleared after one day. The false positive rate ranged from 50% (3/6) to 69% (18/ 26), depending on the hazard classification system used, compared to *in vivo* results. Three substances were false positives when using the EPA and FHSA classification systems and were classified *in vitro* as Category II/III or Irritant, respectively. Seventeen substances were false positives when using the GHS classification system and were classified *in vitro* as Category 2A/2B (n = 16) or Category 1 (n = 1). Eighteen substances were false positives when using the EU classification system and were classified *in vitro* as R36 (n = 17) or R41 (n = 1).

Using INVITTOX Protocol 102 to identify substances not labeled as irritants among the database of 29 nonsurfactant substances, the false negative rate ranged from 24% (5/21) to 40% (8/20), and the false positive rate ranged from 25% (1/4 or 2/8) to 40% (2/5), depending on the hazard classification system used, compared to *in vivo* results.

Intralaboratory reproducibility was assessed based on calculated coefficients of variation (CVs) for MRD_{50} values for two different studies. Mean CVs ranged from 10% to 24% and tended to be slightly higher for surfactant substances than for nonsurfactant substances.

Interlaboratory reproducibility of the CM test method was also assessed using the data from validation studies by the European Commission/Home Office (EC/HO; Balls et al. 1995) and European Cosmetic, Toiletry and Perfumery Association (COLIPA; Brantom et al. 1997), which included four laboratories and two laboratories, respectively. Mean CVs in the EC/HO study ranged

from 16% to 37% for surfactant substances and up to 51% for nonsurfactant substances. For surfactant materials, all four laboratories using the CM test method had 100% agreement for 55% (6/11) of the test substances; 75% of the laboratories had identical results for 27% (3/11) of the test substances; and 50% of the laboratories had agreement for 18% (2/11) of the test substances. For nonsurfactant substances, agreement among the laboratories was 100% for 48% (11/23) of the test substances, 75% for 22% (5/23) of the test substances, 67% for 4% (1/23) of the test substances, and 50% for 13% (3/23) of the test substances.

For the COLIPA study, substances were divided into surfactant materials, surfactant-based formulations and mixtures, and nonsurfactant substances. Two laboratories had mean between-laboratory CVs ranging from 16% to 23% for surfactant materials, approximately 16% for surfactant-based formulations and mixtures, and 32% to 51% for nonsurfactant substances. For surfactant materials, the laboratories had 100% agreement for 90% (9/10) of the test substances and 0% agreement for 10% (1/10) of the test substances. For surfactant-based formulations and mixtures, the laboratories had 100% agreement for 100% (7/7) of the test substances. For nonsurfactant substances, the laboratories had 100% agreement for 78% (7/9) of the test substances and 0% agreement for 22% (2/9) of the test substances.

The Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) Test Method

ICCVAM Recommendations: HET-CAM Test Method Usefulness and Limitations
ICCVAM concludes that the accuracy and reliability of the HET-CAM test method does **not** support its use as a screening test to distinguish substances not labeled as irritants (EPA Category IV, EU Not Labeled, FHSA Not Labeled) from all other hazard categories (EPA Category I, II, or III; EU R41 or R36; FHSA Irritant) when results are to be used specifically to classify and label substances under the EPA, EU, or FHSA classification systems.

The available validation database for the HET-CAM test method has remained unchanged since the original ICCVAM evaluation (ICCVAM 2006b). For the HET-CAM validation database of 60 surfactants and oil/water emulsions, false positive rates were 60% (9/15) to 69% (22/32) and false negative rates were 0% (0/26) to 9% (4/45). Among the four false negatives, 100% (4/4) were EPA Category III substances based on conjunctival redness scores of 2 that required at least three days to resolve. For one of the substances, one of the six rabbits tested had a conjunctival redness score of 2 that required 14 days to resolve. Four of the remaining five rabbits in this study had conjunctival redness scores of 2 that resolved within three days; the last rabbit did not have this lesion. However, there were too few substances in the moderate irritant categories to have sufficient confidence in the ability of HET-CAM to distinguish them from the substances not labeled as irritants category (there were only 2 EPA Category II substances).

