

**Independent Scientific Peer Review Panel Report:
Evaluation of the Validation Status of Alternative Ocular Safety
Testing Methods and Approaches**

July 2009

**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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National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services**

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This document is available electronically at
http://iccvam.niehs.nih.gov/docs/ocutox_docs/OcularPRPRept2009.pdf

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When referencing this document, please cite as follows:

Interagency Coordinating Committee on the Validation of Alternative Methods. 2009.

Independent Scientific Peer Review Panel Report:

Evaluation of the Validation Status of Alternative Ocular Safety Testing Methods and Approaches. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

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List of Abbreviations and Acronyms

AHT	Animal health technician
AMCP	Antimicrobial cleaning product(s)
BCOP	Bovine corneal opacity and permeability
BRD	Background review document
CEC	Commission of the European Communities
CM	Cytosensor Microphysiometer [®]
CPSC	U.S. Consumer Product Safety Commission
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EC/HO	European Commission/British Home Office
ECVAM	European Centre for the Validation of Alternative Methods
EO	EpiOcular [™]
EPA	U.S. Environmental Protection Agency
EU	European Union
FDA	U.S. Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GHS	Globally Harmonized System of Classification and Labeling of Chemicals
GLP	Good Laboratory Practice
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
ITC	Irritation threshold concentration
IVIS	<i>In vitro</i> irritancy score
LVET	Low volume eye test
NEI	National Eye Institute
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NICNAS	National Industrial Chemicals Notification and Assessment Scheme (Australia)

NIH	National Institutes of Health
NSAID	Nonsteroidal anti-inflammatory drug
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OSHA	U.S. Occupational Safety and Health Administration
OTWG	ICCVAM Ocular Toxicity Working Group
SC	By subcutaneous injection
SRD	Summary review document
TG	Test Guideline
TSA	Test substance application
UN	United Nations
V1	First branch of the trigeminal nerve

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Preface

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) convened an international independent scientific peer review panel (hereafter, Panel) meeting on May 19-21, 2009 at the U.S. Consumer Product Safety Commission Headquarters in Bethesda, MD. The Panel, which included 22 expert scientists from six countries, evaluated test methods and approaches that may further reduce and refine the use of animals for ocular safety testing.

These evaluations included the following:

- A proposal for the routine use of topical anesthetics, systemic analgesics, and humane endpoints to avoid and minimize pain and distress during *in vivo* ocular irritation testing
- The use of the bovine corneal opacity and permeability (BCOP), the Cytosensor Microphysiometer[®] (CM), the isolated chicken eye, the isolated rabbit eye, and the hen's egg test – chorioallantoic membrane test methods for identifying moderate and mild ocular irritants and substances not labeled as ocular irritants
- The *in vivo* low volume eye test
- Nonanimal testing strategies that use the BCOP, CM, and/or EpiOcular[™] test methods to assess the eye irritation potential of antimicrobial cleaning products and determine their appropriate U.S. Environmental Protection Agency ocular hazard classification

During the May 2009 public meeting, the Panel discussed each test method and approach, listened to public comments, and developed conclusions and recommendations for ICCVAM. The Panel emphasized its consideration in the following areas: (1) review of the ICCVAM draft background review documents (BRDs) for completeness and identification of errors or omissions of existing relevant data or information that should be included, (2) evaluation of the information in the draft summary review documents (SRDs) and BRDs to determine the extent to which each of the applicable ICCVAM criteria for validation and acceptance of toxicological test methods had been appropriately addressed, and (3) consideration of the ICCVAM draft test method recommendations and comment on the extent to which they are supported by the information provided in the draft BRDs or SRDs for the following:

- Proposed test method uses and limitations
- Proposed recommended standardized protocols
- Proposed future studies

This report details the Panel's independent conclusions and recommendations. ICCVAM will consider this report and all relevant public comments as it develops final test method recommendations. The ICCVAM final test method recommendations will be forwarded to U.S. Federal agencies for their consideration in accordance with the ICCVAM Authorization Act of 2000 (Public Law 106-545).

The Panel gratefully acknowledges the efforts of NICEATM staff in coordinating the logistics of the Panel meeting and in preparing materials for its review. The Panel also appreciates the participation of Drs. Rodger Curren and Arnhild Schrage in the meeting by providing descriptions of several of the test method protocols being considered. Finally, as Panel Chair, I want to thank each Panel member for her or his thoughtful and objective review of these test methods and approaches.

A. Wallace Hayes, Ph.D., DABT, FATS, FIBiol, FACFE, ERT
Chair, Alternative Ocular Safety Testing Methods Peer Review Panel
July 2009

Executive Summary

This report describes the conclusions and recommendations of an international independent scientific peer review panel (hereafter, Panel). The Panel was charged by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) with evaluating the validation status of several proposed test methods and testing approaches. These include:

- A proposal for the routine use of topical anesthetics, systemic analgesics, and humane endpoints to avoid or minimize pain and distress during required *in vivo* ocular irritation safety testing
- Five individual *in vitro* test methods for identifying ocular irritants, including the bovine corneal opacity and permeability (BCOP), Cytosensor Microphysiometer[®] (CM), isolated chicken eye (ICE), isolated rabbit eye (IRE), and the hen's egg test – chorioallantoic membrane (HET-CAM) test methods
- The *in vivo* low volume eye test (LVET), proposed as an alternative to the current *in vivo* rabbit eye test
- Nonanimal testing strategies using three *in vitro* test methods (the BCOP, CM, and EpiOcular[™] [EO] test methods) to assess the eye irritation potential of antimicrobial cleaning products (AMCPs) for U.S. Environmental Protection Agency (EPA) ocular hazard classification and labeling purposes

The Panel evaluated the validation status of each proposed test method and testing strategy according to established Federal and international criteria (ICCVAM 1997, OECD 2005). The Panel also commented on ICCVAM draft recommendations regarding the usefulness and limitations of each proposed test method and testing strategy.

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

The Panel agreed with the ICCVAM draft recommendation that topical anesthetics and systemic analgesics should routinely be used for *in vivo* ocular toxicity studies to avoid or minimize pain and distress. The Panel differed with the ICCVAM draft recommendation on the most appropriate protocol for using topical anesthetics and systemic analgesics in ocular toxicity testing procedures. The Panel proposed an alternative preemptive pain management protocol for all *in vivo* rabbit eye irritation tests intended for regulatory safety testing, unless there is a requirement for monitoring the pain response (e.g., pharmaceutical tolerability testing).

The Panel also recommended that pain assessments should be made immediately after test substance application and recorded daily (i.e., at least twice daily, or more often as necessary).

Use of Humane Endpoints to Minimize Pain and Distress in Ocular Toxicity Testing

The Panel concluded that, based on the available data and information, some humane endpoints as recommended by ICCVAM are adequate to terminate a study. The Panel concluded that the current and proposed humane endpoints are predictive enough of irreversible or severe effects (United Nations Globally Harmonized System of Classification and Labeling of Chemicals [GHS] Category 1, EPA Category I, European Union [EU] R41) that they should routinely be used as humane endpoints to terminate a study as soon as they are observed. However, the Panel emphasized that, while very severe endpoints (i.e., corneal perforation) would be adequate alone to terminate a study, determinations to terminate a study should typically be based on more than one endpoint.

The Hen's Egg Test – Chorioallantoic Membrane Test Method

The Panel agreed with the ICCVAM draft recommendation that, based on an evaluation of available data and corresponding performance (e.g., overall correct classifications that ranged from 40% [23/58] to 41% [24/59]), the HET-CAM test method is not recommended to identify substances from all hazard categories as defined by the GHS (UN 2007), EPA (EPA 2003a), and EU (EU 2001) classification systems.

The Panel did not support the ICCVAM draft recommendation (with one minority opinion) that based on the available data, the HET-CAM IS(A) test method can be used as a screening test to identify substances as not labeled as irritants from all other hazard categories when results are to be used for EU or GHS hazard classifications. The Panel concluded that there were too few surfactants or oil/water emulsions in the mild to moderate irritant categories to have sufficient confidence in the ability of the test to distinguish them from the not labeled as irritant category. However, the Panel did identify possible sources of other existing data that could be analyzed, and they recommended reconsideration of the test method following appropriate analyses.

One Panel member expressed a minority opinion that based on the demonstrated performance, HET-CAM should be recommended to screen substances not labeled as irritants from all other irritant categories for the restricted applicability domain (surfactant-based formulations and oil/water emulsions) for the GHS, EU and EPA hazard classification systems. This Panel member also 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 Isolated Chicken Eye Test Method

The Panel supported the draft ICCVAM recommendations that, based on an evaluation of available data and corresponding performance (e.g., overall correct classifications for ICE test method ranged from 59% [83/141] to 77% [118/153]), 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 also agreed that, based on false negative substances that include at least one substance classified as an ocular corrosive/severe irritant based on Draize rabbit eye data (n = 1 each for the EPA and GHS systems, and n = 6 for the EU system), the ICE test method is not recommended as a screening test to identify substances not labeled as irritants from all other hazard categories as defined by GHS, EPA, and EU classification systems.

The Isolated Rabbit Eye Test Method

The Panel agreed with the ICCVAM draft recommendations that, based on the lack of a standardized protocol and insufficient data using all four recommended IRE endpoints, additional studies are needed before definitive recommendations on the relevance and reliability of the IRE test method can be made.

The Bovine Corneal Opacity and Permeability Test Method

The Panel supported the draft ICCVAM recommendations for the BCOP test method that, based on an evaluation of available data and corresponding performance (e.g., overall correct classifications that ranged from 49% [91/187] to 54% [101/186]), the test method is not recommended to identify substances from all hazard categories as defined by GHS, EPA, and EU classification systems.

The Panel also concluded that the BCOP test method can be used as a screening test to identify substances not labeled as irritants from all other hazard categories when results are to be used for EU or GHS hazard classifications. However, due to the significant lesions associated with 50% (4/8) of the EPA Category III substances that were false negative in the BCOP test method, the BCOP test method cannot be recommended as a screening test to identify EPA Category IV substances.

The Low Volume Eye Test

The Panel concluded 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.

The Cytosensor Microphysiometer® Test Method

The Panel concluded that the available data and performance support the ICCVAM draft recommendations on usefulness and limitations for the CM test method. The Panel concluded that the CM test method can be used as a screening test to identify both ocular corrosive/severe irritants and substances not labeled as irritants, but this use is limited to water-soluble surfactant chemicals and specific types of surfactant-containing formulations (e.g., cosmetics and personal care products). The Panel expressed concern about the availability of the instrument used to conduct the CM test method.

Antimicrobial Cleaning Products Testing Strategies

The Panel agreed with the ICCVAM draft recommendations that there were insufficient data to support the use of the proposed AMCP testing strategy (i.e., using the BCOP, CM, and EO test methods) for classification of substances in all four EPA ocular hazard categories. The Panel also agreed with the ICCVAM draft recommendations that there were insufficient available data on which to base definitive recommendations on an alternate testing strategy (i.e., using the BCOP and EO test methods) for classifying substances in all four EPA ocular hazard categories.

The Panel commented that the absence of data on substances tested in all three *in vitro* test methods (i.e., BCOP, CM, and EO) prevented any definitive recommendation on the AMCP testing strategy. In addition, the availability of only *in vivo* LVET data for some test substances complicated evaluation of *in vitro* test method performance. The Panel recommended that additional EPA-registered AMCPs representing all ocular hazard categories, in particular EPA Categories II and III, be examined in all tests involved in the proposed strategy.

The Panel recognized that the use of histopathological evaluation as an additional endpoint did not improve the accuracy and predictability of the BCOP test method for the limited database of currently tested AMCPs. However, histopathological evaluation may eventually prove to be a useful endpoint, and as such collection of ocular tissue for possible histological evaluation, as well as further efforts to optimize the use of histopathology as an endpoint in BCOP, is recommended.

1.0 Use of Topical Anesthetics and Systemic Analgesics to Minimize Pain and Distress in Ocular Toxicity Testing¹

1.1 Comments on the Proposed Preemptive Pain Management Protocol for the Rabbit in Ocular Toxicity Testing

Following the Panel's review of the background review document (BRD) and draft recommendations developed by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (see **Appendix A** for ICCVAM recommendations), the Panel proposed an alternative preemptive pain management protocol for rabbits used for ocular irritation testing. The Panel's protocol addresses several of the questions that were posed by ICCVAM.

1.1.1 Use of Topical Ocular Anesthetics and Systemic Analgesics to Minimize Pain and Distress in Ocular Toxicity Testing for Rabbits

The Panel offered a balanced preemptive pain management protocol in the event the rabbit is used for ocular irritation testing. This protocol (hereafter, "the alternate protocol" or "the Panel's protocol") is to be applied to all *in vivo* rabbit eye irritation tests intended for regulatory safety testing, unless there is a requirement for monitoring the pain response (e.g., pharmaceutical tolerability testing). Rationale for most of this information is provided below and can be found in Sawyer (2008).

Sixty minutes pre-test substance application (TSA)

Buprenorphine 0.01 mg/kg by subcutaneous injection (SC)

Rationale: Buprenorphine is classified as an opioid agonist-antagonist analgesic that has been found to be effective in managing pain in small animals. The association and disassociation of buprenorphine with mu receptors is much slower than most opioids, so the onset of action may take 30 minutes or longer. In addition, the most effective method of managing pain and distress is to administer the analgesic preemptively to prevent establishment of central sensitization. This drug appears to have a wide margin of safety in rabbits with minimal sedation and also provides a relatively long duration of analgesia (6 – 12 hours). A suggested analgesic dose rate for rabbits is 0.01 – 0.05 mg/kg SC. Clinical experience would suggest using the lowest recommended dose.

¹ While Dr. Rodeheaver participated in the discussions, due to her employment by a manufacturer of anesthetic products she abstained from voting on the Panel's final conclusions and recommendations for this topic.

Fifteen minutes pre-TSA

One or two drops of 0.5% proparacaine hydrochloride (preservative free), a topical ocular anesthetic, applied to the eye three times at 5-minute intervals starting 15 minutes pre-TSA. The last application would be 5 minutes pre-TSA. Anticipated duration of action: 30 - 60 minutes.

Rationale: To relieve pain and distress in the TSA process. Proparacaine is preferred because application to the eye would be less painful than most other topical anesthetics. By only applying it pre-test there would be no impact on hazard classification, variability in rabbit irritation responses, or duration of ocular lesions. The sequence of suggested application is to assure effective penetration of the epithelial layer. Allowing 5 minutes before TSA would preclude any volume dilution by the anesthetic to the test substance.

Eight hours post-TSA²

Buprenorphine 0.01 mg/kg SC and meloxicam 0.5 mg/kg SC.

Rationale: To provide effective systemic analgesia with minimal side effects. The timing is to augment the initial level of analgesia to carry over until the next morning. A well-tested approach to balanced analgesia is to use a combination of an opioid and a cyclooxygenase-sparing nonsteroidal anti-inflammatory drug (NSAID) such as meloxicam. Meloxicam has been used for postoperative or chronic pain in dogs since 1997 and has been found to have effective application in rabbits (Sawyer 2008).

Day 2 through day 4 post-TSA²

Buprenorphine 0.01 mg/kg SC every 12 hours

Meloxicam 0.5 mg/kg SC every 24 hours

Rationale: Continue buprenorphine and meloxicam for 3 days post TSA (i.e., days 2, 3, and 4) unless signs of ocular injury sufficient to cause pain and discomfort are evident. If so, this systemic analgesic protocol would continue until the test is completed.

Rescue Analgesia

Buprenorphine 0.03 mg/kg SC every 8 hours

Meloxicam 0.5 mg/kg SC every 24 hours

Rationale: If a test subject shows signs of physical pain or discomfort during the test interval using the above protocol, a rescue dose of 0.03 mg/kg SC buprenorphine would be given as needed every 8 hours instead of 0.01 mg/kg SC every 12

² Time intervals are +/- 30 minutes.

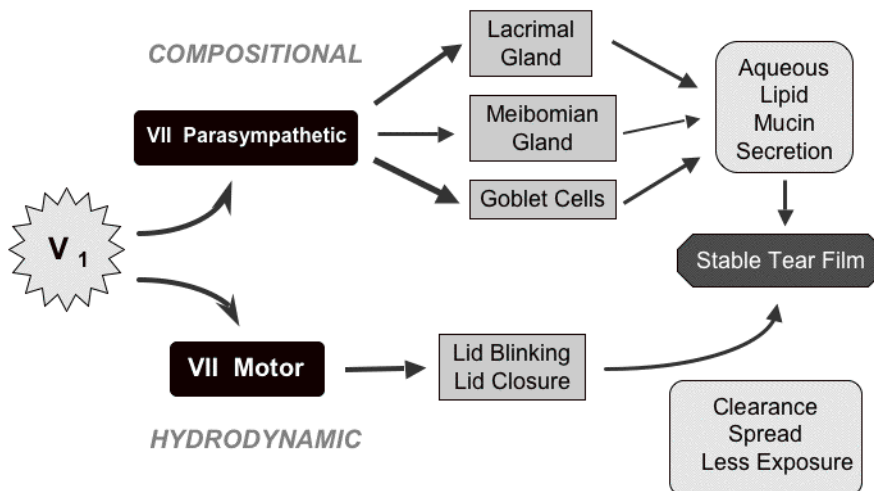
hours. Meloxicam would continue with the same dose and interval. The rescue analgesia could be given immediately post-TSA if pre-emptive analgesia is inadequate.

1.2 Review of the Draft BRD for Errors and Omissions

The Panel was asked if the draft BRD contained any errors that should be corrected, or if there were omissions of relevant data, information, publications, or reports that should be included with regard to the use of topical anesthetics and systemic analgesics to minimize pain and distress in ocular toxicity testing. The Panel members had a variety of observations.

The Panel noted that the BRD should include a more complete description of ocular defense. In short, the ocular defense is controlled by two neural reflexes via sensory input from V1 (i.e., the first branch of the trigeminal nerve) and via two separate (i.e., motor and parasympathetic) branches of the VII facial nerve, which dictates the hydrodynamic and compositional elements of the external adnexae, lids and ocular surface epithelia for maintaining a stable tear film. Hence, a reduction of the ocular surface defense mechanism following topical anesthetics (which abolish the ocular sensitivity) will lead to decreased eyelid blinking, thereby reducing tear clearance and reduction of aqueous (via lacrimal glands), lipid (via meibomian glands) and mucin (via goblet cells). A diagram illustrating such neuroanatomic integration is provided in **Figure 1-1**, as modified from Tseng and Tsubota (1997).

Figure 1-1. A Stable Tear Film is Maintained by a Sound Ocular Surface Defense Governed by Neuroanatomic Integration (Tseng and Tsubota 1997)



The Panel observed that the draft BRD does not consider the presence of preservatives in the solutions proposed as topical anesthetics. The most common preservative is benzalkonium

chloride, typically used at concentrations below 0.05%. This is a Category I irritant, and at doses used in preservatives causes surface epithelial cell damage, with a recent report suggesting a complete breakdown of transcorneal electrical resistance that is linked to a breakdown in barrier function (Chetoni et al. 2003).

The Panel made the following specific comments regarding the text in the draft ICCVAM BRD:

- Table 3-1 of Appendix B of the ICCVAM BRD should include a more relevant test of differences between “equal to or more severe average response” versus “less severe average response”. This is a test of the null hypothesis that anesthetized animals have less severe average response versus all other results, and the null hypothesis would be rejected with statistical significance. This result would effect conclusions given in Appendix B.
- There also appears to be an error in Section 3.2 of Appendix B of the ICCVAM BRD, which should be rewritten to state, “. . .13 formulations produced a *less* severe average response in the rabbits that were (delete *not*) pretreated with tetracaine hydrochloride.”

1.3 Comments on the Use of Topical Anesthetics and Systemic Analgesics

1.3.1 Avoiding or Minimizing Pain and Distress Associated with Initial Application of Test Substances and Initial Chemically-Induced Ocular Injuries

ICCVAM asked the Panel if the proposed topical anesthetics, doses, and time of administration were the most appropriate to optimize local anesthesia of the cornea to avoid pain and distress from the topical administration of test substances. The Panel did not consider them the most appropriate and proposed the alternate protocol outlined in **Section 1.1.1**. As described in the Panel’s protocol, the expected duration of topical anesthesia would be 30 to 60 minutes. As noted in **Section 1.2**, the Panel expressed two concerns about the preservatives used in the topical anesthetic formulations and the potential for test substance dilution. Topical anesthetics would need to be preservative-free to ensure that there is no effect of the preservatives on the ocular response. Therefore, the Panel recommended that 0.5% preservative-free proparacaine solution be required.

The draft BRD described previous studies and current understanding of the effects of proposed topical anesthetics on ocular physiology. The Panel members’ views on (1) the potential for topical anesthetics to alter the ocular injury response for the range of substances that might be tested, and (2) the potential effect, if any, of these changes on the outcome of the test with regard to current hazard classification categories (i.e., U.S. Environmental Protection Agency [EPA], United National Globally Harmonized System of Classification

and Labeling of Chemicals [GHS], European Union [EU]) are addressed in their alternate protocol (**Section 1.1.1**).

Furthermore, the Panel considered that the study conducted by ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) described in the draft BRD on tetracaine pretreatment may have lacked statistical power because the variation in the original test was not known. However, clinical experience indicates that minimal to no impact on either outcome is anticipated.

As indicated previously, an additional statistical analysis should be conducted for the evaluation of the topical anesthetics described in the draft BRD to further characterize the differences (or lack thereof) between pretreated and untreated animals.

The Panel was queried about testing situations in which it would be inadvisable to administer topical anesthetics before treatment because of potential interference with the outcome of the test in terms of current hazard classification categories (i.e., EPA, GHS, EU). The Panel responded that drugs intended to be used for ocular effects, such as eye drops, need to be tested by other means. However, the focus of this evaluation is specific to eye irritation hazard classification, and therefore the proposal would be relevant to all such testing.

ICCVAM asked the Panel if the proposed pretreatment with systemic analgesics—in terms of the selected analgesic, dose, and time of administration—is the most appropriate to optimize analgesia in order to avoid or minimize pain and distress resulting from initial injuries resulting from topical administration of test substances. The Panel responded that the proposed pretreatment is not the most appropriate method in this situation, and the protocol it proposed is outlined in **Section 1.1.1**. The Panel's protocol addresses a number of ICCVAM's questions, including whether opioids are the most appropriate class of analgesics for this type of testing and if NSAIDs should be avoided. As indicated in the Panel's protocol, which includes an NSAID (meloxicam), the Panel does not consider the use of NSAIDs to be a problem.

The Panel was also asked what duration of ophthalmic analgesia could be expected. As indicated in the Panel's protocol (**Section 1.1.1**), the duration of analgesia would be adequate with buprenorphine given every 12 hours and meloxicam given once every 24 hours, when these two drugs are used in combination.

The Panel concluded that the combination of buprenorphine and meloxicam is the most appropriate regimen for managing any ocular pain associated with chemically-induced ocular injuries.

The Panel expressed concerns regarding the use of transdermal patches for analgesic dosing, due to the need for shaving prior to patch application and the possibility that skin irritation

will occur. With multiple applications, the availability of irritation-free skin sites may be limited. In clinical practice, analgesic patches have proven to be somewhat unreliable, with significant animal-to-animal variation as well as species-to-species variation in both effectiveness and duration of effect. For this reason the Panel's protocol requires subcutaneous injection.

Based on current understanding of the effect of proposed systemic analgesics on ocular physiology, the Panel was asked to comment on (1) the potential for systemic analgesics to alter the ocular injury response for the range of substances that might be tested, and (2) the potential effect of these changes on the outcome of the test with regard to current hazard classification categories (i.e., EPA, GHS, EU). The Panel responded that the effect of meloxicam in combination with buprenorphine on these endpoints is unknown. Based on clinical experience, ocular pain probably does have an effect on response to injury. However, considering both the time frame and the effects measured in the Draize rabbit eye test, change to the results would not be expected. Additionally, self-mutilation as a result of ocular pain may lead to severe ocular damage and infection that would lead to spurious overinterpretation of ocular irritation test results. The use of analgesics and a local anesthetic would decrease the likelihood of this complication, thereby producing less severe (and thereby more accurate) results.

The Panel did not consider there to be any ocular irritation testing situations in which it would be inadvisable to administer analgesics before treatment because of the potential to interfere with the outcome of the test in terms of current hazard classification categories (i.e., EPA, GHS, EU).

The Panel was asked what specific observations should be recorded immediately after test substance application in order to assess the effectiveness of the pretreatment topical anesthesia and systemic analgesia. The Panel recommended that pain assessments should be made immediately after TSA and recorded daily (i.e., at least twice daily, or more often as necessary). A number of pain scoring systems are available for this purpose. For example Sawyer (2008) provides a numerical system that assigns scores of 0 (no pain) to 10 (severe pain).

ICCVAM asked the Panel if the database and/or information available on these anesthetics/analgesics were sufficient to warrant their inclusion in Draize rabbit eye tests for any of the types of chemicals and products that are typically tested for ocular irritation potential. The Panel did not consider there to be any ocular irritation testing situations in which their protocol should not be used.

NICEATM based its evaluation of the effect of topical anesthetics on reversibility of ocular lesions on studies that used tetracaine as the topical anesthetic (see Appendix A of the draft

BRD). The Panel was asked if there is any reason that these results should not also be applied to other similar topical anesthetics (e.g., proparacaine).

The Panel considered that there was no clinical reason identified to suggest that another topical anesthetic would behave differently than tetracaine. However, as noted above, the study reported in the draft BRD needs an additional statistical analysis to further characterize any differences in response between rabbits pretreated or not treated with tetracaine. For this reason, the study should not at this time be used to support the use of other topical ocular anesthetics.

1.3.2 Avoiding or Minimizing Pain and Distress Associated with Post-Application Chemically-induced Ocular Injuries

The Panel concluded that the ocular lesions that would be expected to cause ophthalmic pain were adequately described in the draft BRD. In the Panel's opinion, there are no other lesions that should be added.

The Panel also concluded that the clinical signs of post-application pain and distress are adequately described and that no other clinical signs should be added.

As indicated in the Panel's protocol (**Section 1.1.1**), the Panel differed with ICCVAM in some aspects of the post-application systemic analgesics treatment regimen (i.e., selected analgesic, dose, and time of administration). The Panel considered its protocol appropriate to avoid or minimize pain and distress associated with ocular irritation testing.

ICCVAM asked the Panel about other systemic analgesics that might have greater efficacy in relieving ophthalmic pain associated with chemically-induced injuries. As indicated in its protocol, the Panel considered a combination of buprenorphine and meloxicam to have greater efficacy than buprenorphine alone (as was recommended by ICCVAM [**Appendix A**]).

The Panel responded to an ICCVAM question about the duration that can be expected of ophthalmic analgesia. As indicated in the Panel's protocol (**Section 1.1.1**), the duration of analgesia would be adequate with buprenorphine given once every 12 hours and meloxicam given once every 24 hours (Sawyer 2008).

The Panel did not consider there to be any specific pain-related chemically-induced ocular injuries that its protocol would not cover.

The Panel was asked to comment on the potential effectiveness and use of transdermal patches to deliver analgesia after treatment with the test substance, and on the optimal time for application in order to achieve optimal tissue levels to address pain from injuries if a pre-application injection of analgesic was used. As indicated in **Section 1.3.1**, the Panel

expressed concerns regarding the use of transdermal patches for analgesic dosing due to the need for shaving prior to patch application, and the possibility that skin irritation will occur. With multiple applications, the availability of irritation-free skin sites may be limited. In clinical practice, analgesic patches have proven to be unreliable, with significant animal-to-animal variation as well as species-to-species variation in both effectiveness and duration of effect. For this reason, the Panel's protocol requires subcutaneous injection.

ICCVAM asked the Panel, based on current understanding of the proposed systemic analgesics on ocular physiology, to comment on (1) the potential for use of systemic analgesics beyond the initial preapplication treatment to alter the ocular injury response for the range of substances that might be tested, and (2) the potential effect of these changes on the outcome of the test with regard to current hazard classification categories (i.e., EPA, GHS, EU). The Panel indicated that, while it is beneficial to select the analgesic with the least likelihood of altering the *in vivo* score, this potential issue should not be a consideration for using the analgesics.

The Panel was asked about any ocular irritation testing situations in which it would be inadvisable to administer postapplication analgesics because of the potential to interfere with the outcome of the test in terms of current hazard classification categories (i.e., EPA, GHS, EU). The Panel noted that administration of postapplication analgesics is not a concern if a standard dosing regimen is used throughout (per the Panel's alternate protocol outlined in **Section 1.1.1**) and not adjusted according to each animal to avoid overdosing side effects as described above. The Panel is sensitive to the issue of the anti-inflammatory effect of meloxicam on the interpretation of the ocular irritation testing process. However, based on clinical experience, any deviations in the *in vivo* results due to analgesics would be less likely to cause misclassification than would the variability and erroneous responses of the animal test itself.

Because a corneal abrasion can become infected, and one test animal with a severe effect can drive the regulatory classification of a test substance, ICCVAM asked the Panel whether measures should be taken to prevent secondary infections and avoid a potential overclassification. The Panel noted that ocular infection secondary to ocular irritation testing would cause increased pain, and prophylactic antibiotics should be considered. Obvious signs of eye infections (e.g., presence of purulent discharge) should be documented and animal health technicians (AHTs) trained in their detection. Once an eye infection is confirmed or strongly suspected, the animal should be immediately removed from the study. If ocular infection is introduced by self-mutilation, the Panel's protocol should alleviate the problem.

1.3.3 Consideration of All Available Data and Relevant Information

The Panel was asked whether the draft BRD had adequately considered all the relevant data identified in published or unpublished studies. To the best of its knowledge, the Panel considered that all relevant data have been adequately considered. Data are not available regarding the use of NSAIDs and opioids in combination in ocular irritation testing, as proposed in the Panel's protocol.

1.4 Comments on the Draft ICCVAM Test Method Recommendations on the Use of Topical Anesthetics and Systemic Analgesics to Minimize Pain and Distress³

1.4.1 Test Method Usefulness and Recommendations

The Panel was asked if the available data and information supported the ICCVAM test method recommendations on the routine use of topical anesthetics and systemic analgesics. The Panel did not support the ICCVAM recommendations, but instead offered an alternate protocol (**Section 1.1.1**), and other comments and supporting information.

1.4.2 Test Method Protocol

The Panel was asked if it agreed that the available data and information support the ICCVAM draft recommendations on the type and frequency of dosing for topical anesthetics and systemic analgesics. The Panel considered its proposal (**Section 1.1.1**) to be more appropriate in terms of the type and frequency of dosing for topical anesthetics and systemic analgesics.

The Panel was asked if it considered the available guidance on measuring fluorescein staining to be adequate for laboratories to obtain consistent results. The Panel responded that the available guidance on measuring fluorescein staining as presented in the draft ICCVAM recommendations is not adequate for laboratories to obtain consistent results, and the method of fluorescein staining will have to be standardized in order to be useful. In addition, the guidelines lack details about potential preservatives in the dye, anesthesia requirements, or physical restraint that may need to be considered.

1.4.3 Future Studies

In response to ICCVAM's question regarding whether it agreed that the available data supported the draft recommendations for use of anesthetics/analgesics in terms of the proposed future studies, the Panel agreed with both recommendations on future studies related to the use of topical anesthetics and systemic analgesics.

³ See **Appendix A** for draft ICCVAM test method recommendations reviewed by the Panel.

ICCVAM asked the Panel if there were knowledge gaps regarding the severity and duration of pain associated with the range and severity of ocular lesions in animals. The Panel responded by noting that, when pertaining to rabbits in ocular testing procedures, any gaps in their knowledge did not influence the offering of the alternate protocol.

The Panel was queried about additional research that should be considered to support the development and validation of improved treatment strategies to avoid pain and distress from ocular injuries during testing without altering hazard classification outcome. In response, the Panel emphasized that the Draize rabbit eye test has both significant scientific and ethical limitations and it recommended refinement of the current *in vivo* test system to evaluate ocular irritation utilizing contemporary/novel technologies to address both concerns.

To improve treatment strategies, the Panel recommended the following guidance. The Panel recognized that many of the following recommendations are research proposals and not necessarily directly related to regulatory safety testing.

- New animal studies should only be considered when absolutely necessary in developing new strategies for testing.
- Products overpredicted when pretreatment with anesthetics and analgesics is used should be identified.
- The diligent collection of animal responses in testing currently being conducted for regulatory testing purposes could help determine whether the proposed ICCVAM schemes are adequate, or whether refinements in the dosing and timing of anesthetic, analgesic, and antibiotic treatments are warranted. AHT training requirements are an important part of a successful pain reduction program.
- 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 it is due to variability in the ocular defense linking to the neuroanatomic integration or not.
- Studies should be conducted to determine whether the timing and dosing of systemic analgesics together with topical anesthetics might alter the ocular defense sufficient 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 that would refine/change it to make it a more humane test that is also more reliable.

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2.0 Use of Humane Endpoints to Minimize Pain and Distress in Ocular Toxicity Testing

2.1 Review of the Draft BRD for Errors and Omissions

The Panel was asked if the draft BRD contained any errors that should be corrected or omissions of relevant data, information, publications, or reports that should be included with regard to the use of humane endpoint to minimize pain and distress in ocular toxicity testing. The Panel members responded that there were no errors or omissions.

2.2 Comments on Proposed Humane Endpoints for *In Vivo* Ocular Irritation Testing

The Panel concluded that the current and proposed humane endpoints are predictive enough of irreversible or severe effects (i.e., GHS Category 1, EPA Category I, EU R41) that they should routinely be used as humane endpoints to terminate a study as soon as they are observed.

ICCVAM asked the Panel how often these lesions should be looked for, and their presence or absence recorded, in order to ensure that termination decisions are made in a timely manner. The Panel recommended that test animals be examined, and the presence or absence of these lesions recorded, at least daily to ensure that termination decisions are made in a timely manner. Test animals should be examined at least twice daily, or more often as necessary, for the first three days. The Panel emphasized that a slit lamp examination would be necessary to ensure accurate measurement of most of the proposed endpoints.

The Panel did not consider there to be sufficient data in the BRD to determine the adequacy of pannus as a recommended humane endpoint for terminating a test. Therefore, the Panel recommended that data be collected to support this decision.

The Panel was asked whether it considered fluorescein staining at each observation time point to be an appropriate and practical measure for determining severe ocular lesions, and whether the area of fluorescein staining could be monitored effectively enough that one could accurately determine that staining had not diminished over time. The Panel noted that fluorescein staining may be an important tool. However, the technique needs to be better described before a reasonable conclusion regarding its value can be made.

The Panel was asked about other observations or lesions that would suggest that the proposed endpoints might completely reverse, thereby discouraging use of the proposed endpoints to terminate the study. The Panel did not consider there to be any other observations or lesions that would suggest that the proposed endpoints might completely reverse.

ICCVAM asked the Panel if other objective and/or more sensitive biomarkers (e.g., extent and depth of corneal damage) are, or would be, considered sufficiently predictive of severe or irreversible effects that they should be used as routine humane endpoints. The Panel suggested that extent of epithelial loss (which would require measurement by fluorescein staining), limbal ischemia (i.e., blanching), and deep stromal loss (which would require slit-lamp examination) could be detected. Therefore, they could be used as routine humane endpoints.

The Panel was asked if there are other, earlier biomarkers/criteria indicating that painful lesions could be expected to fully reverse to EPA Category II (< 21 days) or III lesions (< 7 days), and which could be used as a basis for early termination of studies and classification in these reversible injury categories. The Panel noted that EPA classification relies on “clearing of corneal/iris score *or* conjunctival score (redness/chemosis)” in “time”. While acknowledging that eyes with conjunctival scores but without corneal/iris scores would most likely recover, the Panel did not consider these criteria to justify early study termination, given the possibility that these lesions might not reverse.

The Panel was asked for recommendations of additional data that might be collected during future animal studies to help identify earlier, more humane endpoints for ocular testing. The Panel emphasized that, when possible, determinations to terminate a study should be based on more than one endpoint. Only very severe endpoints (i.e., corneal perforation) would be adequate alone to terminate a study. The Panel also recommended that additional data should be collected on the use of fluorescein staining to monitor wound healing. In this regard, the Panel recommended that guidelines for the following be developed:

1. Frequency of fluorescein staining with respect to potential impacts on wound healing
2. Association of fluorescein staining with hazard categories

The Panel also recommended that study results with test animals that develop ocular infections be evaluated to determine how often ocular infection results in overclassification to better justify the use of ocular infection as an early humane endpoint.

ICCVAM asked the Panel if the additional endpoints provided for termination of a Draize rabbit eye test were adequate and whether any should be added or omitted. In light of the Panel’s protocol for using topical anesthetics and systemic analgesics (**Section 1.1.1**), pain and distress associated with chemically-induced ocular lesions will be reduced. While the Panel did not consider some of the endpoints adequate for early study termination when taken individually, they could be considered together to reach such a conclusion.

Destruction of more than 25% of the limbus will result in irreversible pannus in humans. However, since rabbits heal faster and have more regenerative reserve, irreversible pannus will not likely occur until more than 50% of the limbus has been destroyed, as indicated by blanching. The Panel provided several references to support this conclusion (Chen and Tseng 1990, 1991; Kruse et al. 1990). The Panel considered destruction of 50% of the limbus to be an important criterion in the decision process but felt that it would not suffice as a single criterion. It was felt that a criterion of destruction of 75% of the limbus (as recommended by ICCVAM [**Appendix A**]) might be excessive.

The Panel recommended the occurrence of a severe eye infection (i.e., purulent discharge) to be used as a single criterion for study termination. The continuation of a study under these circumstances would compromise the study results.

The Panel concluded that the timeframes for consideration of termination of a study based on the use of these endpoints are adequate to ensure that reversal would not be expected. Based on the available Draize rabbit eye test data, the Panel indicated that some ocular lesions are not reversible.

2.3 Comments on the Draft ICCVAM Test Method Recommendations on the Use of Humane Endpoints to Minimize Pain and Distress⁴

2.3.1 Test Method Usefulness and Recommendations

The Panel was asked if the available data and information supported the ICCVAM test method recommendations on the use of humane endpoints to justify early study termination. The Panel responded that some endpoints are adequate to terminate a study:

- Endpoints currently accepted for study termination (OECD 2002)
- Severe depth of injury (e.g., corneal ulceration extending beyond the superficial layers) (ICCVAM/NICEATM/ECVAM Scientific Symposium, 2005: Minimizing Pain and Distress in Ocular Toxicity Testing [ICCVAM draft BRD, Appendix A])
- Limbus destruction > 50% (as evidenced by blanching of the conjunctival tissue)
- Severe eye infection (purulent discharge)

Used in combination, many of the other ICCVAM-recommended endpoints should be considered as potentially useful to influence the clinical decision on early study termination. However, there are insufficient data to use these endpoints (e.g., pannus) individually to

⁴ See **Appendix A** for draft ICCVAM test method recommendations reviewed by the Panel.

justify terminating a study. The Panel emphasized that once an AHT or study director has identified severe ocular effects, a veterinarian should be consulted for a clinical exam to determine if the combination of these effects warrant study termination.

2.3.2 *Future Studies*

The Panel was also queried about additional research that should be considered to support the development and validation of improved treatment strategies directed towards humane endpoints to avoid pain and distress from ocular injuries during testing without altering hazard classification outcome.