ICCVAM Recommendations: HET-CAM Test Method Protocol
The updated ICCVAM-recommended HET-CAM test method protocol is included as an appendix to this report. The protocol has been modified from a generic description of the Irritation Score (IS) analysis method to include a more detailed IS(A) analysis method to be used for prospective studies. However, a description of the IS(B) method is included for retrospective analyses, where IS(B) analysis method data could be converted to fixed time points similar to those used for the IS(A) analysis method. All future studies intended to further characterize the usefulness and limitations of the HET-CAM test method should be conducted using this protocol.

ICCVAM Recommendations: HET-CAM Future Studies
ICCVAM recommends additional studies to further characterize and potentially improve the usefulness and applicability of the HET-CAM test method to distinguish ocular irritants from all hazard categories:

- Additional studies should be conducted to further optimize the HET-CAM test method decision criteria that would be used to identify ocular corrosives and severe irritants (EPA Category I, EU R41, GHS Category 1), as well as moderate irritants (EPA Category II, EU R36, GHS Category 2A) and mild irritants (EPA Category III, GHS Category 2B), as defined by the EPA, GHS, or EU classification systems. Such studies could potentially improve the usefulness of the HET-CAM test method for identifying these types of substances.
- The types of substances appropriate for testing should be expanded to include a broader range of chemical and product classes.
- Users of the HET-CAM test method should provide all data that are generated from future studies, because they could help to further characterize the usefulness and limitations of the HET-CAM test method to identify all ocular hazard categories.

ICCVAM Recommendations: HET-CAM Performance Standards
Based on the available data and associated performance described above, ICCVAM recommends that the development of performance standards for the HET-CAM test method is not warranted at this time.

Validation Status of the HET-CAM Test Method
ICCVAM reviewed HET-CAM performance compared to the Draize rabbit eye test for each classification system (EPA, EU, and GHS) using each of the six HET-CAM protocols (IS[A], IS[B], Q-Score, S-Score, IS, and ITC protocols). With the exception of the IS(A) and IS(B) protocols, all protocols classified at least one *in vivo* moderate or severe irritant substance as a substance not labeled as an irritant (EPA Category IV, EU Not Labeled, GHS Not Classified). The IS(B) overpredicted more than 90% (39/42) of the GHS Not Classified substances. Therefore, more extensive analyses of HET-CAM were restricted to the IS(A) protocol.

No new HET-CAM data have been obtained since the ICCVAM evaluation of the HET-CAM test method for identifying ocular corrosives and severe irritants (ICCVAM 2006b). Overall accuracy in distinguishing substances not labeled as irritants (EPA Category IV, EU Not Labeled, FHSA Not Labeled, GHS Not Classified) from all other categories ranged from 62% (36/58) to 80% (44/55), depending on the hazard classification system used. False positive rates were 60% (9/15) to 69% (22/32) and false negative rates were 0% (0/26) to 9% (4/45). Among the four false negatives, 100% (4/4, all oil/water emulsion cosmetic formulations) were EPA Category III substances based on conjunctival redness scores of 2 that required at least three days to resolve. For one of the substances, one out of the six rabbits tested had a conjunctival redness score of 2 that required 14 days to resolve. Four of the remaining five rabbits in this study had conjunctival redness scores of 2 that resolved within three days; the last rabbit did not have this lesion.

Quantitative and qualitative evaluations of HET-CAM test method reliability have been conducted previously (ICCVAM 2006b). Because the database used for the current evaluation of the HET-CAM test method has not changed, the quantitative evaluation of test method reliability remains unchanged. Additional qualitative analyses of interlaboratory reproducibility were conducted to evaluate how well the HET-CAM hazard classifications agreed among the five laboratories that participated in the interlaboratory validation study (Hagino et al. 1999). These evaluations were based on the use of the HET-CAM test method (1) to identify all ocular hazard categories according to the EPA, EU, or GHS systems, and (2) to distinguish substances not labeled as irritants from all other ocular hazard

categories. For both approaches, there was 100% agreement among the multiple laboratories in each study for a majority of the correctly identified ocular irritant hazard categories. Because the performance of the HET-CAM test method was similar for the EPA and FHSA hazard classification systems, additional reliability analyses were not conducted for the FHSA hazard classification system.