In response, the Panel recommended the following guidance:

- Studies should be conducted to identify better and earlier endpoints such as those seen with fluorescein staining.
- Consideration should be given to incorporating these endpoints into current testing and data collected to develop a database.
- As noted above, the Panel did not consider there to be sufficient data in the BRD to determine the adequacy of pannus as a recommended humane endpoint for terminating a test. Therefore, the Panel recommended that data be collected to support pannus as a potential humane endpoint.

ICCVAM asked the Panel to identify the knowledge gaps that should be addressed in research, development, and validation efforts regarding predictive early humane endpoints. The Panel noted that it was necessary to analyze the variability in rabbit wound healing responses to determine whether or not it is due to variability in the ocular defense linking to the neuroanatomic integration. The Panel also recommended the need to improve the identification of signs of pain in rabbits and the collection of cytology samples from the surface of the eye. The Panel emphasized that AHT training requirements are an important part of a successful humane endpoint program.

3.0 The Hen's Egg Test - Chorioallantoic Membrane Test Method⁵

3.1 Review of the Draft Background Review Document for Errors and Omissions

The Panel was asked if the draft BRD for the hen's egg test – chorioallantoic membrane (HET-CAM) test method contained any errors that should be corrected or omissions of relevant data or information that should be included. The Panel noted a number of typographical errors that would be corrected by NICEATM in the final version of the document. In addition, HET-CAM studies from the Australian NICNAS database (available at <http://www.nicnas.gov.au/publications/car/New.asp>), relevant published HET-CAM articles missing from the ICCVAM BRD [ICCVAM 2006a] or current ICCVAM draft BRD, and HET-CAM data from the forthcoming BASF publication by Schrage et al. (2009; submitted for publication) should be included in the final draft. Data should also be solicited from any companies that have published articles using HET-CAM data (e.g., L'Oreal).

The articles listed below are not cited in either of the HET-CAM BRDs (i.e., the ICCVAM BRD [ICCVAM 2006a] or current ICCVAM draft BRD) and contain (1) data from possibly key HET-CAM studies, (2) data that might be useful in evaluating the HET-CAM test method for different classes of chemicals, and (3) modifications to the standard HET-CAM test method that some investigators have found useful in enhancing its performance.

- Boue-Grabot M, Bernardin G, Chaumond S, Pinon JF. 1995. Alternative methods: Hen's egg chorioallantoic membrane and *in vitro* cytotoxicity – a complementary approach. *Int J Cosmet Sci* 17:207-215.
- Budai P, Várnagy L, Fejes S, Somlyay IM, Linczmayer K, Pongrácz A. 2004. Irritative effects of some pesticides and a technical component on tissue structure of the chorioallantoic membrane. *Commun Agric Appl Biol Sci* 69:807-809.
- Dahl JE. 2007. Potential of dental adhesives to induce mucosal irritation evaluated by the HET-CAM method. *Acta Odontol Scand* 65:275-83.
- De Silva O, Rougier A, Dossou KG. 1992. The HET-CAM test: a study of the irritation potential of chemicals and formulations. *ATLA* 20:432-437.
- Gettings SD, Lordo RA, Demetrlias J, Feder PI, Hintze KL. 1996. Comparison of low-volume, Draize and *in vitro* eye irritation test data. Part I. Hydroalcoholic formulations. *Food Chem Toxicol* 34:737-749.

⁵ While Dr. Vanparys participated in the discussions, due to the performance of this test method in the laboratory managed by Dr. Vanparys, he abstained from voting on the Panel's final conclusions and recommendations for this topic.

- Kramer A, Below H, Behrens-Baumann W, Müller G, Rudolph P, Reimer K. 2002. New aspects of the tolerance of the antiseptic povidone-iodine in different *ex vivo* models. *Dermatology* 204 Suppl 1:86-91.
- Mancebo A, Hernández O, González Y, Aldana L, Carballo O. 2008. Assessment of skin and eye irritation of 14 products under the stepwise approach of the OECD. *Cutan Ocul Toxicol* 27:173-85.
- Moldenhauer F. 2003. Using *in vitro* prediction models instead of the rabbit eye irritation test to classify and label new chemicals: a post hoc data analysis of the international EC/HO validation study. *ATLA* 31:31-46.
- Tavaszi J, Budai P. 2006. Toxicity study of agrochemicals on chorioallantoic membrane of the egg. *Commun Agric Appl Biol Sci* 71:101-5.
- Tavaszi J, Budai P. 2007. The use of HET-CAM test in detecting the ocular irritation. *Commun Agric Appl Biol Sci* 72:137-41.
- Tavaszi J, Budai P, Pálovics A, Kismányoki A. 2008. An alternative test battery in detecting ocular irritancy of agrochemicals. *Commun Agric Appl Biol Sci* 73:891-895.

3.2 Comments with Specific References to the Text

The Panel recommended the following specific corrections and revisions to the draft HET-CAM BRD.

- Pgs xxvi and xxvii: Clarify the descriptions relevant to the phrase “100% agreement.”
- Table 6-1:
 1. Gettings et al. (1994)—severe, underestimated should be “0/1” instead of “0/0”
 2. Hagino et al. (1999)—severe, underestimated should be “0/8” instead of “0/0”
 3. Hagino et al. (1999)—mild, actual should be “0/3” instead of “0/0”
 4. Hagino et al. (1999)—mild, underestimated should be “0/3” instead of “0/0”
 5. Hagino et al. (1999)—Not Labeled, actual should be “0/4” instead of “0/0”

- Table 7-1: When referring to the 2006 HET-CAM BRD (ICCVAM 2006a), Table 7-1 corresponds to intralaboratory reproducibility, whereas in the 2009 BRD, it refers to interlaboratory reproducibility. Therefore, the correct version of the BRD must be cited when referring to Table 7-1 of either HET-CAM BRD.
- Lines 650-653: several confusing lines and numerical errors:
 - Line 650: The accuracy stated as 62% (41/59) in the text is listed as 62% (36/58) in Table 2.
 - Line 653: “36/58” should be 62% and “47/60” should be 78% as shown in Table 2.
 - Lines 650-651: “overall accuracy ranged from 62% ... classification system used.” should be deleted, because this is repeated on line 653. It should be rewritten as “As indicated in Table 2, overall accuracy for the identification of...”
- Lines 728-729: Clarify the statement “none of the GHS Not Classified substances were correctly identified by HET-CAM...”
- Lines 1090, 1114, and others: These lines identify the focus of the BRD as identification of mild to moderate irritants which seems contradictory to the executive summary (line 566-567) and other places throughout the document that focus on distinguishing substances not labeled as irritants from all other classes. These terms may not have been corrected after the focus of the evaluation was changed.
- Line 1216: Change “113/27” to “13/27”; verify that this statement is relevant to Table 6-1, as these numbers do not appear in Table 6-1.
- Line 1216-1217: Text refers to 48% (13/27) and 52% (14/27), but Table 6-1 lists 50% (13/26) and 50% (13/26).
- Lines 1220-1222: Clarify and/or reword: no Category 2A substances were included according to *in vivo* criteria, yet the BRD indicates that “the HET-CAM test method did not identify any substances as moderate ocular irritants (i.e., GHS Cat 2A).”
- Line 1347: Strike the word “on” or “from”.

- Pgs 4-3, 5-4, and other parts of the BRD that cite compliance with Good Laboratory Practices (GLPs): The actual number of studies that were or were not compliant should be stated rather than citing the references to the studies.

3.3 Evaluation of the Validation Status of the HET-CAM Test Method

3.3.1 *Test Method Protocol*

The Panel was asked if the protocol was sufficiently detailed that it could be conducted reproducibly in other laboratories. The Panel concluded that the protocol is sufficiently detailed that it could be conducted reproducibly in other laboratories. However, the protocol should reflect any restrictions of the current applicability domain (e.g., solids were not considered). In addition, the protocol reflects the IS(A) analysis method, which was the subject of the ICCVAM draft recommendation, but additional data derived using the IS(B) analysis method could be collected and extrapolated to the IS(A) analysis method.

ICCVAM also asked the Panel whether critical aspects of the test method protocol, as outlined in the ICCVAM Submission Guidelines (ICCVAM 2003), had been adequately justified and described in the BRD. The Panel concluded that the test method protocol had been adequately justified and described, reiterating that the protocol should reflect any restrictions of the applicability domain. The protocol should also reflect details specific to the testing of certain types of substances, such as washing off of solids.

3.3.2 *Substances Used for the Validation Studies*

ICCVAM asked the Panel whether it considered the HET-CAM test method database to be representative of a sufficient range of chemical classes and physicochemical properties that it was applicable to the types of chemicals and products that are typically tested for ocular irritation potential. The Panel indicated that the majority of data were for cosmetics, consumer products, and surfactant chemicals. The range of chemical classes where the test method showed utility was limited to cosmetic and personal care formulations that are oil/water emulsions or surfactant-containing formulations. However, to properly assess the applicability domain of the HET-CAM test method for the range of irritancy with these substances, more materials representing mild and moderate hazard classes should be tested. In addition, a larger number of substances representing a wider variety of chemical and product classes should be tested. These would include materials such as solids, gels, nanoparticles, pesticides, pesticide formulations, polymer-type dispersions, preservatives, solids, viscous products, fertilizers, amines, and substances with various ranges of osmolarity.

3.3.3 Test Method Accuracy

The current accuracy analysis is based on overall concordance with the Draize rabbit eye test. The Panel was asked whether these data were adequate for assessing the accuracy of the test method. The Panel concluded that these data were adequate for assessing the accuracy of the test method. Any *in vitro* test currently under regulatory consideration as a replacement test method is specifically targeted to replace the Draize test; therefore, accuracy analysis on the basis of concordance with the Draize test is appropriate. While the data are adequate for assessing the accuracy of the test method in general, the data set for substances in mild/moderate *in vivo* categories include only a very small sample size, and thus the current assessment is not conclusive. For example, there are too few substances under “Mild” in Tables 6-1 and 6-3 of the draft BRD, and under “Moderate” in Tables 6-6 and 6-8. Furthermore, the accuracy analysis should be repeated in the future on a larger data set including additional compounds in these categories.

The Panel was then asked whether the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of these test methods had been adequately evaluated and compared to the Draize rabbit eye test (refer to Table 6-1 of the draft BRD), and, if not, what other analyses should be performed. In response, the Panel noted important exceptions regarding the relevance of the HET-CAM test method in terms of domain restrictions and some hazard category omissions. For the purposes of distinguishing not labeled as irritants from all other classes, the data evaluated showed acceptable accuracy (78%), high sensitivity (91%), low specificity (40%), moderate false positive rate (60%), and 0% (GHS, EU) or 9% (EPA) false negative rate. Increased accuracy was only realized by excluding various chemical classes, which then reduced the number of samples, and different chemical classes had to be excluded to achieve improved classifications. Inclusion of a larger number of known mild and moderate *in vivo* irritants (i.e., GHS Category 2A and 2B, EPA Category II and III, and EU R36) would increase confidence in the test method accuracy calculation.

The Panel recommended a statistical power analysis to estimate the minimum number of materials needed to be tested in each classification category and chemical class to reach a predetermined level of statistical significance. The tendency of test substances in the *in vivo* validation data (reported in Section 6.1.3 of the HET-CAM BRD) to be classified as strong irritants (38%) or as substances not labeled as irritants (51%), with a lack of moderate irritants (3%), is likely a reflection of the typical range among commercial products being tested. Therefore, the Panel concluded it might be difficult to expand the dataset sufficiently. The hazard categorization schemes being used to assess HET-CAM’s predictive capacity are

not generally used with reference to cosmetics. Therefore a reanalysis of the data for cosmetics using a more appropriate categorization scheme may be helpful.

3.3.4 Test Method Reliability (Intra- and Interlaboratory Reproducibility)

The Panel was asked if it considered the intralaboratory reproducibility of the HET-CAM test method to have been adequately evaluated and compared to the Draize rabbit eye test and, if not, what other analyses should be performed.

The Panel noted that the draft BRD, which utilizes the IS(A) analysis method, does not include an analysis of intralaboratory reproducibility. However, the 2006 BRD contains a comprehensive study of the IS(B) analysis method (Gilleron et al. 1997). The Panel concluded that the Gilleron intralaboratory reproducibility study for IS(B) would provide a reasonable estimate of intralaboratory reproducibility for IS(A) because the IS(B) reproducibility (due to the nature of the scoring) is expected to be more challenging than that for IS(A).

The Panel was also asked if the interlaboratory reproducibility of the HET-CAM test method has been adequately evaluated and compared to the Draize rabbit eye test (refer to Tables 7-2 and 7-3 of the draft HET-CAM BRD). If not, the Panel was asked what other analyses should be performed, and if there were any limitations apparent based on this interlaboratory reproducibility assessment. There was 100% agreement among five laboratories for classification of the majority of substances tested as defined by GHS, EU, and EPA, according to the data in Hagino et al. (1999) using HET-CAM IS(A) analysis. However, the Panel concluded that the data were insufficient to draw a conclusion of interlaboratory reproducibility due to the low overall number of substances tested (16 to 17), particularly in the mild to moderate irritant range and in limited chemical classes. This low overall number of substances limits the interlaboratory reproducibility analysis.

The draft HET-CAM BRD analyzed data from studies that used coded substances, as well as studies that were not coded. The Panel was asked whether the lack of coding of test substances adversely impact or bias the current evaluation. The Panel responded that coded substances should always be used if possible, but retrospective studies cannot control the data available for analysis. If sufficient data of both types are available, a statistical comparison could be conducted to determine if the results are significantly different. If the results were not statistically different, there would be greater confidence in the data that were not coded.

3.3.5 *Data Quality*⁶

Not all of the studies evaluated in the draft ocular BRD were conducted in accordance with GLP guidelines (OECD 1998; EPA 2003b, 2003c; FDA 2003). The Panel was asked to discuss the impact this might have on the evaluation of the ocular test methods. The Panel stated that GLP guidelines help ensure the quality of the data. However, studies not performed under GLP can increase knowledge about the applicability domain of a method. Eliminating non-GLP data should be considered if audits suggest quality problems, or if the original data are not available for audit. Once a preferred protocol and method of analysis have been established, it will be easier to justify the rigor and additional cost of GLP studies.

The original records for these studies are available upon request but have not yet been obtained. As a result, an independent audit has not been conducted to confirm that the reported data are the same as the data recorded in laboratory notebooks. ICCVAM asked the Panel whether any recommendations from ICCVAM should be contingent upon the completion of such an audit and findings that there were no significant errors in data transcription. The Panel concluded that data audits should be completed before final ICCVAM recommendations are made, especially since some studies were not conducted under GLP. Significant errors in the data should cause the study to be excluded. However, errors will be found in even the best studies; these should be corrected before the data and statistical analyses are conducted. Any ICCVAM recommendations should be contingent upon the completion of an audit. While the overall data set on which the Panel put forward recommendations may not have been from studies conducted under GLP or not as representative of all chemical classes as is ideal, there should be steps taken to note (and correct if appropriate), significant errors in the records upon which recommendations are based.

3.3.6 *Consideration of All Available Data and Relevant Information*

The Panel was asked if the draft HET-CAM BRD adequately considered all the relevant data identified in published or unpublished studies that employ the test method. The Panel concluded that all available relevant data have been adequately considered, but some additional references that may not have been included in the HET-CAM BRDs were provided to NICEATM (see **Section 3.1**). NICEATM should again contact testing laboratories and companies known to use the HET-CAM (e.g., L'Oreal and BASF) to inquire about existing *in vivo* and *in vitro* data. Also, NICEATM will be working to consolidate

⁶ The Panel emphasized that comments regarding data quality requirements are relevant to all of the test methods being reviewed. Therefore, these comments are not repeated in each section of this report.

databases using the IS(A) and IS(B) analysis methods where feasible to obtain additional data for analyses.

3.4 Comments on the Draft ICCVAM Test Method Recommendations on the Use of the HET-CAM Test Method to Identify Nonsevere Irritants

3.4.1 Test Method Usefulness and Recommendations

The Panel was asked if the available data and test method performance (accuracy and reliability) support the ICCVAM draft recommendations for the ocular test methods and testing strategy in terms of the proposed test method usefulness and limitations. The Panel concluded that the HET-CAM test method cannot identify substances from all hazard categories. The Panel disagreed with the ICCVAM draft recommendation (with one minority opinion) that the HET-CAM using the IS(A) analysis method can be used as a screening test to distinguish substances as not labeled as irritants (i.e., EU Not Labeled, GHS Not Classified) from all other hazard categories (i.e., EU R41 or R36; GHS Category 1, 2A, or 2B) when results are to be used for EU or GHS hazard classifications. The Panel reached this conclusion because there were too few surfactants or oil/water emulsions in the mild to moderate irritant categories to have sufficient confidence in the ability of the test to distinguish them from the not labeled as irritants category.

Minority Opinion (Ms. Alison McLaughlin): 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.

ICCVAM stated that when evaluating the HET-CAM for its ability to distinguish substances not labeled as irritants from all other irritant classes, the false negative rate for the EU and GHS systems is 0% (0/26 or 0/31) and therefore the HET-CAM is recommended for such testing purposes. By comparison, the false negative rate was 9% (4/45) for the EPA classification system. 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. For one of the substances, one out of the six test animals tested had a conjunctival redness score of two that required 14 days to resolve. Four of the remaining five test animals in this study had conjunctival redness scores of two that resolved within three days; one test animal did not have this lesion. The Panel was asked if it agreed that the severity and number of ocular lesions noted *in vivo* do not present a significant hazard to the user, and as such, whether the HET-CAM test method could be considered useful as a screening test for EPA Category IV substances.

The Panel concluded that HET-CAM can not identify substances labeled as EPA Category IV, because four EPA Category III (22% [4/18]) substances were underclassified by HET-CAM as Category IV, and because too few chemical classes and mild/moderate irritants were included in the data evaluated. Among the four false negatives under the EPA system, 100% (4/4, all oil/water emulsion cosmetic formulations) were EPA Category III substances based on Draize rabbit eye tests that produced conjunctival redness scores of two that required at least three days to resolve. For one of the substances, one of the six test animals had a conjunctival redness score of two that required 14 days to completely resolve, although it was considered cleared with a score of one within seven days. Four of the remaining five test animals in this study had conjunctival redness scores of two that resolved within three days; one test animal did not have this lesion. The Panel commented that guidance for acceptable false positive and false negative rates would be helpful in its assessment of test applicability.

The Panel noted that the validation database does not include substances currently regulated by EPA and that collection of additional data would be needed. Therefore, the Panel concluded that additional testing of EPA-regulated substances in HET-CAM would be necessary before definitive recommendations can be made on its usefulness for identifying EPA Category IV substances.

The Panel was asked if it considered it necessary to conduct additional validation studies on which to base expanding the applicability domain of HET-CAM beyond cosmetic and

personal care formulations that are oil/water emulsions or surfactant-containing formulations. The Panel responded that it would be useful to expand the HET-CAM applicability domain. Furthermore, because HET-CAM cannot be used as a screen to eliminate severe irritants (based on the current dataset), its use seems limited to a screening test to identify substances not labeled as irritants.

In the 2009 draft HET-CAM BRD, there are discordant data related to the applicability domain of surfactant-containing formulations due to the high number of false positives. The Panel recommended references that may be relevant to this discordant data:

- De Silva O, Rougier A, Dossou KG. 1992. The HET-CAM test: a study of the irritation potential of chemicals and formulations. *ATLA* 20: 432-437.
- Debbasch et al. 2005. Eye irritation of low-irritant cosmetic formulations: correlation of *in vitro* results with clinical data and product composition. *Food Chem Toxicol* 43:155-165.

Additional validation studies should be conducted that include a sufficient number of products of mild and moderate irritant categories. There was insufficient representation of a range of chemical classes and physicochemical properties in the current validation studies. The Panel noted that there was a need to recognize different classes of surfactants (anionic, cationic, non-ionic, and zwitterionic) and to ensure that they are adequately represented. Additional validation studies should target select chemicals (or polymers) representative of the product type and the concentrations that would need to be tested in order to expand the applicability domain.