The Isolated Chicken Eye (ICE) Test Method

ICCVAM Recommendations: ICE Test Method Usefulness and Limitations
ICCVAM concludes that the accuracy and reliability of the ICE test method does **not** support its use as a screening test to distinguish substances not labeled as irritants (EPA Category IV, EU Not Labeled, FHSA Not Labeled) from all other hazard categories (EPA Category I, II, or III; EU R41 or R36; FHSA Irritant) when results are to be used specifically to classify and label substances under the EPA, EU, or FHSA classification systems.

The available validation database for the ICE test method has remained unchanged since the original ICCVAM evaluation (ICCVAM 2006c). For the ICE validation database of 175 substances, false positive rates were 11% (10/93) to 34% (27/79) and false negatives rates were 6% (4/62) to 22% (13/60). Among the false negatives, at least one substance was classified as an ocular corrosive/severe irritant based on Draize rabbit eye test data (n = 1 each for the EPA and GHS systems, and n = 6 for the EU system). Considering the public health impact of misclassifying a corrosive substance as Not Labeled, these false negative results cannot be minimized.

ICCVAM Recommendations: ICE Test Method Protocol
For use of the ICE test method as a screening test to identify substances as ocular corrosives and severe irritants (EPA Category I, GHS Category 1, EU R41), ICCVAM recommends using the updated ICCVAM ICE test method protocol that is included as an appendix to this report. All future studies intended to further characterize the usefulness and limitations of the ICE test method should be conducted using this protocol.

ICCVAM Recommendations: ICE Future Studies
ICCVAM recommends additional studies to further characterize and potentially improve the usefulness and applicability of the ICE test method to distinguish ocular irritants from all hazard categories:

- Additional optimization studies should be conducted to improve the correct classification of mild and moderate ocular irritants and substances not labeled as irritants. After optimization, additional studies to further assess the reliability and accuracy of the test method are recommended.
- Histopathological evaluation of the corneal tissue, using standardized procedures, should be included when the ICE test method is used. Such data will help develop 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.
- Users of the ICE test method should provide all data that are generated from future studies, because they could help to further characterize the usefulness and limitations of the ICE test method to identify all ocular hazard categories.

ICCVAM Recommendations: ICE Performance Standards
Based on the available data and associated performance described above, ICCVAM recommends that the development of performance standards for the ICE test method is not warranted at this time.

Validation Status of the ICE Test Method

No new ICE data have been obtained since the ICCVAM evaluation of the ICE test method for identifying ocular corrosives and severe irritants (ICCVAM 2006c). Overall accuracy in distinguishing substances not labeled as irritants (EPA Category IV, EU Not Labeled, FHSA Not Labeled, GHS Not Classified) from all other categories ranged from 78% (110/141) to 85% (130/153), depending on the hazard classification system used. False positive rates were 11% (10/93) to 34% (27/79) and false negative rates were 6% (4/62) to 22% (13/60). Among these false negatives, at least one substance was classified as an ocular corrosive/severe irritant based on Draize rabbit eye test data (n = 1 each for the EPA and GHS systems, and n = 6 for the EU system). Considering the public health impact of misclassifying a corrosive substance as Not Labeled, these false negative results cannot be minimized.

Quantitative and qualitative evaluations of ICE test method reliability have been conducted previously (ICCVAM 2006c). Because the database used for the current evaluation of the ICE test method has not changed, the quantitative evaluation of test method reliability remains unchanged. Additional qualitative analyses of interlaboratory reproducibility were conducted to evaluate how well the ICE hazard classifications agreed among the four laboratories that participated in the interlaboratory validation study (Balls et al. 1995). These evaluations were based on the use of the ICE test method (1) to identify all ocular hazard categories according to the EPA, EU, or GHS systems, and (2) to distinguish substances not labeled as irritants from all other ocular hazard categories. For both approaches, there was 100% agreement among the multiple laboratories in each study for a majority of the correctly identified ocular irritant hazard categories. Because the performance of the ICE test method was similar for the EPA and FHSA classification systems, additional reliability analyses were not conducted for the FHSA classification system.