When evaluating the HET-CAM test method for its ability to distinguish substances not labeled as irritants from all other irritant classes, the false negative rate for the EU and GHS systems is 0% (0/26 or 0/31) and therefore the HET-CAM test method is recommended by ICCVAM for such testing purposes. By comparison, the false negative rate was 9% (4/45) for the EPA system using this approach. 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. For one of the substances, one of the six test animals had a conjunctival redness score of two that required 14 days to resolve. Four of the remaining five test animals in this study had conjunctival redness scores of two that resolved within three days. One test animal did not have this lesion.

The Panel was asked if the severity and number of ocular lesions noted *in vivo* do not present a significant hazard to the user, and as such whether the HET-CAM test method could be considered useful as a screening test for EPA Category IV substances. The Panel noted that

these lesions indicate mild ocular irritation (i.e., EPA Category III is defined as corneal involvement or irritation clearing in seven days or less where a positive response is defined as a Draize rabbit eye test opacity or iritis score ≥ 1 or a conjunctival redness or chemosis score ≥ 2). Under the guidelines of the EPA classification system, these four false negative substances would be labeled as EPA Category III irritants. Under the GHS and EU system they would be not labeled or not classified, respectively. However, based on prior discussions regarding the limitations in the validation database, the Panel concluded that the HET-CAM test method could not at this time be considered useful as a screen for Category IV substances in the EPA classification system.

The validation database does not include any substances currently regulated by EPA. The Panel was asked if additional testing should be required before a recommendation on the usefulness of HET-CAM for identifying Category IV substances is made. The Panel agreed with ICCVAM that the validation database does not include any substances currently regulated by EPA and concluded that additional testing should be required before a recommendation on the usefulness of HET-CAM for identifying Category IV substances is made.

3.4.2 Test Method Protocol

The Panel was asked whether or not it agreed that the available data support the ICCVAM draft recommendations for the HET-CAM test method procedure in terms of the proposed test method standardized protocols. The Panel responded that the available data support the ICCVAM draft recommendations, but only when considering the IS(A) analysis method. NICEATM updated the protocol to reflect the most recent HET-CAM method, which includes the use of IS(A) decision criteria as well as collection of data adequate for an IS(B) analysis. NICEATM determined that the IS(B) data can be converted to fixed time points similar to those used for the IS(A) analysis method.

3.4.3 Future Studies

ICCVAM asked the Panel whether it agreed that the available data support the ICCVAM draft recommendations for the HET-CAM test method in terms of the proposed future studies. If not, the Panel was asked what recommendations it would make.

The Panel disagreed with the ICCVAM recommendations for additional studies for using the HET-CAM test method to identify all categories of ocular irritants. The method has been extensively evaluated for this task and proven incapable. However, in order to further optimize the protocol and more adequately characterize the usefulness of the HET-CAM test method for identifying substances not labeled as irritants, the Panel recommended that:

- Additional data be collected on mild and moderate irritants
- The applicability domain be expanded to include a broader range of chemical and product classes

Most of the single ingredients tested in the HET-CAM performed poorly, whereas formulations had better performance. Hence, the effect of increasing the concentration of single ingredients on accuracy and sensitivity should be assessed in the HET-CAM to determine if there are test substance concentration limits for specific chemical classes.

The Panel concluded that its recommendation on the HET-CAM test method should be re-evaluated if additional data (e.g., NICNAS chemical reports, BASF data, solicited HET-CAM data, and NICEATM conversion of IS[B] to IS[A]) become available. The Panel stated that ICCVAM should conduct an expedited peer review if new HET-CAM data warrants re-evaluation.

3.4.4 Performance Standards

The Panel was asked if it agreed that the results described above do not warrant the development of performance standards for the HET-CAM test method at this time. The Panel responded that it is premature to develop performance standards for HET-CAM. A fixed HET-CAM protocol is needed before this could be considered.

4.0 The Isolated Chicken Eye Test Method

4.1 Review of the Draft Background Review Document for Errors and Omissions

The Panel stated that the isolated chicken eye (ICE) test method BRD appeared to be accurate, with no errors that should be corrected or omissions of existing relevant data or information.

4.2 Evaluation of the Validation Status of the ICE Test Method

4.2.1 Test Method Protocol

According to the Panel, the test method protocol included in the draft BRD includes adequate detail to conduct the test. However, interlaboratory reproducibility and transferability are problematic. The Panel stated that it is not possible to make a conclusion about the adequacy of the test method protocol for assigning ocular irritation hazard categories based upon the limited data.

4.2.2 Substances Used for the Validation Studies

ICCVAM asked the Panel whether it considered the ICE test method database to be representative of a sufficient range of chemical classes and physicochemical properties such that the analysis using the data was applicable to any of the types of chemicals and products typically tested for ocular irritation potential. If the Panel felt otherwise, it was asked to suggest (1) the relevant chemical classes/properties that should either be tested with caution or not evaluated using this test method (other than those that are identified as limitations in the previous ICCVAM BRD [ICCVAM 2006b]), and (2) chemicals or products that should be evaluated to fill the data gap. In the Panel's opinion, an insufficient number and range of chemicals had been tested. The false negative rate will need to be addressed to make the ICE test method useful for identifying all ocular hazard categories.

4.2.3 Test Method Accuracy

The current accuracy analysis was based on overall concordance with the Draize rabbit eye test. The Panel considered the Draize test data adequate for assessing the accuracy of the ICE test method. However, Draize test data quality should be reviewed, and false negatives arising from problematic Draize test data could be excluded from the assessment. There is less confidence in test results with high variability, and Draize test data producing gross discrepancies in different classification schemes (e.g., EPA versus GHS) may signal anomalous results in a single test animal. Draize test data conducted on substances such as solids or highly viscous materials are expected to produce anomalous results and could be excluded from the validation data. Likewise, the Panel considered that the relevance (e.g.,

accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of the ICE test method had been adequately evaluated and compared to the traditional rabbit test.

4.2.4 Test Method Reliability (Intra- and Interlaboratory Reproducibility)

The Panel was asked if it considered the intralaboratory reproducibility of the ICE test method to have been adequately evaluated and compared to the Draize rabbit eye test (refer to the ICCVAM ICE BRD, Section 7.2 [ICCVAM 2006b]). The Panel concurred that the intralaboratory reproducibility had been adequately addressed.

According to the Panel, the interlaboratory reproducibility of the ICE test method has been adequately assessed. Acceptable concordance, particularly among substances not labeled as irritants (n=2 EPA Category IV in the validation database), was not achieved among the four laboratories.

The draft ICE BRD analyzed data from validation studies that used coded substances (Balls et al. 1995), as well as from studies that used uncoded substances (Prinsen and Koëter 1993, Prinsen 1996, Prinsen 2005). ICCVAM asked the Panel whether the lack of coding of test substances adversely impacted or biased the current evaluation. In the opinion of the Panel, coding of substances precludes any biases and is necessary for subjective evaluations. Lack of coding is acceptable and not as critical as adherence to GLP guidelines. However, the Panel recommended coding in future validation studies.

4.2.5 Data Quality

The Panel concluded that data quality requirements are relevant to all of the test methods being reviewed (see **Section 3.3.4**).

4.2.6 Consideration of All Available Data and Relevant Information

The Panel was asked if the draft ICE BRD adequately considered all the relevant data identified in published or unpublished studies that employ the test method. The Panel responded that all available relevant data made public has been included.

4.3 Comments on the Draft ICCVAM Test Method Recommendations on the ICE Test Method to Identify Nonsevere Ocular Irritants and Substances Not Labeled as Irritants⁷

4.3.1 Test Method Usefulness and Recommendations

The Panel concluded that the available data and test method performance (accuracy and reliability) supported the ICCVAM draft recommendation 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 concluded that the ICE test method is not recommended as a screening test to identify substances as not labeled as irritants from all other hazard categories as defined by GHS, EPA, and EU classification systems.

In the evaluation of the ICE for its ability to distinguish substances as not labeled as irritants from all other irritant classes, the false negative rate for the GHS system was 6% (4/62). However, among these false negatives was a Category 1 substance. ICCVAM asked the Panel if this result should result in a recommendation that the ICE not be used as a screening test to identify GHS Not Classified substances. The Panel concluded that the ICE test method should not be used as a screening test to identify GHS Not Classified substances.

4.3.2 Test Method Protocol

The Panel was asked if the available data supported the ICCVAM draft recommendations for the ICE test method procedure in terms of the proposed test method standardized protocol. The Panel concluded that the proposed test method standardized protocol appeared acceptable. However, it suggested that the protocol could be improved by adding objective endpoints for corneal opacity and fluorescein staining.

4.3.3 Future Studies

ICCVAM asked the Panel whether it agreed that the available data support the ICCVAM draft recommendations for the ICE test method in terms of the proposed future studies. If not, the Panel was asked for recommendations. The Panel responded 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 to evaluate corneal tissue might add to the accuracy and determination of the test.

4.3.4 Performance Standards

The Panel asserted that the results described above did not warrant development of ICE test method performance standards at this time.

⁷ See **Appendix A** for draft ICCVAM test method recommendations reviewed by the Panel.

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5.0 The Isolated Rabbit Eye Test Method

5.1 Review of the Draft Background Review Document for Errors and Omissions

The Panel noted that lines 747, 748, 750, and 766 of the draft proposed ICCVAM recommendations for the isolated rabbit eye (IRE) test method read as “ICE” instead of “IRE” and must be corrected.

5.2 Evaluation of the Validation Status of the IRE Test Method⁸

5.2.1 Test Method Usefulness and Recommendations

The Panel concluded that there are insufficient data from all four recommended IRE test method endpoints (i.e., corneal opacity, fluorescein penetration, corneal swelling, and observations of significant effect on corneal epithelium) to evaluate the accuracy and reliability of the test method when all four are evaluated in a single study. The Panel concluded that additional optimization and validation studies are needed to further evaluate the relevance and reliability of the IRE test method, and in turn develop more definitive recommendations.

If it has not been done, the Panel recommended a validation study to compare the utility of shipped rabbit eyes versus freshly collected rabbit eyes. Specifically, the Panel recommended inclusion of the study of shipping effects on ocular tissues into the planned validation study by GlaxoSmithKline and SafePharm. In general, the Panel felt there should be rigid criteria on the handling and storage of the eyes. For example, the length of time between death and study initiation should be tightly controlled to account for any postmortem effects on the eye that could compromise the study. In addition, the Panel recommended development of appropriate inclusion/exclusion criteria for eyes. Finally, the Panel recommended that criteria on test article administration/washout (e.g., viscous substances) should be developed.

5.2.2 Test Method Protocol

The Panel was asked if it agreed that the available data supported the ICCVAM draft recommendations for the proposed IRE test method standardized protocol. The Panel reiterated its concerns about the proposed protocol. It recommended that there should be rigid criteria specifying the handling and storage of the eyes, including control of the length of time between death and study initiation to account for any postmortem effects on the eye. The Panel also recommended development of criteria for appropriate inclusion/exclusion of ocular tissue and on test article administration/washout (e.g., viscous substances).

⁸ See **Appendix A** for draft ICCVAM test method recommendations reviewed by the Panel.

5.2.3 *Future Studies*

ICCVAM asked the Panel whether it agreed that the available data support the ICCVAM draft recommendations for proposed future studies on the IRE test method. If not, the Panel was asked for recommendations. The Panel stated that additional studies including all four recommended IRE endpoints are required to assess the ability of the IRE test method to distinguish among hazard categories.

5.2.4 *Performance Standards*

The Panel asserted that development of IRE test method performance standards was not recommended at this time.

6.0 The Bovine Corneal Opacity and Permeability Test Method⁹

6.1 Review of the Draft Background Review Document for Errors and Omissions

6.1.1 Comments with Specific References to the Text

The Panel was asked if there were any errors in the draft BRD for the bovine corneal opacity and permeability (BCOP) test method that should be corrected, if omissions of existing relevant data had been identified, and if there was additional information that should be included.

The Panel recommended the following specific corrections and revisions to the draft BCOP BRD.

- Line 650: Why is ICE being discussed under BCOP accuracy section?
- Line 654: Indicates 54% in the text but Table 1 shows 55%.
- Line 660: Data in Table 1 and text do not agree.
- Line 660: Change “101/186” to “101/187”.
- Lines 683-690: Values in text do not correspond to those in Table 2, and there is some redundancy (false positive rate) with Table 6-2, p. 6.5.
- Line 1216: Change “29/123” to “29/124”.
- Table 1: The Overall Correct Classification for EPA is 54%.
- Table 2: The first column should be “EPA, EU and GHS”.

6.2 Evaluation of the Validation Status of the BCOP Test Method

6.2.1 Test Method Protocol

ICCVAM asked the Panel if the protocol was sufficiently detailed that it could be conducted reproducibly in other laboratories. ICCVAM also asked the Panel whether critical aspects of the test method protocol, as outlined in the ICCVAM Submission Guidelines (ICCVAM 2003), had been adequately justified and described in the BRD. The Panel responded that the BCOP test method had been validated during the ICCVAM 2006 review and, based upon this review, the test method protocol was considered sufficiently detailed that it could be conducted reproducibly in other laboratories. However, while the BCOP test method protocol was previously reviewed for use in identifying ocular corrosives/severe irritants, the use of

⁹ While Dr. Vanparys participated in the discussions, due to the performance of this test method in laboratory managed by Dr. Vanparys, he abstained from voting on the Panel’s final conclusions and recommendations for this topic.

this protocol to identify mild/moderate ocular irritants should include (1) methods for harvest and storage of eyes, (2) time frame from harvest to use of eyes, (3) consistent animal age, (4) screening for existing corneal lesions prior to use, (5) concurrent positive and negative controls of the same chemical class/formulation, (6) inclusion of an untreated negative control, and (7) refinement of histopathological methodology.

6.2.2 Substances Used for the Validation Studies

ICCVAM asked the Panel whether it considered the BCOP test method database to be representative of a sufficient range of chemical classes and physicochemical properties such that the analysis using the database was applicable to any of the types of chemicals and products typically tested for ocular irritation potential. If the Panel felt otherwise, it was asked to suggest (1) the relevant chemical classes/properties that should either be tested with caution or not evaluated using these test method (other than those that are identified as limitations in the previous ICCVAM BRD [ICCVAM 2006c]), and (2) chemicals or products that should be evaluated to fill this data gap.

In the Panel's opinion, the chemical database appeared adequate; however, additional chemicals in certain chemical classes will provide a more robust statistical inference as these data become available. A power analysis was conducted to enable a non-inferiority test (Chen et al. 2000) to show that the BCOP test is not inferior to the Draize rabbit eye test. For each hazard category, a sample size of 13 substances in each particular chemical class represented in each of the 4 hazard categories (i.e., 13 x 4 for each chemical class for a 4-category hazard classification system) is required to achieve 80% power using a two-group normal approximation test for proportions. This sample size is obtained using a one-sided 0.05 significance level to reject the null hypothesis that the BCOP test is inferior to the Draize 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, assuming that the expected accuracy of the BCOP test is 0.6 and the accuracy of the Draize test is 0.9.

6.2.3 Test Method Accuracy

The current accuracy analysis is based on overall concordance with the Draize rabbit eye test. The Panel was asked (1) whether this data was adequate for assessing the accuracy of the test method, and (2) whether the draft BCOP BRD had adequately evaluated and compared the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of the test method compared to the Draize rabbit eye test (refer to Tables 6-1, 6-3, 6-8, and 6-13 of the draft BCOP BRD).

In response to the initial question, the Panel responded that the Draize rabbit eye test is the only standard accepted by regulatory agencies for assessing the accuracy of the test method. However, Draize test data quality should be reviewed, and false negatives arising from problematic Draize test data could be excluded from the assessment. There is less confidence in test results with high variability, and Draize test data producing gross discrepancies in different classification schemes (e.g., EPA versus GHS) may signal anomalous results in a single animal. Draize test data conducted on substances such as solids or highly viscous materials are expected to produce anomalous results and could be excluded from the validation data. As for the BCOP test method's relevance, the Panel felt that it had been adequately evaluated.

6.2.4 Test Method Reliability (Intra- and Interlaboratory Reproducibility)

The Panel was asked if it considered the intralaboratory reproducibility of the BCOP test method to have been adequately evaluated compared to the Draize rabbit eye test (refer to the ICCVAM BCOP BRD, Section 7.2 [ICCVAM 2006c]) and, if not, what other analyses should be performed. The Panel concluded that the intralaboratory reproducibility appeared to have been adequately evaluated, as it was previously addressed in the ICCVAM BCOP BRD (ICCVAM 2006c). The Panel also concluded that the interlaboratory reproducibility of the BCOP appeared to be adequate.

The draft BCOP BRD analyzed data from validation studies that used coded substances, as well as from studies that used uncoded substances. ICCVAM asked the Panel whether the lack of coding of test substances adversely impacted or biased the current evaluation. In the opinion of the Panel, coding of substances precludes any biases and is necessary for subjective evaluations. However, lack of coding is acceptable and not as critical as adherence to GLP guidelines. The Panel recommended that test substances be coded in future validation studies.

6.2.5 Data Quality

The Panel concluded that data quality requirements are relevant to all of the test methods being reviewed (see **Section 3.3.4**).

6.2.6 Consideration of All Available Data and Relevant Information

The Panel was asked if the draft BCOP BRD adequately considered all the relevant data identified in published or unpublished studies that employ the test method. The Panel responded that all available relevant data made public have been included.

6.3 Comments on the Draft ICCVAM Test Method Recommendations on the BCOP Test Method to Identify Nonsevere Irritants¹⁰

6.3.1 Test Method Usefulness and Limitations

ICCVAM asked the Panel whether or not the available data and test method performance (accuracy and reliability) support the ICCVAM draft recommendations for the BCOP test method in terms of the proposed test method usefulness and limitations. The Panel concluded that the available data and test method performance support the ICCVAM draft recommendations that the BCOP test method is not recommended to identify substances from all hazard categories as defined by GHS, EPA, and EU classification systems. 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 negative, the BCOP test method cannot be recommended as a screening test to identify EPA Category IV substances.

6.3.2 Test Method Protocol

The Panel was asked if the available data supported the ICCVAM draft recommendations for the BCOP test method procedure in terms of the proposed test method standardized protocol. The Panel responded that while the BCOP test method protocol was previously reviewed for use in identifying ocular corrosives/severe irritants, it emphasized the importance of protocol elements. Use of this protocol to identify mild/moderate ocular irritants should include (1) methods for harvest and storage of eyes, (2) time frame from harvest to use of eyes, (3) consistent animal age, (4) screening for existing corneal lesions prior to use, (5) concurrent positive and negative controls of the same chemical class/formulation, (6) inclusion of an untreated negative control, and (7) refinement of histopathological methodology.

When evaluating the BCOP test method for its ability to distinguish substances not labeled as irritants from all other irritant classes, the false negative rate for the EU and GHS systems was 0% (0/54 or 0/97); therefore, the BCOP test method was recommended for such testing purposes. 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 data. For 38% (3/8) of these substances, the categorization was based on at least one test animal with a corneal opacity score of one that was not resolved until day three of the study. Another substance was categorized based on all six test animals with a conjunctival redness scores of three that were not resolved until day seven of the study. The Panel was asked whether it agreed that the severity and number of ocular lesions

¹⁰ See **Appendix A** for draft ICCVAM test method recommendations reviewed by the Panel.

noted in the Draize rabbit eye test presented a significant risk to the user and that, consequently, the BCOP test method should not be recommended as a screening for EPA Category IV substances. In agreement, the Panel did not recommend that the BCOP test method be utilized as a screening for EPA Category IV substances.

The Panel concluded that consideration of differing recommendations among hazard classification systems on test method usefulness was justified. The BCOP test method was recommended as a screening for severe irritants. Concern exists over BCOP being used to classify mild/moderate (EPA, EU, and GHS) and EPA Category II and I irritants.

6.3.3 *Future Studies*

ICCVAM asked the Panel whether it agreed that the available data support the ICCVAM draft recommendations for the BCOP test method in terms of the proposed future studies. The Panel concluded that the available data support the ICCVAM draft recommendations and asserted that no further testing is necessary.

The Panel believed that the current BCOP test method lacks the sensitivity to discriminate between all hazard categories. However, the Panel encouraged continued method development and refinement to achieve more accurate classification of mild/moderate irritants. The Panel recommended identification of problematic classes within these hazard categories. The Panel also recommended refinement of the test method protocol (see Sections 5.2.1 and 5.3.2 of the ICCVAM draft BRD). For each hazard category, a sample size of 13 substances in each particular chemical class should be represented in each of the four hazard categories (see Section 5.2.2 of the ICCVAM draft BRD).

6.3.4 *Performance Standards*

The Panel members concurred that, because the method is recommended as valid for identifying substances not labeled as irritants for the GHS and EU schemes, performance standards should be developed as this might facilitate development of related methods.

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7.0 The Low Volume Eye Test¹¹

The Panel concluded that, based on the available data, the low volume eye test (LVET) seems to be more accurate in predicting the human response than the Draize rabbit eye test for some substances for which comparative Draize test, LVET, and human data (both accidental and ethical human testing) are available. However, in the absence of known data, including the BRD prepared by the European Centre for the Validation of Alternative Methods (ECVAM), the Panel 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 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. Therefore, the Panel's discussions that led to the conclusions and recommendations are provided below.

7.1 Review of the Draft Summary Review Document for Errors and Omissions

The Panel was asked if the ICCVAM draft LVET summary review document (SRD) contained any errors that should be corrected or omissions of relevant data or information that should be included. The Panel suggested that the following published papers be included in the SRD:

- Gettings SD, Lordo RA, Demetrulias J, Feder PI, Hintze KL. 1996. Comparison of low-volume, Draize and *in vitro* eye irritation test data. I. Hydroalcoholic formulations. *Food Chem Toxicol.* 34:737-49.
- Jester JV. 2006. Extent of corneal injury as biomarker for hazard assessment and the development of alternative models to the Draize rabbit eye test. *Cutan Ocul Toxicol.* 25:41-54.
- Maurer JK, Molai A, Parker RD, Li L, Carr GJ, Petroll WM, Cavanagh HD, Jester JV. 2001. Pathology of ocular irritation with bleaching agents in the rabbit low-volume eye test *Toxicologic Pathology.* 29: 308–319.

It was also noted that the Cosmetic, Toiletry, and Fragrance Association Evaluation of Alternatives Program series may contain data relevant to the LVET SRD.

Another reference included in the SRD, *Toxicology of the Eye* (Grant 1974), was not the most recent edition. The last edition was published in 1993 under the same title, with Grant and Schuman as authors.

¹¹ While Dr. Ward participated in the discussions, due to a consulting relationship she abstained from voting on the Panel's final conclusions and recommendations for this topic.

Although the LVET SRD states that there are limited LVET data for severe irritants, the Panel noted published sources of such data. Gettings et al. (1996) was cited, which identified three severe irritants. Additionally, there are reports in the literature that have used the LVET to test several irritation categories, slight to severe, for a wide range of materials, including surfactants, acids, alkalis, alcohols, bleaches, and aldehydes (Maurer et al. 2001; Gettings et al. 1998; Jester 2006). Furthermore, the Panel noted that unpublished references can be considered as sources of potentially useful information relevant to the use of the LVET, particularly with regards to its classification of known human corrosives.

7.2 Evaluation of the Validation Status of the LVET

7.2.1 Test Method Protocol

The Panel concluded that the LVET test method is adequate for its intended use as a test method for identification of ocular hazards.

The LVET has primarily been used to test surfactants and surfactant-containing products. ICCVAM asked the Panel if this limited database was adequate to determine its validity for use as an *in vivo* reference test in general or if such consideration should be relevant to only this limited applicability domain. The Panel concluded that, based on the limited database provided in the ICCVAM LVET SRD, there was adequate information to determine the validity of the LVET as an *in vivo* reference test. Furthermore, the Panel noted that the LVET is more relevant in predicting irritancy to the human eye than the Draize rabbit eye test and it indicated that the LVET has been used on materials other than surfactants and surfactant-containing products, such as acids, alkali, bleaches, alcohols, aldehydes, and acetone. The irritation response seems to be dependent on the amount of injury, not necessarily the type of irritant. Therefore, the fact that most studies reporting on the LVET have used surfactants is most likely not important. The Panel noted that the LVET does detect the full range of ocular irritation using a full range of materials, and therefore it would seem that the LVET is a reasonable test.

The Panel was asked whether there should be concern that direct application of the test substance to the cornea causes additional pain and distress relative to the Draize rabbit eye test (where the substance is applied into the conjunctival sac). Pain is a concern; therefore, the Panel recommended the routine use of a topical anesthetic and systemic analgesics in any protocol that employs these types of tests.

7.2.2 Substances Used for the Validation Studies

ICCVAM asked the Panel whether it considered the LVET database, which is limited primarily to surfactants and surfactant-containing materials, representative of a sufficient

range of chemical classes and physicochemical properties of chemicals and products that are typically tested for ocular irritation potential. The Panel concluded that the LVET database was sufficient, though some of the classes have a limited number of chemicals tested. Furthermore, it was noted that if any additional historical data were obtained, there might be sufficient data to further determine the performance of the LVET for several other chemical classes.

The Panel was asked if it was aware of any other data available in the published literature that could expand the applicability domain of the database. As indicated in **Section 7.1**, the Panel suggested additional references that could address this issue. Gettings et al. (1996) includes hydroalcoholic formulations tested in the LVET, Maurer et al. (2001) includes various bleaching agents tested in the LVET, and Jester (2006) includes LVET data on a range of substances (surfactants, acids, bases, aldehydes, alcohols, and bleaches). These references were provided as supplementary information to Panel members. The Panel also noted the potential utility of the unpublished ECVAM BRD, which could further expand the applicability domain for the LVET.

7.2.3 Test Method Accuracy

The current accuracy analysis is based on overall concordance with the Draize rabbit eye test. The Panel was asked if the data were adequate for assessing the accuracy of the test method. The Panel responded that the current accuracy analysis is adequate; however, the small sample size was noted. The Panel concluded that both the Draize test and the LVET are overpredictive of the human response. However, the LVET has been reported to overpredict the human response less than the Draize test.

The Panel concluded that the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive, and false negative rates) of this test method had been adequately evaluated and compared to the traditional rabbit test. The Panel considered that the test might be useful even if the category scores differ, but data are needed to make such predictions. As indicated in Table 4-1 of the ICCVAM SRD, the LVET produces scores that are lower than that of the Draize eye test. This is also shown in Gettings et al. (1996) where regression analyses showed lower scores. It should be noted that the full range of irritation was measured in the LVET.

The Panel concluded that the currently utilized Draize rabbit eye test scoring system is not considered relevant since only 10% of the volume is being used in the LVET. In this regard, the development of a more appropriate scoring/classification system is recommended. The Panel also recommended using existing data for a statistical analysis to develop such a classification system.

As demonstrated by Gettings et al. (1996), the LVET produced less severe responses than the Draize eye test. Based on other information comparing the Draize rabbit eye test, the LVET, and human testing, both the Draize test and LVET appear to overpredict human responses, but the LVET is less overpredictive than the Draize test. Furthermore, the rabbit seems to be more sensitive to irritants than other species including dog, primate, and man (Durham et al. 1992).

Substances tested in humans are limited for ethical reasons to mild ocular irritants and substances not labeled as irritants. Accidental exposure data with more severe irritants are vague with respect to concentration of the test substance and to the volume of exposure. Thus, the LVET data are being compared to human data where the severity of the irritants may be limited, and there is concern that the LVET has not been shown to be capable of detecting a severe irritant or corrosive test substance. ICCVAM asked whether this concern was justified. The Panel felt that the LVET can predict the full range of irritancy potential, and therefore the concern is not justified. Furthermore, there is not sufficient information associated with the human accidental exposure data to articulate the concern.

It is difficult to compare LVET data with Draize rabbit eye test data because the LVET has been reported to underpredict relative to the Draize test and overpredict relative to human experience data. For example, a Draize test EPA Category I test substance might be labeled as an EPA Category II or III when tested in the LVET. The Panel was asked if there is a statistically meaningful way to compare these data.

The Panel concluded that there is no statistically meaningful way to compare the data, as the numbers of studies is small and the predictive results are often derived from one animal out of three making statistical analyses difficult, if not impossible. Table 4-1 of the ICCVAM draft SRD shows a trend in the ability of the LVET to predict the Draize rabbit eye test response. In this regard, the development of a more appropriate scoring/classification system is recommended. The Panel also recommended the use of existing human data for a statistical analysis to develop a classification system to demonstrate that the LVET is equal to (if not more accurate than) the Draize test at predicting the human response.

The Panel was asked if it was aware of any instances in which the Draize rabbit eye test failed to predict a severe irritant/corrosive response in the human. The Panel responded that, to the best of its knowledge, it was not aware of appropriately documented examples where the Draize test failed to predict a severe irritant/corrosive response in the human.

7.2.4 Data Quality

The Panel concluded that data quality requirements are relevant to all of the test methods being reviewed (see **Section 3.3.4**).

7.2.5 Consideration of All Available Data and Relevant Information

The Panel was asked if the LVET draft SRD adequately considered all the relevant data identified in published or unpublished studies that employ this test method. A related question was asked regarding whether any other comparative test method data available for consideration had nevertheless not been considered in the draft BRD for the proposed strategy for classification of antimicrobial cleaning products (AMCPs) or ECVAM LVET BRD. The Panel concluded that it is necessary to obtain data from all sources. The Panel emphasized the need to examine Jester (2006) and further inquire about the existence of any additional historical data the participating companies have on the LVET – for example, in-house or external studies they have supported, or research and testing studies. While the ECVAM BRD has not been provided and therefore the Panel cannot comment on this document, the Panel concluded that the AMCP BRD appears to contain all available relevant data.

7.3 Comments on the Draft ICCVAM Test Method Recommendations on the LVET¹²

As indicated above, the Panel concluded that based on the available data, the LVET seems to be more accurate in predicting the human response than the Draize rabbit eye test for some substances for which comparative Draize, LVET, and human data (both accidental and ethical human testing) are available. However, in the absence of all data, including the ECVAM BRD, the Panel 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 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.

¹² See **Appendix A** for draft ICCVAM test method recommendations reviewed by the Panel.

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8.0 *In Vitro* Testing Strategies for Ocular Hazard Categorization of Antimicrobial Cleaning Products¹³

8.1 Review of the Draft SRD for Errors and Omissions

8.1.1 *General Comments*

The Panel was asked if the ICCVAM draft AMCP SRD contained any errors that should be corrected or omissions of relevant data or information that should be included.

The Panel requested that additional discussion on L929 cells be added to the AMCP SRD, specifically addressing the relevance of using a non-ocular cell line in ocular irritation testing. The Panel noted that human ocular cell lines are available (e.g., SV40 transformed cell lines from Lonza and SkinEthic, or HuCL cell line of Ilene Gipson).

The Panel also noted that the EpiOcular™ (EO) test method does not use ocular cells. While this test is mechanistically better than the Cytosensor Microphysiometer® (CM) test method in that it uses a multilayered construct, there are no data showing that the cells used in the EO test method have metabolic and molecular pathways similar to cornea. The Panel suggested that corneal epithelial cells noted above could be considered as *in vitro* models for toxicity testing.

The Panel suggested that the following papers be referenced in the AMCP SRD:

- Cater KC, Harbell JW. 2006. Prediction of eye irritation potential of surfactant-based rinse-off personal care formulations by the bovine corneal opacity and permeability (BCOP) assay. *Cutan Ocul Toxicol.* 25: 217-33.
- Cater KC, Harbell JW. 2008. Comparison of *in vitro* eye irritation potential by bovine corneal opacity and permeability (BCOP) assay to erythema scores in human eye sting test of surfactant-based formulations. *Cutan Ocul Toxicol.* 27:77-85.

8.1.2 *Comments with Specific References to the Text*

The Panel recommended that the following specific corrections and revisions be made to the AMCP SRD.

- Page ix: The List of Abbreviations and Acronyms should include the abbreviation SRD (summary review document).

¹³ While Dr. Ward participated in the discussions, due to her consulting relationship with a company that manufactures antimicrobial cleaning products, she abstained from voting on the Panel's final conclusions and recommendations for this topic.

- Line 589: Change “MRD₅₀ <2 = EPA...” to “MRD₅₀ <2 mg/mL”.
- Line 590: A brief explanation should be provided for the exclusion of EPA Category II classification for the CM test method.
- Line 594: In the phrase “...EPA Category III; ET₅₀ >70 mg/mL...”, the expression “>70 mg/mL” should be changed to “>70 min”.
- Line 595: A brief explanation should be provided for the exclusion of Category II classification for the EO test method.
- Line 600: A brief explanation should be provided for exclusion of Category IV classification for the BCOP test method.
- Line 756: Change “MRD₅₀ <2 = EPA...” to “MRD₅₀ <2 mg/mL”.
- Line 759: Change “ET₅₀ >70 mg/mL...” to “ET₅₀ >70 min...”.
- Line 764: Change “ET₅₀ >70 mg/mL...” to “ET₅₀ >70 min...”.
- Line 798: The superscripts 1, 2, and 3 in Table 2 need clarification.
- Line 958: In Table 1-2, remove the abbreviations for CPSC, FDA, and OSHA from the footnotes. The abbreviations are not used in the table.
- Line 1009: Change “MRD₅₀ <2 = EPA...” to “MRD₅₀ <2 mg/mL”.
- Line 1013: Change “ET₅₀ >70 mg/mL...” to “ET₅₀ >70 min”.
- Line 1055: Increase font size.
- Label diagram on page 9 as Figure 2-2, and move to page 10.
- Page 12: Change the "box" symbol in footnote 3 to μL.
- Line 1277: Change “MRD₅₀ <2 = EPA...” to “MRD₅₀ <2 mg/mL”.
- Line 1280: Change “ET₅₀ >70 mg/mL...” to “ET₅₀ >70 min”.
- Line 1285: Change “ET₅₀ >70 mg/mL...” to “ET₅₀ >70 min”.

8.2 Evaluation of the Validation Status of the AMCP Testing Strategy

8.2.1 *Comments on the Test Methods Used in the AMCP Testing Strategy*

8.2.1.1 The BCOP Test Method

The Panel was asked if the protocol was sufficiently detailed such that the test method could be conducted reproducibly in other laboratories. The Panel concluded that the BCOP test method protocol was sufficiently detailed and commented that these issues have been addressed in previous evaluations of the use of the BCOP test method as a screening test to identifying ocular corrosives and severe irritants. However, the Panel noted the lack of some details in the BCOP test method protocol (e.g., preparation and use of media).

ICCVAM asked the Panel whether critical aspects of the test method protocol, as outlined in the ICCVAM Submission Guidelines (ICCVAM 2003), had been adequately justified and described in the AMCP SRD. The Panel concluded that critical aspects of the BCOP test method protocol are adequately justified and described. However, in order to use the BCOP test method in a testing strategy to identify severe irritants (EPA Category I) and moderate irritants (EPA Category II), positive controls that represent these hazard categories should be included in any future validation studies.

The Panel was asked whether the current histopathology database for the BCOP test method justifies the use of histopathological evaluation, or whether additional data are needed before a recommendation for the use of histopathological evaluation in the BCOP test method for hazard classification of AMCPs can be made.

The Panel recognized that the use of histopathological evaluation as an additional endpoint did not improve the accuracy and predictability for the limited database of currently tested AMCPs. However, histopathological evaluation may prove to be a useful endpoint and, as such, collection of ocular tissue and further efforts to optimize histopathological evaluation is strongly encouraged. Therefore, routine collection and fixation of tissue during performance of the BCOP test method for possible histopathological evaluation is recommended.

8.2.1.2 The EO Test Method

The Panel was asked if the EO test method protocol was sufficiently detailed that the EO test method could be conducted reproducibly in other laboratories. The Panel noted that quality control should be assessed for each batch of EO and the manufacturer should provide a “certificate of quality”.

ICCVAM asked the Panel whether critical aspects of the EO test method protocol, as outlined in the ICCVAM Submission Guidelines (ICCVAM 2003), had been adequately justified and described in the AMCP SRD. The Panel concluded that the test method protocol

has been adequately justified and described. However, the Panel noted that in order to use EO in a testing strategy to identify mild irritants (EPA Category III) and substances not labeled as irritants (EPA Category IV), positive controls that represent these hazard categories should be included in any future validation studies. In addition, due to the small number of Category III substances in the AMCP database that have been tested, the Panel concluded that there are insufficient data with which to adequately demonstrate that the EO test method can currently distinguish EPA Category III from Category IV.

The EO test method protocol included in the AMCP SRD, which uses a time-to-toxicity protocol, differs from one included in a recent EO test method submission to ECVAM, which uses a threshold of relative viability at a single time point. A validation study based on the EO test method protocol submitted to ECVAM is planned. ICCVAM asked the Panel whether it considered one of these protocols more appropriate than the other for the hazard classification of AMCPs. The Panel did not consider one protocol to be more appropriate than the other. However, if these studies are going to be acceptable to regulatory bodies, then improved standardization of endpoints and identical experimental designs will likely be required.

The Panel was asked if the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) for the EO test method had been adequately evaluated and compared to the traditional rabbit test and/or the LVET for the types of substances included in the AMCP database (i.e., surfactants, solvents, bases, acids, oxidizers). If not, the Panel was asked what other analyses should be performed. The Panel indicated that, based on the data provided, the total number of products and their distribution across hazard categories were not sufficient. The Panel also noted that, although the *in vitro* results for the three AMCPs that were tested more than once in a single laboratory were consistently reproducible, these results often differed from those *in vivo* (i.e., for two of the three materials tested more than once in a single laboratory, the *in vivo* hazard category based on Draize rabbit eye test data differed from the predicted *in vitro* hazard category).

The Panel was asked if intralaboratory reproducibility of the EO test method had been adequately evaluated for the types of substances included in the AMCP database (i.e., surfactants, solvents, bases, acids, oxidizers). If not, the Panel was asked what other analyses should be performed and if there are any limitations apparent based on this intralaboratory reproducibility assessment. The Panel concluded that the intralaboratory reproducibility of the EO test method had not been adequately evaluated and that the data set used for assessing intralaboratory reproducibility was limited in number and chemical classes.

The Panel was asked if the interlaboratory reproducibility of the EO test method had been adequately evaluated and compared to the traditional rabbit test and/or the LVET. If not, the

Panel was asked what other analyses should be performed and if there are any limitations apparent based on this interlaboratory reproducibility assessment. The Panel concluded that the interlaboratory reproducibility of this test method had been adequately evaluated.

8.2.1.3 *The CM Test Method*

The Panel was asked if the protocol for the CM test method was sufficiently detailed that the test method could be conducted reproducibly in other laboratories. The Panel concluded that the protocol was sufficiently detailed. However, the CM test method is unlikely to be widely used because manufacture of the instrument required to conduct the test method has been discontinued. If a new, similar, or redesigned instrument were to be developed, revalidation would be required.

ICCVAM asked whether critical aspects of the CM test method protocol, as outlined in the ICCVAM Submission Guidelines (ICCVAM 2003), have been adequately justified and described in the AMCP SRD. The Panel concluded that critical aspects of the CM test method had been adequately justified and described. However, because the method is limited to testing water-soluble surfactants and certain types of surfactant formulations (specified below), the following additional consideration was added. The Panel recommends that a range of surfactant concentrations should be tested, because surfactants form micelles at higher concentrations, which reduce the number of surfactant molecules available to react with the target tissue (ECVAM 2008). The Panel also recommended that if the CM test method is used in a testing strategy to identify mild irritants (EPA Category III) and substances not labeled as irritants (EPA Category IV), then positive controls that represent these hazard categories should be included in any future validation studies.