The Isolated Rabbit Eye (IRE) Test Method

ICCVAM Recommendations: IRE Test Method Usefulness and Limitations

The available validation database for the IRE test method has remained unchanged since the original ICCVAM evaluation (ICCVAM 2006d). Because of the lack of a standardized protocol and insufficient data using all four recommended IRE endpoints, ICCVAM concludes that additional studies are needed before definitive recommendations on the accuracy and reliability of the IRE test method can be made.

ICCVAM Recommendations: IRE Test Method Protocol

An ICCVAM-recommended test method protocol for the IRE test method that should be used for all future IRE studies is included as an appendix to this report. The recommended protocol remains unchanged from the previous ICCVAM evaluation (ICCVAM 2006e) and includes four endpoints that should be measured: maximal corneal opacity (opacity x area), maximal corneal swelling, fluorescein penetration (intensity x area), and assessment of epithelial integrity (at 0.5, 1, 2, 3, and 4 hours after test substance administration.

ICCVAM Recommendations: IRE Future Studies

ICCVAM recommends additional studies to further characterize and potentially improve the usefulness and applicability of the IRE test method to distinguish ocular irritants from all other hazard categories:

- Additional evaluation studies should be conducted to increase the current IRE database and optimize the IRE test method decision criteria. Once these studies are conducted, ICCVAM recommends that additional validation studies be conducted to further evaluate the relevance and reliability of the IRE test method.
- Histopathological evaluation of the corneal tissue, using standardized procedures, should be included when the IRE test method is used. Such data will help develop 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.

- Users of the IRE test method should provide all data that are generated from future studies, because they could help to further characterize the usefulness and limitations of the IRE test method to identify all ocular hazard categories.

ICCVAM Recommendations: IRE Performance Standards

Based on the available data described above, ICCVAM recommends that the development of performance standards for the IRE test method is not warranted at this time.

Validation Status of the IRE Test Method

The performance section of the IRE BRD (ICCVAM 2006d) uses data from Balls et al. (1995), Gettings et al. (1996), and Guerriero et al. (2004). These references were examined for decision criteria that would help classify moderate and mild irritants. There are insufficient data using all four recommended IRE endpoints (corneal opacity, fluorescein penetration, corneal swelling, and observations of significant effect on corneal epithelium) to assess the accuracy and reliability of the IRE test method when all of these endpoints are evaluated in a single study. Furthermore, among the studies that included each endpoint, decision criteria focused on distinguishing ocular corrosives and severe irritants from all other ocular hazard categories (moderate and mild irritants and substances not labeled as irritants) and did not specify decision criteria for each ocular hazard category. For these reasons, an adequate evaluation of the IRE test method for its ability to distinguish substances not labeled as irritants from all other ocular hazard categories is not feasible at this time.

Because of the lack of quantitative IRE test method data for replicate experiments within an individual laboratory, the intralaboratory repeatability and reproducibility of the IRE test method could not be evaluated. However, multilaboratory qualitative and quantitative IRE test data were available for a collaborative study by the Commission of European Communities (CEC 1991) involving three laboratories and a validation study conducted by Balls et al. (1995) involving four laboratories. In the CEC (1991) study, each substance tested was assigned a EU classification (R41, R36, or nonirritant [EU 2001]) based on Draize rabbit eye test results. However, due to the lack of individual rabbit Draize scores, a reliability assessment for the CEC (1991) study using the GHS (UN 2007) or EPA (EPA 2003) classification criteria was not possible. The Balls et al. (1995) data were used for an evaluation of the interlaboratory reproducibility of the IRE test method according to the GHS (UN 2007), EPA (EPA 2003), and EU (EU 2001) classification systems.

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 7-1** lists the nine different opportunities for public comments that were provided during the ICCVAM evaluation of the validation status of alternative ocular safety testing methods and approaches. A total of 37 public comments were received. Comments received in response to or related to the *Federal Register* notices are also available on the NICEATM-ICCVAM website.[8]

[8] Available at http://ntp-apps.niehs.nih.gov/iccvambp/searchPubCom.cfm

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