The ICCVAM draft position is that the LVET predictivity for the Draize rabbit eye test, and the lack of LVET data for substances known to cause moderate and severe irritation and ocular corrosion, make the LVET inadequate to serve as a reference test method to support the validity of *in vitro* test methods. For this reason, the CM test method, for which there exists only LVET reference data for AMCPs, was considered inadequate to support the proposed testing strategy. The Panel concluded that the LVET data can be used to support the validity of the CM test method in the proposed testing strategy.

In addition, data from the Draize rabbit eye test on 53 surfactant and surfactant-containing formulations were provided in a BRD prepared by ECVAM with which to assess the accuracy of the CM test method.¹⁴ These substances were not claimed as AMCPs, but they

¹⁴ The CM test method is currently undergoing separate peer review by an ECVAM Scientific Advisory Committee Peer Review Panel, which includes two members of the ICCVAM Ocular Peer Review Panel (Drs. Hayes and Wilson).

were surfactant-containing formulations, as are many AMCPs. Based on the performance of the CM test method using these 53 substances, ICCVAM has proposed that the CM test method can be used 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 EPA Category I, GHS Category 1, or EU Category R41; or as EPA Category IV, GHS Not Labeled, EU Not Classified, in a tiered-testing strategy, as part of a weight-of-evidence approach. ICCVAM asked the Panel whether these results with non-AMCPs suggest that the CM test method could be useful in a testing strategy. The Panel concluded that the additional data with surfactant-containing formulations suggest that the CM test method can be used as a screening test to identify water-soluble surfactant chemicals and certain types of surfactant-containing formulations (e.g., cosmetics and personal care products). In addition, the Panel recommended that the performance of the CM test method for different classes of surfactants (i.e., nonionic, anionic, cationic, and zwitterionic) be evaluated for the existing data and in future studies.

The Panel also noted that the equipment required to conduct the CM test method has been discontinued and is not currently supported by the manufacturer. While it could be purchased as a used piece of equipment, concerns regarding software, disposables, and other necessary accessories were expressed. These deficiencies may also impact GLP compliance.

The Panel expressed reservations about the use of the CM test method for classification and labeling of AMCPs for EPA registration. The Panel recommended that additional EPA-registered AMCPs representing all ocular hazard categories, in particular EPA Categories II and III, should be tested to expand the database.

Molecular Devices Corporation (Sunnyvale, CA) has stopped production of the CM instrument, although the Transwell™ inserts and other test method-specific materials are expected to be available for some time to current CM test method users. The Panel was asked if this would affect any recommendation on the usefulness of the CM test method in the testing strategy. The Panel responded that currently available CM instruments can still be used for the recommended purpose. If a new instrument to replace the existing CM instrument were to be designed and manufactured, then a “catch-up” validation study (i.e., not a full validation study) would be required. Until CM performance standards are developed, a full validation study (OECD 1996; ICCVAM 1997) is required.

The Panel was asked if the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) for the CM test method had been adequately evaluated and compared to the traditional rabbit test and/or the LVET. If not, the Panel was asked what other analyses should be performed. The Panel concluded that the CM test method had been adequately evaluated and compared to the Draize rabbit eye test and/or the LVET for water-

soluble substances, including surfactants and surfactant-containing formulations (e.g., cosmetics and personal care products). However, all classes of surfactants (i.e., nonionic, anionic, cationic, and zwitterionic) should be evaluated to further characterize the usefulness of the CM test method for these types of substances.

The Panel was asked if the intra- and interlaboratory reproducibility of the CM test method has been adequately evaluated. If not, the Panel was asked what other analyses should be performed, and if there are any limitations apparent based on this intralaboratory reproducibility assessment. The Panel concluded that both the intra- and interlaboratory reproducibility has been adequately evaluated. However, the Panel noted that the available data are limited to specific chemical/product classes (i.e., water-soluble substances, including surfactants and surfactant-containing formulations such as cosmetics and personal care products).

8.2.2 *Comments on the Draft ICCVAM Test Method Recommendations on the CM Test Method to Identify All Categories of Ocular Irritation*¹⁵

ICCVAM asked the Panel whether it agreed that the available data and test method performance (accuracy and reliability) supported the ICCVAM draft recommendations for the CM test method in terms of the proposed test method usefulness and limitations. The Panel concluded that the CM test method is recommended as a screening test to identify water-soluble surfactant substances as ocular corrosives and severe irritants and not labeled as irritants in a tiered-testing strategy, as part of a weight-of-evidence approach. However, major concerns are the continued availability of the instrument used to conduct the CM test method, and what new manufacturing processes, including the subsequent required revalidation, might mean to already existing CM test method data.

When the CM test method was used to identify substances not labeled as irritants among the database of 53 surfactant-containing substances, the false negative rate ranged from 0-2% (0/27 to 1/46) when compared to Draize rabbit eye test results. The one false negative substance was Category III based on *in vivo* data when using the EPA classification system. 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) that cleared after one (n=1) or two days (n=2), and two test animals had corneal opacity (score = 1) that cleared after one day. The Panel was asked if it considered the type and number of lesions observed in this study to be reason for concern regarding the use of CM to identify EPA Category IV substances. The Panel concluded that the type and number of lesions associated with this study could be a reason for concern regarding the use of the CM test method to

¹⁵ See **Appendix A** for draft ICCVAM test method recommendations reviewed by the Panel.

identify EPA Category IV substances. Based on the *in vivo* data, this substance would be classified as EPA Category III and therefore may cause irritation in the eyes of humans.

When using the CM test method to distinguish substances not labeled as irritants from all other irritant classes, the false negative rate for the EU and GHS systems was 0% (0/27 or 0/28) while EPA is 2% (1/46); therefore, the CM test method was recommended for such testing purposes. The CM test method validation database does not include any substances currently regulated by EPA. ICCVAM asked the Panel if additional testing should be required before a recommendation is made on the usefulness of cell function-based test methods for identifying Category IV substances. The Panel recommended that further studies using the CM test method are needed, in particular for EPA Categories III and IV. The available data restrict the applicability domain of the CM test method to water-soluble surfactants and surfactant based formulations (e.g., cosmetics and personal care products).

The false positive rates for the CM test method ranged from 50% to 69% (3/6 to 18/26) when compared to *in vivo* results. Three substances were false positives when using the EPA classification system and were classified *in vitro* as Category II/III. 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). The Panel was asked whether these high false positive rates raised concern about the usefulness of the CM test method as a screening test for substances not labeled as irritants, even if the false negative rate is near 0%. The Panel concluded that the high false positive rates are of concern, and therefore recommended a tiered-testing strategy, as part of a weight-of-evidence approach.

8.3 Comments on the AMCP Testing Strategies

ICCVAM asked the Panel whether it considered the database supporting the original AMCP testing strategy (using the BCOP, CM, and EO test methods) or the alternate testing strategy (using only BCOP and EO test methods) to be adequate for the classification and labeling of AMCPs for EPA registration. The Panel concluded that the database supporting the original and alternate AMCP testing strategies are not sufficient. The Panel recommended that a more robust database be established, comprised of *in vitro* test data and paired Draize/LVET data evaluating AMCPs.

The Panel was asked if there are other test methods that should be considered in a testing strategy that would be expected to improve classification and labeling of AMCPs for EPA registration. The Panel is not aware of other test methods that have been explored specifically for AMCPs, but there may be other test methods that could be considered.

8.3.1 Substances Used for the Validation Studies

The AMCP SRD included data for 228 substances tested using one or two of the three *in vitro* test methods proposed for use in the testing strategy. However, none of the substances had been tested using all three *in vitro* test methods. Therefore, there are no data available with which to characterize the actual performance of a testing strategy that includes the BCOP, CM, and EO test methods. The Panel was asked if it agreed that this limitation prevents any definitive recommendation on the AMCP testing strategy. The Panel commented that the absence of data on substances tested in all three test methods (i.e., BCOP, CM, and EO) prevents any definitive recommendation on the AMCP testing strategy.

Of the 228 substances, 28 are EPA-registered AMCPs; eight additional materials are in-use dilutions of EPA-registered antimicrobial concentrates. The Panel was asked if this small proportion of the total database was problematic with regard to any conclusions that might be reached on the usefulness of the testing strategy for classification and labeling of AMCPs. The Panel concluded that the database of AMCPs used to evaluate the testing strategy was too limited. The Panel recommended that additional EPA-registered AMCPs representing all ocular hazard categories, in particular EPA Categories II and III, should be tested in all tests involved in the strategy.

ICCVAM asked the Panel if the database for each test method used in the AMCP testing strategy was representative of a sufficient range of chemical classes and physicochemical properties that it would apply to any of the types of chemicals and products that are typically tested for ocular irritation potential. The Panel stated that the database for each test method used in the AMCP testing strategy was not representative of a sufficient range of chemical classes and physicochemical properties. Therefore, the Panel concluded that the AMCP testing strategy was not applicable to the types of chemicals and products that are typically tested for ocular irritation. The Panel recommended the following additional categories of compounds for evaluation: acids, alcohols, organics, esters, dyes, fixatives, sensitizers, water-insoluble substances, solids, and semisolids.

8.3.2 Test Method Accuracy

The current accuracy analysis is based on overall concordance with the Draize rabbit eye test. The Panel was asked if these data are adequate for assessing the accuracy of the test methods. According to the Panel, the Draize test data are adequate for assessing the accuracy of the testing strategy. However, the Panel felt that LVET data can also be included because based on the available data, the LVET seems to be more accurate in predicting the human response than the Draize test for some substances for which comparative Draize test, LVET, and human data (from both accidental exposures and ethical human testing) are available.

The Panel was asked if the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of these test methods (i.e., BCOP, CM, and EO) had been adequately evaluated and compared to the traditional rabbit test and/or the LVET for the types of substances included in the AMCP database (i.e., surfactants, solvents, bases, acids, and oxidizers). If not, the Panel was asked what other analyses should be performed. The Panel concluded that, based on the data provided, the total number of products and their distribution across hazard and chemical categories were not sufficient. The Panel recommended that additional EPA-registered AMCPs representing all ocular hazard categories, in particular EPA Categories II and III, should be tested in all tests involved in the strategy.

8.3.3 *Data Quality*

The Panel concluded that data quality requirements are relevant to all of the test methods being reviewed (see **Section 3.3.4**).

8.3.4 *Test Method Reliability (Intra- and Interlaboratory Reproducibility)*

The Panel was asked if it considered the intra- and interlaboratory reproducibility of these test methods (i.e., BCOP, CM, and EO) to have been adequately evaluated and compared to the traditional rabbit eye test and/or LVET for the types of substances included in the AMCP database (i.e., surfactants, solvents, bases, acids, oxidizers) and, if not, what other analyses should be performed. For intra- and interlaboratory reproducibility of the individual test methods, refer to those specific sections. For the AMCP testing strategy, the Panel indicated that studies to address the intra- and interlaboratory reproducibility have not been performed.

8.3.5 *Consideration of All Available Data and Relevant Information*

The Panel was asked if the draft AMCP SRD adequately considered all the relevant data identified in published or unpublished studies that employ these test methods. The Panel was not aware of additional data that should be considered, but stated that it is possible that additional data could be identified.

8.4 **Comments on the Draft ICCVAM Test Method Recommendations on the AMCP Testing Strategies¹⁶**

8.4.1 *Test Method Usefulness and Recommendations*

The Panel was asked if the available data and test method performance (accuracy and reliability) supported the ICCVAM draft recommendations for the ocular test methods and AMCP testing strategy (i.e., using the BCOP, CM, and EO test methods) in terms of the

¹⁶ See **Appendix A** for draft ICCVAM test method recommendations reviewed by the Panel.

proposed test method usefulness and limitations. The Panel concluded that there were 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 ocular hazard categories).

The Panel was asked if it agreed that there are insufficient available data on which to base definitive recommendations on the alternate testing strategy (i.e., using the BCOP and EO test methods). The Panel concluded that there were insufficient available data on which to base definitive recommendations on the alternate testing strategy for classifying substances in all four ocular hazard categories.

ICCVAM asked the Panel if a retrospective evaluation of results in more than one test method could suffice as an adequate performance evaluation, even if the same substances were not tested in each method proposed in a strategy. The Panel responded that a retrospective evaluation of results 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.

8.4.2 Test Method Protocol

The Panel was asked if it agreed that the available data supported the ICCVAM draft recommendations for the ocular test method procedures in terms of the proposed standardized test method protocols. The Panel concluded that the available data support the ICCVAM draft recommendations for the ocular test method procedures in terms of the proposed standardized test method protocols. The Panel recommended routine fixation of tissue collected during conduct of the BCOP test method for possible histopathological evaluation. The Panel supported the use of the INVITTOX Protocol 102 (ECVAM 2008) for future studies to further characterize the usefulness and limitations of the CM test method.

8.4.3 Future Studies

The Panel was asked if it agreed that the available data support the ICCVAM draft recommendations for each of the ocular test methods in terms of the proposed future studies and, if not, what recommendations it would make. The Panel concluded that additional testing would expand existing databases and could be used to optimize test method decision criteria. The following additional recommendations were made:

1. Appropriate positive and negative controls should be identified for each hazard category.
2. Microscopic analysis has the potential to add value to the BCOP test method. However, future studies are needed to develop objective and quantifiable endpoints that can be used to differentiate hazard categories.
3. Future test methods should consider cells and tissue constructs of cornea/conjunctiva origins.
4. Future studies should consider prospective testing (a preferred and scientifically more valid approach compared to retrospective testing) with GLP compliance, coded samples, and pre-established evaluation criteria. Such testing should include a list of reference substances tested in each method to determine the optimum scheme to be followed. The testing scheme could then be validated through appropriate evaluations.
5. Future BCOP studies should consider quantitative fluorescent readings instead of OD₄₉₀ readings to provide more accurate quantification of permeability.

ICCVAM asked the Panel whether, given that the EO test method alone would provide the same performance as the alternate testing strategy (i.e., BCOP and EO test methods) based on the 28 substances evaluated, additional studies should focus on using the EO test method alone instead of the testing strategy. The Panel concluded that additional studies should not focus on the use of the EO test method alone because it considered the use of a testing strategy more promising. The Panel stated that the BCOP test method was useful in the alternate testing strategy. In general, the Panel concluded that at this time no single *in vitro* test method constitutes an evaluation system applicable to all types of test materials and for all EPA classes.

8.4.4 Performance Standards

ICCVAM asked the Panel if it agreed that the results described above do not warrant the development of performance standards for the CM test method at this time. Based on the available data, the Panel concluded that the development of performance standards was not warranted at this time. However, if the applicability domain is restricted to surfactants (with the limitations stated above), then performance standards could and should be developed.

ICCVAM asked the Panel if it agreed that the results described above do not warrant the development of performance standards for the AMCP testing strategy at this time. The Panel concluded that the development of performance standards for the AMCP testing strategy was

not warranted at this time. The Panel indicated that a new approach for comparing testing strategies needs to be defined.

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Appendix A
Draft ICCVAM Test Method Recommendations

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**Draft Proposed ICCVAM Test Method Recommendations:
Evaluation of the Validation Status of Alternative Ocular
Safety Testing Methods and Approaches**

April 1, 2009

The draft Background Review Documents supporting these draft recommendations are available at <http://iccvam.niehs.nih.gov/methods/>. The draft Background Review Documents and the draft recommendations will be considered by an independent scientific peer review panel that will meet in public session on May 19-21, 2009 at the Consumer Product Safety Commission headquarters in Bethesda, MD. Public comments are welcome. More information is available in the *Federal Register* Notice of the meeting, available at <http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/>. ICCVAM will finalize these recommendations after consideration of comments from the peer review panel, the public, and its scientific advisory committee.

These draft recommendations do not represent the official position of any Federal agency.

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

1.1 Draft Proposed ICCVAM Recommendations: Use of Topical Anesthetics Systemic Analgesics in Ocular Toxicity Testing to Avoid or Minimize Pain and Distress

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) proposes the following draft test method recommendations on the use of topical anesthetics and systemic analgesics to avoid or minimize pain and distress in acute eye irritation testing. ICCVAM developed the draft recommendations after considering available relevant data, information, and analyses, which are provided in the draft Background Review Document (BRD) for this topic (<http://iccvam.niehs.nih.gov/methods/ocutox/pretreat/BRD.pdf>). This section provides a brief summary of the background and rationale for the draft proposed recommendations, followed by the specific draft recommendations on proposed usefulness and limitations, proposed modifications to the current standardized test method protocol, and proposed future studies and activities.

Background and Rationale for the Draft Proposed ICCVAM Recommendations

Since 1984, the U.S. Consumer Product Safety Commission has recommended preapplication of tetracaine ophthalmic anesthetic for all rabbit eye toxicity studies. However, current U.S. Environmental Protection Agency (EPA) and Organisation for Economic Co-operation and Development (OECD) test guidelines for the rabbit eye test state that topical anesthetics can only be used if the user demonstrates that such pretreatments do not interfere with the results of the tests.¹⁷ Therefore, they often are not used because a separate study would likely be necessary to provide such information.

The use of topical ophthalmic anesthetics and/or systemic analgesics during the conduct of the Draize rabbit eye irritation test was evaluated at a recent scientific symposium entitled “Minimizing Pain and Distress in Ocular Toxicity Testing” (cosponsored by ICCVAM, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods [NICEATM], and the European Centre for the Validation of Alternative Methods [ECVAM]). While invited experts acknowledged that a single treatment with a topical anesthetic to anesthetize the surface of the cornea prior

¹⁷ OECD TG 405 (OECD 2002) states, "The type, concentration, and dose of a local anesthetic should be carefully selected to ensure that differences in reaction to the test substance will not result from its use." Similarly, EPA (1998) states that, "The type and concentration of the local anesthetic should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use."

to the application of the test article to the eye could potentially cause slight physiologic changes, the consensus was that such alterations to the irritant response would be slight if any. Furthermore, the predominant view was that if there were any effects on the irritant response, it would tend to slightly increase the severity of the response. Therefore, the routine use of topical anesthetics was recommended, since the anesthetics at least avoid the discomfort experienced from instillation of the test article on the eye, and temporarily avoid or minimize pain and distress that might result from immediate ocular damage. Experts also recommended that pretreatment with topical anesthetics combined with systemic analgesics should be routinely used to avoid pain, and that animals exhibiting clinical signs of pain or distress or with ocular lesions associated with painful conditions should continue to be treated with systemic analgesia.

A recent evaluation by NICEATM of the effects of pretreatment with tetracaine hydrochloride (0.5% w/v) on the ocular irritancy potential of 97 formulations indicate that such pretreatments had no statistically significant impact on the hazard classification severity category of observed ocular irritation (Choksi et al. 2007). For a majority of the formulations tested, topical anesthetic pretreatment had no or minimal impact on:

- The hazard classification severity category of observed ocular irritation
- The variability in ocular irritation responses among animals treated with the same test article
- The number of days required for an ocular lesion to clear.

When a difference in ocular irritation response was observed in animals pretreated with topical anesthesia compared to animals that were not pretreated, the more severe response was more frequently observed in the pretreated animals. However, none of the observed differences were statistically significant. The observed differences occurred in both directions (increasing and decreasing the level of irritancy), which suggests that they are likely related to the inherent interindividual biological variability of response rather than topical anesthetic pretreatment.

The draft proposed ICCVAM recommendations that follow were developed based on available data in conjunction with clinical experience and expert judgment.

Usefulness and Limitations

In order to avoid or minimize potential pain and distress caused by test article administration and initial injuries in the Draize rabbit eye test, ICCVAM proposes the routine use of a topical anesthetic (i.e., tetracaine or proparacaine, 1-2 drops of 0.5% w/v solution) and an opioid systemic analgesic (i.e., buprenorphine, 0.05 mg/kg) prior to

instillation of a test substance, unless there is an adequate scientific rationale for not using these substances. Anti-inflammatory analgesics (e.g., nonsteroidal anti-inflammatory drugs) are not recommended because of their possible influence on study results due to demonstrated effects on the wound healing process. In addition, treatment with an opioid systemic analgesic (i.e., buprenorphine, 0.05 mg/kg, every 12 hr) should continue as long as a test animal displays clinical signs of more than momentary or slight pain or distress (e.g., blepharospasm, excessive lacrimation, pawing at the treated eye) or has ocular injuries expected to cause or be associated with pain or distress (e.g., opacity, iritis, conjunctival redness, chemosis scores ≥ 2). Users should also consider the humane endpoints detailed in **Section 1.2**, which could justify early termination of the study.

Test Method Protocol

When required for ocular safety testing, the current Draize eye test protocol used for regulatory safety assessments of potential ocular hazards (EPA 1998, OECD 2002) should be conducted with the ICCVAM proposed modifications for the use of topical anesthetics and systemic analgesics. These modifications include the following procedures. Prior to instillation of a test substance, the animal is given a single dose of a systemic opioid analgesic (i.e., buprenorphine, 0.05 mg/kg, subcutaneous or intramuscular injection) and a topical anesthetic (i.e., tetracaine or proparacaine, 2 drops of 0.5% w/v solution). After test substance application, the animal is carefully observed for any clinical signs of pain and distress. Treatment with a systemic analgesic (i.e., buprenorphine, 0.05 mg/kg SC, IM, q 12 hr) should continue after instillation of the test substance if a test animal displays clinical signs of more than momentary or slight pain or distress (e.g., blepharospasm, excessive lacrimation, pawing at the treated eye) or ocular injuries expected to cause pain or distress; in this case a regular treatment regimen (i.e., every 12 hr) should proceed until such signs or injuries are no longer present. While the choice of analgesic and its dosage should be made by the attending veterinarian because of the many variables associated with pain management, the recommended analgesic and associated dose (buprenorphine, 0.05 mg/kg) is based on its long history of successful veterinary use as an analgesic for moderate to severe pain in rabbits (Kohn et al. 2007). Buprenorphine is also available in a transdermal patch that provides up to four days of controlled release drug, and this could be considered as an option to more frequent dosing.

Proposed Future Studies

Routine observation and recording of lesions and clinical signs is recommended during ocular irritation safety studies to evaluate efficacy in order to optimize analgesic dose and treatment schedule. Periodic review of these data should be performed to determine if

adjustments are needed to improve the effectiveness of pretreatment and posttreatment analgesia. Ideally, data should be collected during routine safety testing that could be analyzed periodically to determine the efficacy for specific types of lesions and clinical signs of pain and distress associated with ocular irritation/corrosivity testing.

ICCVAM encourages users to provide all data generated using the modified test method protocols to NICEATM to create a database that can be periodically evaluated to further characterize the usefulness and limitations of topical anesthetics and systemic analgesics for avoiding or minimizing pain and distress in ocular safety assessments.

1.2 Draft Proposed ICCVAM Recommendations on the Use of Humane Endpoints in Ocular Toxicity Testing

ICCVAM proposes the following draft test method recommendations on the use of humane endpoints to avoid or minimize pain and distress in ocular toxicity testing. ICCVAM developed the draft recommendations after considering available relevant data, information, and analyses, which are provided in the draft BRD for this topic (<http://iccvam.niehs.nih.gov/methods/ocutox/pretreat/BRD.pdf>). This section provides a brief summary of the background and rationale for the draft proposed recommendations, followed by the specific draft recommendations on proposed usefulness and limitations, proposed modifications to the current standardized test method protocol, and proposed future studies and activities.

Background and Rationale for the Draft Proposed ICCVAM Recommendations

U.S. Public Health Service policy and U.S. Department of Agriculture regulations on pain and distress in laboratory animals state that more than momentary or slight pain and distress:

- Should be limited to that which is unavoidable for the conduct of scientifically valuable research or testing
- Should be conducted with appropriate pain relief medication unless justified in writing by the principal investigator
- Should continue for only the necessary amount of time required to attain the scientific objectives of the study

These regulations also state that animals suffering severe or chronic pain or distress that cannot be relieved should be humanely killed after or, if appropriate, during the procedure, and finally, that Institutional Animal Care and Use Committees must ensure that the principal investigator complies with the requirements.

Participants at the recent symposium “Minimizing Pain and Distress in Ocular Toxicity Testing” also discussed early adverse responses predictive of ocular lesions associated with severe irritant or corrosive substances (United Nations Globally Harmonized System of Classification and Labeling of Chemicals [GHS] Category I [UN 2007], European Union [EU] Category R41 [EU 2001], or EPA Category I [EPA 1998]) that could be used routinely as humane endpoints to terminate a study. Among the invited participants were human and veterinary ophthalmologists and anesthesiologists, scientific experts in ocular hazard testing, research scientists, and industrial toxicologists. Subsequent to these discussions, the endpoints described below were recommended for routine use.

Usefulness and Limitations

ICCVAM recognizes that current ocular testing guidelines include guidance that allow for certain types of severe ocular injuries, or evidence of severe pain and distress, to be used as criteria for study termination for humane reasons (OECD 2000, 2002; EPA 1998). In addition there is international guidance on general humane endpoints that can be used as the basis for ending an experiment (OECD 2002). ICCVAM recommends that the following ocular lesions, which are considered to be predictive of a severe irritant or corrosive response and are not expected to fully reverse by the end of the 21-day posttreatment observation period, should be considered and used as humane endpoints to terminate studies early where determined appropriate:

- Endpoints currently accepted for study termination (OECD 2000):
 - Draize corneal opacity score of 4 that persists for 48 hr
 - Corneal perforation or significant corneal ulceration including staphyloma
 - Blood in the anterior chamber of the eye
 - Absence of light reflex that persists for 72 hr
 - Ulceration of the conjunctival membrane
 - Necrosis of the conjunctiva or nictitating membrane
 - Sloughing
- Vascularization of the corneal surface (i.e., pannus)
- Greater than 75% of the limbus destroyed
- Area of fluorescein staining not diminishing over time based on daily assessment
- Lack of re-epithelialization five days after application of the test substance
- Extent of depth of injury to the cornea (routinely using slit-lamp and fluorescein staining) where corneal ulceration extends beyond superficial layers of the stroma or the depth of injury increases over time

Given the many years of clinical experience represented by the Symposium participants, ICCVAM considers that consideration and use of the recommended humane endpoints where determined appropriate can aid in further minimizing the duration and severity of pain and distress for animals used in ocular toxicity testing. However, while these

endpoints are recommended for consideration as additional humane endpoints, a minority view expressed by some members of the ICCVAM Ocular Toxicity Working Group (OTWG) is that some of the recommended endpoints should not automatically be used as a basis to terminate a study (i.e., pannus, fluorescein staining).

Test Method Protocol

Ocular safety assessment studies should be conducted using the ICCVAM recommended modifications to the current Draize eye test protocol for regulatory safety assessments of potential ocular hazards (EPA 1998; OECD 2002). These include incorporation of the recommended humane endpoints and the following language.

As described in EPA (1998) and OECD (2002), eyes should be examined at 24, 48, and 72 hours after treatment with a test substance. Evaluations can be facilitated by use of a hand slit-lamp or other appropriate ophthalmologic device. After recording observations at 24 hr post-treatment, the eyes can be examined with the aid of fluorescein at each observation time point. Accordingly, one drop of sodium fluorescein (or equivalent) is dropped directly onto the corneal surface. After flushing out excess fluorescein with sodium chloride solution (or equivalent) injured areas of the cornea appear yellow. Digital photographs during all fluorescein staining observations may add clarity toward accurately evaluating changes in the extent or depth of staining corresponding to a lesion that is not likely to reverse.

Proposed Future Studies

ICCVAM encourages users to provide to NICEATM all data that are generated using these modifications so NICEATM can create a database that can be periodically evaluated to further characterize the usefulness and limitations of using the proposed humane endpoints to avoid or minimize pain and distress in ocular safety assessments.

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2.0 ***In Vitro* Alternative Test Methods for Identifying Ocular Hazard Categories**

ICCVAM previously evaluated the validation status of the hen's egg test—chorioallantoic membrane (HET-CAM), isolated chicken eye (ICE), isolated rabbit eye (IRE), and bovine corneal opacity and permeability (BCOP) test methods for their ability to identify ocular corrosives and severe irritants, and considered BCOP and ICE to have sufficient performance to substantiate their use for regulatory hazard classification testing of some types of substances. The IRE and HET-CAM assays lacked sufficient performance and/or sufficient data to substantiate their use for regulatory hazard classification. ICCVAM subsequently recommended that the BCOP and ICE should be used in a tiered-testing strategy, where positive substances can be classified as ocular corrosives or severe irritants without the need for animal testing. ICCVAM is now reviewing the validation status of these *in vitro* test methods for identifying nonsevere ocular irritants (i.e., those that induce reversible ocular damage) and substances not labeled as irritants.

2.1 **The HET-CAM Test Method**

ICCVAM proposes the following draft test method recommendations on the HET-CAM test method. ICCVAM developed the draft recommendations after considering available relevant data, information, and analyses, which are provided in the draft BRD for this topic (<http://iccvam.niehs.nih.gov/methods/ocutox/mildmod/HETCAM-BRD.pdf>). This section provides a brief summary of the background and rationale for the draft proposed recommendations, followed by the specific draft recommendations on proposed usefulness and limitations, a proposed test method protocol, and proposed future studies and activities.

Background and Rationale for the Draft Proposed ICCVAM Recommendations

Performance analyses for the HET-CAM test method, compared to the Draize rabbit eye test, were performed for each classification system (i.e., GHS, EPA, EU) using each of the six HET-CAM protocols (i.e., IS[A], IS[B], Q-Score, S-Score, irritation score [IS], and irritation threshold concentration [ITC] protocols). With the exception of the IS(A) and IS(B) protocols, all analysis methods had at least one *in vivo* moderate or severe irritant substance classified *in vitro* as not labeled as an irritant (i.e., EPA Category IV, EU Not Labeled, GHS Not Classified). The IS(B) overclassified over 90% (39/42) of the Not Classified (GHS) substances. Therefore, more extensive analyses of the HET-CAM test method were restricted to the IS(A) protocol.

The test method recommendations described herein are based upon two analyses of ICE test method performance:

- Overall correct classifications that ranged from 40% (23/58) to 41% (24/59), depending on the hazard classification system evaluated when using the entire database; and 62% (5/8) to 78% (7/9) depending on the hazard classification system evaluated when discordant classes are removed.
- Overall accuracy for identification of substances not labeled as irritants (i.e., EPA Category IV, EU Not Labeled, GHS Not Classified) from all other categories ranged from 58% (36/58) to 60% (47/60) depending on the hazard classification system used. False positive and false negative rates ranged from approximately 60% (9/15) to 69% (22/32) and 0% (0/26) to 9% (4/45), respectively. The lowest false negative rate (0% [0/26 or 0/31]) was noted for the EU or GHS systems, respectively, followed by 9% (4/45) for the EPA system. For all three systems, the substances correctly identified as not labeled as irritants (i.e., EPA Category IV, EU Not Labeled, GHS Not Classified) were cosmetic formulations that were either oil/water emulsions or surfactant containing formulations. 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. For one of the substances, one out of the six test animals tested had a conjunctival redness score of two that required 14 days to resolve. Four of the remaining five test animals in this study had conjunctival redness scores of two that resolved within three days; one test animal did not have this lesion.

The available validation database for the HET-CAM test method has remained unchanged since the original ICCVAM evaluation (ICCVAM 2006a). Therefore, the original ICCVAM recommendation for the use of the HET-CAM test method to identify substances as ocular corrosives/severe irritants remains unchanged (i.e., “Based on these rates, the use of these analyses methods and decision criteria for screening and identifying ocular corrosives and severe irritants [i.e., EPA Category I, GHS Category 1, EU R41] in a tiered-testing strategy, as part of a weight-of-evidence approach, is not recommended.”)

Usefulness and Limitations

Based on an evaluation of available data and corresponding performance (sensitivity and specificity), ICCVAM proposes that the HET-CAM test method is not recommended to identify substances from all hazard categories as defined by the GHS, EPA, and EU classification systems (EPA 2003a; EU 2001; UN 2007). However, based on an analysis of 60 compounds (25 surfactant based formulations, 18 oil/water emulsions and 17 individual substances), the HET-CAM IS(A) test method can be used as a screening test to identify substances as not labeled as irritants (i.e., EU Not Labeled, GHS Not Classified), from all other hazard categories (i.e., EU R41 or R36; GHS Category 1, 2A, or 2B) when results are to be used for EU or GHS hazard classifications. However, based on the limited database for HET-CAM IS(A), this recommended use is limited to cosmetic and personal care formulations that are oil/water emulsions or surfactant containing formulations. Furthermore, while the limited database also indicates that the HET-CAM test method could identify substances labeled as EPA Category IV, the database does not include substances that are actually regulated by EPA (e.g., pesticide formulations). For this reason, additional testing of such products in the HET-CAM test method may be necessary before definitive recommendations can be made on its usefulness for identifying Category IV substances.

Test Method Protocol

An ICCVAM recommended test method protocol for the HET-CAM test method is included in the ICCVAM test method evaluation report (ICCVAM 2006d). This same protocol should be used for all future HET-CAM studies with the modification of including decision criteria for all categories of ocular irritation as described in the current HET-CAM BRD. 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 the HET-CAM test method for the identification of all ocular hazard categories.

Proposed Future Studies

ICCVAM recommends that additional studies should be conducted to further optimize the HET-CAM prediction models and the 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 (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.

2.2 The ICE Test Method

ICCVAM proposes the following draft test method recommendations on the ICE test method. ICCVAM developed the draft recommendations after considering available relevant data, information, and analyses, which are provided in the draft BRD for this topic (<http://iccvam.niehs.nih.gov/methods/ocutox/mildmod/ICE-BRD.pdf>). This section provides a brief summary of the background and rationale for the draft proposed recommendations, followed by the specific draft recommendations on proposed usefulness and limitations, a proposed test method protocol, and proposed future studies and activities.

Background and Rationale for the Draft Proposed ICCVAM Recommendations

The test method recommendations described herein are based upon two analyses of ICE test method performance:

- The overall correct classifications for the ICE test method ranged from 59% (83/141) to 77% (118/153), depending on the hazard classification system evaluated when using the entire database; and 64% (49/77) to 80% (66/82), depending on the hazard classification system evaluated when discordant classes are removed.
- Overall accuracy for identification of substances not labeled as irritants (i.e., EPA Category IV, EU 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 and false negative rates ranged from approximately 11% (10/93) to 34% (27/79) and 6% (4/62) to 22% (13/60), respectively, whether or not discordant classes were included in the evaluation. The lowest false negative rate (6% [4/62]) was noted for the GHS system, followed by 14% (11/81) for the EPA system, and 22% (13/60) for the EU system. However, among these false negatives, at least one substance was classified as an ocular corrosive/severe irritant based on Draize 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.

The available validation database for the ICE test method has remained unchanged since the original ICCVAM evaluation (ICCVAM 2006b). Therefore, the original ICCVAM recommendation for the use of the ICE test method to identify substances as ocular corrosives/severe irritants remains unchanged (i.e., that there are sufficient data to

support the use of the ICE test method, in appropriate circumstances and with certain limitations, as a screening test to identify substances as ocular corrosives and severe irritants [i.e., EPA Category I, UN GHS Category 1, EU R41] in a tiered-testing strategy, as part of a weight-of-evidence approach.)

Usefulness and Limitations

The ICE test method has been previously recommended for identification of ocular corrosives and severe irritants (i.e., EPA Category I, EU R41, GHS Category 1) in appropriate circumstances and with certain limitations. Based on an evaluation of available data and corresponding performance (sensitivity and specificity), ICCVAM proposes that the ICE test method not be recommended to identify all categories of ocular hazard classification as defined by the GHS, EPA, and EU classification systems (EPA 2003a; EU 2001; UN 2007). Furthermore, the ICE test method is not recommended as a screening test to identify substances as not labeled as irritants (i.e., EPA Category IV, EU Not Labeled, GHS Not Classified) from all other hazard categories (i.e., EPA Category I, II, or III; EU R41 or R36; GHS Category 1, 2A, or 2B) as defined by the GHS, EPA, and EU classification systems (EPA 2003a; EU 2001; UN 2007).

Test Method Protocol

An ICCVAM recommended test method protocol for the ICE test method is included in the ICCVAM test method evaluation report (ICCVAM 2006d). This same protocol should be used for all future ICE studies with the modification of including decision criteria for all categories of ocular irritation as described in the current ICE BRD. 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 the ICE test method for the identification of all ocular hazard categories.

Proposed Future Studies

To further the use of this test method and to evaluate the use of the ICE test method as a potential replacement for the *in vivo* rabbit eye test method or for the identification of mild and moderate ocular irritants and substances not labeled as irritants (e.g., EPA Category II, III, and IV; GHS Category 2A, 2B, and Not Classified; EU R36 and Not Labeled), ICCVAM recommends additional studies be considered and undertaken.

- Additional optimization studies/evaluations should be conducted in an attempt 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.

- ICCVAM recommends that a histopathological evaluation of the corneal tissue, using standardized procedures, be included when the ICE 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.

2.3 The IRE Test Method

ICCVAM proposes the following draft test method recommendations on the IRE test method. ICCVAM developed the draft recommendations after considering available relevant data and information. This section provides a brief summary of the background and rationale for the draft proposed recommendations, followed by the specific draft recommendations on proposed usefulness and limitations, a proposed test method protocol, and proposed future studies and activities.

Background and Rationale for the Draft Proposed ICCVAM Recommendations

Currently, there is no widely accepted standardized IRE test method for detecting ocular irritants. Evaluation of the IRE test method for its usefulness as a partial or full replacement for the Draize rabbit eye test has been confounded by the lack of a standardized protocol. As an indication of the diversity among IRE protocols used, consider the following list of endpoints evaluated among published IRE studies:

- Commission of the European Communities (CEC 1991): Corneal opacity, corneal swelling, and fluorescein retention (1 and 4 hours)
- Balls et al. (1995): Corneal opacity and corneal swelling (1 and 4 hours)
- Gettings et al. (1996): Mean extent of corneal swelling across time (1 to 4 hours)
- Guerriero et al. (2004): Maximal corneal opacity (opacity x area), maximal corneal swelling, fluorescein penetration (intensity x area) and assessment of epithelial integrity (0.5, 1, 2, 3, and 4 hours)

Although initially developed by Burton et al. (1981) for the assessment of severe eye irritants using a relatively small set of eleven test substances, the IRE test method has been modified for use in the assessment of either selective types of irritants (e.g., severe irritants) or for specific classes of chemical substances or products (e.g., surfactant-containing chemicals, cosmetic and hair care products) (Gettings et al. 1996; Chamberlain et al. 1997; Cooper et al. 2001; Jones et al. 2001). In other studies, protocols were geared to evaluate a wider range of chemical classes over the entire range of irritancy for test method assessment or validation purposes (Price and Andrews 1985; Koeter and Prinsen 1985; CEC 1991; Balls et al. 1995; Gettings et al. 1996) or for interlaboratory trials (Whittle et al. 1992). Guerriero et al. (2004) modified the original IRE test method protocol to refine assessment of pharmaceutical worker safety by using decision criteria designed to identify severe eye irritants using a chemical database of 30 pharmaceutical ingredients, chemical intermediates, and raw materials and an additional

14 reference chemicals from the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC 1998).

The available validation database for the IRE test method has remained unchanged since the original ICCVAM evaluation (ICCVAM 2006e). Therefore, the original ICCVAM recommendation for the use of the IRE test method to identify substances as ocular corrosives/severe irritants remains unchanged (i.e., the use of the IRE test method for screening and identifying ocular corrosives and severe irritants [i.e., EPA Category I, GHS Category 1, EU R41] in a tiered-testing strategy, as part of a weight-of-evidence approach, is not recommended. There also 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 test method accuracy and reliability when all these endpoints are evaluated in a single study.)

Usefulness and Limitations

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 test method accuracy and reliability when all these endpoints are evaluated in a single study. Furthermore, among the studies that included each endpoint, decision criteria are focused on distinguishing ocular corrosives and severe irritants from all other ocular hazard categories (i.e., moderate and mild irritants and substances not labeled as irritants), and do not specify decision criteria for each ocular hazard category. For these reasons, an adequate evaluation of the IRE test method for its ability to identify all ocular hazard categories is not feasible at this time.

Test Method Protocol

An ICCVAM recommended test method protocol for the IRE test method is included in the ICCVAM test method evaluation report (ICCVAM 2006d). This same protocol should be used for all future IRE studies with the modification of including decision criteria for all categories of ocular irritation as described in the current IRE BRD. ICCVAM encourages users to provide all data that are generated from future studies to NICEATM, as they could be used to further characterize the usefulness and limitations of the IRE test method for the identification of all ocular hazard categories.

Proposed Future Studies

To further the use of this test method and to evaluate the use of the IRE test method as a potential replacement for the *in vivo* rabbit eye test method or for the identification of all ocular hazard categories (e.g., EPA Category I-IV; GHS Category 1, 2A, 2B, and Not

Classified; EU R41, R36 and Not Classified), ICCVAM recommends additional studies be considered and undertaken.

- 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.
- ICCVAM recommends that a histopathological evaluation of the corneal tissue, using standardized procedures, be included when the IRE 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.

2.4 The BCOP Test Method

ICCVAM proposes the following draft test method recommendations on the BCOP test method. ICCVAM developed the draft recommendations after considering available relevant data, information, and analyses, which are provided in the draft BRD for this topic (<http://iccvam.niehs.nih.gov/methods/ocutox/mildmod/BCOP-BRD.pdf>). This section provides a brief summary of the background and rationale for the draft proposed recommendations, followed by the specific draft recommendations on proposed usefulness and limitations, a proposed test method protocol, and proposed future studies and activities.

Background and Rationale for the Draft Proposed ICCVAM Recommendations

The test method recommendations described herein are based upon two analyses of BCOP test method performance:

- Overall correct classifications that ranged from 49% (91/187) to 54% (101/186), depending on the hazard classification system evaluated when using the entire database; and 47% (31/66) to 54% (35/65) depending on the hazard classification system evaluated when discordant classes are removed. Using alternative decision criteria for the identification of corrosive/severe ocular irritants (i.e., *in vitro* irritancy score [IVIS] ≥ 75 as the cutoff to define such substances [used in the protocol included in the submission for an *in vitro* testing strategy for ocular hazard classification of antimicrobial cleaning products (AMCPs)] instead of IVIS ≥ 55.1 as the cutoff to define such substances [as per the ICCVAM-recommended BCOP protocol]) does not improve test method performance.
- Overall accuracy for identification of substances not labeled as irritants (i.e., EPA Category IV, EU Not Labeled, GHS Not Classified) from all other categories ranged from 64% (76/118) to 83% (154/186) 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 (6% [8/141] for the EPA system, and 0% [0/54 or 0/97] for the EU and GHS systems, respectively). Among the eight false negatives for the EPA system, 100% (8/8) were EPA Category III substances based on Draize data. For 38% (3/8) of these substances, the categorization was based on at least one test animal with a corneal opacity score of one that was not resolved until day three of the study. Another substance was categorized based on six test animals with a conjunctival redness score of three that was not resolved until day seven of

the study. Considering the severity and number of ocular lesions noted *in vivo*, these false negative results cannot be minimized as they present a significant risk to the user that could be exposed to these materials.

In the original ICCVAM evaluation of the BCOP test method, which was based on 145 substances, overall accuracy, false positive, and false negative rates were 79% (113/143) to 81% (119/147), 19% (20/103) to 21% (22/103), 16% (7/43) to 25% (10/40) depending on the hazard classification system evaluation (i.e., EPA, EU, or GHS). Based on the current BCOP validation database, which has increased to 211 substances, overall accuracy, false positive, and false negative rates are 77% (91/118) to 79% (147/186), 24% (20/85 to 29/123), 15% (10/65) to 21% (7/33). Based on these similar performance statistics, the original ICCVAM recommendation for the use of the BCOP test method to identify substances as ocular corrosives/severe irritants remains unchanged (i.e., that there are sufficient data to support the use of the BCOP test method, in appropriate circumstances and with certain limitations, as a screening test to identify substances as ocular corrosives and severe irritants [i.e., EPA Category I, UN GHS Category 1, EU R41] in a tiered-testing strategy, as part of a weight-of-evidence approach.)

Usefulness and Limitations

The BCOP test method has been previously recommended for identification of ocular corrosives and severe irritants (i.e., EPA Category I, EU R41, GHS Category 1) in appropriate circumstances and with certain limitations. Based on an evaluation of available data and corresponding performance (sensitivity and specificity), ICCVAM proposes that the BCOP test method is not recommended to identify substances from all hazard categories as defined by the GHS, EPA, and EU classification systems (EPA 1996; EU 2001; UN 2007). The BCOP test method can be used as a screening test to identify substances as not labeled as irritants (i.e., EU Not Labeled, GHS Not Classified), from all other hazard categories (i.e., EU R41 or R36; GHS Category 1, 2A, or 2B) 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 were false negative in the BCOP test method (i.e., identified as Category IV), the BCOP cannot be recommended as a screening test to identify EPA Category IV substances.

Test Method Protocol

An ICCVAM-recommended test method protocol for the BCOP test method is included in the ICCVAM test method evaluation report (ICCVAM 2006d). This same protocol should be used for all future BCOP studies, with the modification of including decision criteria for all categories of ocular irritation as described in the current BCOP BRD. ICCVAM encourages users to provide all data that are generated from future studies to

NICEATM, as they could be used to further characterize the usefulness and limitations of the BCOP test method for the identification of all ocular hazard categories

Proposed Future Studies

To further the use of this test method and to evaluate the use of the BCOP test method as a potential replacement for the *in vivo* rabbit eye test method or for the identification of mild and moderate ocular irritants (e.g., EPA Category II and III; GHS Category 2A and 2B; EU R36), ICCVAM recommends additional studies be considered and undertaken.

- Additional optimization studies/evaluations should be conducted in an attempt 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.
- ICCVAM recommends that a histopathological evaluation of the corneal tissue, using standardized procedures, 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.

3.0 Draft Proposed ICCVAM Recommendations: The Low Volume Eye Test

ICCVAM proposes the following draft test method recommendations on the low volume eye test (LVET). ICCVAM developed the draft recommendations after considering available relevant data, information, and analyses, which are provided in the draft BRD for this test method (<http://iccvam.niehs.nih.gov/methods/ocutox/antimicro/LVET-BRD.pdf>). This section provides a brief summary of the background and rationale for the draft proposed recommendations, followed by the specific draft recommendations on proposed usefulness and limitations, standardized test method protocol, and proposed future studies and activities.

Background and Rationale for the Draft Proposed ICCVAM Recommendations

The review of the validity of the LVET was undertaken because LVET data are used to support the validity of one if the *in vitro* test methods proposed in the *in vitro* testing strategy for AMCPs. The accuracy of the LVET was compared to the Draize test and to available human data and experience.

The LVET data, as well as the comparative traditional Draize rabbit data with which to evaluate the accuracy of the LVET, are only available for limited types and numbers of substances (i.e., surfactant-containing personal and household cleaning products). The available comparative LVET and human (clinical studies and accidental exposures) data proposed to support its accuracy are largely with substances that are mild irritants or nonirritating (which also are predominantly surfactant containing cosmetic and personal care product formulations). Ethical considerations have limited the types of substances that can be tested in human clinical studies. As a result, LVET comparisons to human clinical study data are based on tests with mild irritants or substances not labeled as irritants. Such data provide little assurance to the regulatory agencies charged with protecting public health that the LVET can provide adequate protection from substances that may cause moderate or severe ocular injuries in humans.

Accidental exposures are not generally considered to be a reliable source of the true ocular hazard potential since such exposures are likely immediately followed by flushing the eyes with large volumes of water, and may not represent the most severe lesions that might be produced by such an exposure. Such accidental exposures as human reference data do not allow definitive quantitative measures of amount and time of exposure.

Thus while the LVET is proposed as more likely to approximate the volume of a substance that could enter the human eye experimentally, there is limited data to indicate whether it can accurately identify the ocular hazard of substances known to cause

moderate, severe, or permanent human ocular injuries. In contrast, there are no documented instances where a substance with a hazard category determined in the Draize eye test produced a more severe hazard category response in humans following accidental exposures or ethical human studies.

Usefulness and Limitations

A review of available data regarding the usefulness and limitations of the LVET (see ICCVAM BRD available at <http://iccvam.niehs.nih.gov/methods/ocutox/antimicro/LVET-BRD.pdf>) determined that:

- The LVET underpredicts severe irritants compared to the Draize.
- There are insufficient data to evaluate the extent of underprediction relative to known human severe ocular irritants.
- There is an inconsistent relationship between LVET and Draize results (i.e., time-to-clear) for substances with available human data.

Accordingly, ICCVAM proposes that the LVET has not been adequately validated and does not have adequate demonstrated performance (sensitivity and specificity) to serve as an acceptable reference test method against which to determine the validity of *in vitro* alternative test methods for hazard classification and labeling purposes.

Test Method Protocol

Any future validation studies conducted to further evaluate the usefulness and limitations of the LVET should use the LVET protocol as originally developed by Griffith et al. (1980). The LVET differs from the Draize rabbit eye test by applying 10 μ L instead of 100 μ L volume of the test substance, and applying the test substance directly on the cornea instead of in the conjunctival sac. Scoring of corneal, iridal, and conjunctival lesions in the LVET is identical to that of the Draize rabbit eye test (EPA 1998; OECD 2002). In addition, due to the increased potential for pain from administering the test article directly onto the corneal surface, routine pretreatment with topical anesthetics and systemic analgesics is recommended unless there is an adequate scientific rationale for withholding such pretreatments.

Proposed Future Studies

If an organization or sponsor desires to more adequately characterize the usefulness and limitations of the LVET, ICCVAM recommends that a comprehensive set of reference substances be tested and compared to Draize eye test results and human responses, where available. This reference list should be representative of the many types of substances

that are evaluated for their ocular toxicity potential and include substances that are known to cause moderate, severe, and corrosive responses in humans.

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4.0 Draft Recommendations for the Cytosensor Microphysiometer® Test Method: Uses and Limitations

4.1 Use of the Cytosensor Test Method (INVITTOX Protocol Number 102) to Identify Ocular Corrosives and Severe Irritants

The database of 53 water-soluble surfactants tested using INVITTOX Protocol 102 (ECVAM 2008) includes 21 surfactant chemicals and 32 surfactant-containing formulations tested across seven different laboratories. Most of the 32 formulations, which are limited to cosmetic and personal care products, contain one or more surfactants at a final concentration of greater than five percent. There were no pesticide formulations included in the validation database. Using INVITTOX Protocol 102 to identify ocular corrosives and severe irritants among these surfactant-containing substances, the false positive rate ranged from 3-10% (1/29 to 3/29) when 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 Not Classified/Not Labeled based on *in vivo* data. The false negative rate ranged from 9-22% (2/23 to 5/23) when compared to *in vivo* results. In each case, these substances were classified as mild or moderate irritants *in vitro* based on the EPA, EU, and GHS classification systems (i.e., Category II/III, R36, or Category 2A/2B, respectively).

The nonsurfactant substances database for INVITTOX Protocol 102 consisted of 29 water-soluble nonsurfactant chemicals (n=27), which included a range of chemical classes (e.g., acids, alcohols, alkalis, and ketones), and nonsurfactant formulations (n=2) tested in seven laboratories. Using INVITTOX Protocol 102 to identify ocular corrosives and severe irritants among these nonsurfactant substances, the false positive rate ranged from 0-6% (0/18 to 1/18) when compared to *in vivo* results. The one false positive when using the EPA or EU classification systems was Category III and R36 respectively based on *in vivo* data. There were no false positives when using the GHS classification system. The false negative rate ranged from 43-55% (3/7 to 6/11) when compared to *in vivo* results. Three substances were false negatives when using the EPA classification system and were classified *in vitro* as either Category II/III (n=2) 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).

Based on these data and test method performance, ICCVAM proposes that the Cytosensor Microphysiometer® (CM) test method can be used as a screening test to identify water-soluble substances as ocular corrosives and severe irritants (i.e., EPA Category I, GHS Category 1, EU R41) in a tiered-testing strategy, as part of a weight-of-evidence approach. A

substance that tests negative with the CM test method would need to be tested in another test method that is capable of identifying possible *in vitro* false negative severe irritants and ocular corrosives and to distinguish between moderate and mild ocular irritants. Currently, the *in vivo* rabbit eye test is the only test method capable of making such a distinction.

4.2 Use of the Cytosensor Test Method (INVITTOX Protocol Number 102) to Identify Substances Not Labeled as Irritants

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 0-2% (0/27 to 1/46) when compared to *in vivo* results. The one false negative, which occurred only for the EPA classification system, was classified as Category III 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) that cleared after one (n=1) or two days (n=2), and two test animals had corneal opacity (score = 1) that cleared after one day. The false positive rate ranged from 50-69% (3/6 to 18/26) when compared to *in vivo* results. Three substances were false positives when using the EPA classification system and were classified *in vitro* as Category II/III. 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 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 24-38% (5/21 to 8/21) when compared to *in vivo* results. The false positive rate ranged from 25-40% (1/4 to 2/5) when compared to *in vivo* results.

Based on these data, ICCVAM proposes that the CM test method can be used 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 substances not labeled as irritants (i.e., EPA Category IV, GHS Category Not Classified, EU Category Not Labeled) in a tiered-testing strategy, as part of a weight-of-evidence approach. However, based on the false positive rate, a substance that tests positive with the CM test method would need to be tested in another test method that is capable of correctly identifying possible *in vitro* false positives. Positives would also need to be additionally tested with methods that can correctly identify severe, moderate, and mild ocular irritants.

Because of the high false negative rate 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.

4.3 Use of the Cytosensor Test Method (INVITTOX Protocol 102) to Identify Either Ocular Corrosives/Severe Irritants or Substances Not Labeled as Irritants

Given that the CM test method (INVITTOX Protocol 102) is proposed for use as a screening test to identify both ocular corrosive/severe irritants and substances not labeled as irritants, specifically for water-soluble surfactant chemicals and specific types of surfactant-containing formulations (e.g., cosmetics and personal care product formulations, but not pesticide formulations), users may want to consider using the CM test method prior to another *in vitro* ocular test method for testing these types of substances. However, water-soluble surfactant chemicals and surfactant formulations that are not identified as ocular corrosive/severe irritants or as substances not labeled as irritants with the CM test method would need to be tested in another test method(s) capable of correctly classifying substances into each of the four hazard classification categories for EPA or GHS. Currently, the only test method accepted for these purposes is the *in vivo* Draize test. Because of the high false positive rate (> 50%) for the substances not labeled as irritants decision criteria, users may not want to use the CM test method if the intended use is to start with identifying substances not labeled as irritants.

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5.0 ***In Vitro* Testing Strategies for Ocular Hazard Classification of Antimicrobial Cleaning Products**

ICCVAM proposes the following draft test method recommendations on *in vitro* testing strategies for ocular hazard classification of antimicrobial cleaning products. ICCVAM developed the draft recommendations after considering available relevant data, information, and analyses, which are provided in the draft Summary Review Document (SRD) for this topic (<http://iccvam.niehs.nih.gov/methods/ocutox/antimicro/BRD.pdf>). This section provides a brief summary of the background and rationale for the draft proposed recommendations, followed by the specific draft recommendations on proposed usefulness and limitations, proposed test method protocols, and proposed future studies and activities.

Background and Rationale for the Draft Proposed ICCVAM Recommendations

The AMCP BRD included data for 228 substances tested in one or two of the three *in vitro* test methods proposed for use in the testing strategy. However, none of the substances had been tested in all three *in vitro* test methods. Therefore, there are no data available for the proposed substances with which to characterize the actual performance of a testing strategy that includes the BCOP, CM, and EpiOcular™ (EO) test methods. Of the 228 substances, 28 are EPA registered antimicrobial cleaning products, with eight additional materials being in-use dilutions of EPA registered antimicrobial concentrates.

In addition, the test method protocol used to generate the *in vivo* reference data varied among the 228 substances included in the validation database. Most of the substances tested with the BCOP test method (85% [58/68]) were tested in the traditional Draize rabbit eye test protocol (i.e., EPA 1998; OECD 2002). Approximately half (54% [29/54]) of the substances tested with the EO test method were tested in the Draize rabbit eye test, while the remaining substances (46% [25/54]) were tested in the LVET. All 105 of the substances tested with the CM test method were tested in the LVET. The 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. As noted in **Section 3.0**, the draft OTWG position is that the LVET predictivity for the Draize test and the lack of LVET data for substances that are known to cause moderate and severe irritation and ocular corrosion makes it inadequate to serve as a reference test method to support the validity of *in vitro* test methods. For this reason, the CM data and some EO data for which only LVET reference data exists were not considered adequate to support the proposed testing strategy.

However, additional data on 53 surfactant and surfactant-containing formulations were provided in a BRD prepared by ECVAM where there was data from the traditional Draize rabbit test available to assess the accuracy of the CM test method. These substances were not claimed as AMCPs, but they were surfactant-containing formulations with similar composition to many AMCPs. The database of 53 water-soluble surfactants tested in CM includes 21 surfactant chemicals and 32 surfactant-containing formulations tested across seven different laboratories. Based on the performance of CM using these 53 substances, ICCVAM has proposed¹⁸ that the CM test method can be used 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 EPA Category I, GHS Category 1, or EU Category R41; or as EPA Category IV, GHS Not Labeled, EU Not Classified, in a tiered-testing strategy, as part of a weight-of-evidence approach. A substance that is not classified into one of these two categories would need to be tested in another test method that is capable of correctly identifying possible *in vitro* false positives. Positives would also need to be additionally tested with methods that can correctly identify severe, moderate, and mild ocular irritants. Analyses performed to identify the ocular hazard potential of these non-AMCP test substances based on Draize reference data suggest that the CM test method could be useful in a testing strategy.

An alternative testing strategy, which would include only the BCOP and EO test methods, was also evaluated using two approaches: 1) test with the BCOP test method first and then with the EO test method, or 2) test with the EO test method first and then with the BCOP test method. For the first approach, the BCOP test method was evaluated for its ability to identify substances as either EPA Category I or II. All substances that were classified as Category I or II with the BCOP test method (n=15) were removed from the database and the remaining 13 substances were evaluated based on EO test method results 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 identify substances as either Category III or IV and all substances that were classified as Category III or IV with the EO test method (n=13) were removed from the database and the remaining 15 substances were evaluated based on BCOP test method results for identifying Category I or II substances. Regardless of which approach was used, the performance of the proposed BCOP/EO testing strategy was the same. The BCOP/EO testing strategy correctly classifies 79% (22/28) of the substances, which includes identifying 100%

¹⁸ This evaluation is currently undergoing separate peer review by an ECVAM Scientific Advisory Committee Peer Review Panel, which includes two members of the ICCVAM Ocular Peer Review Panel (Drs. Hayes and Wilson).

(14/14) of the Category I substances, 100% (4/4) of the Category III substances, and 44% (4/9) of the Category IV substances. The one Category II substance in the database was underclassified as a Category III. None of the irritant categories (i.e., Category I, II, or III) were underclassified as Category IV substances.

Usefulness and Limitations

Given the limitations of the available database for three *in vitro* test methods (the CM, EO, and the bovine corneal opacity and permeability BCOP test methods), there are currently insufficient data with which to adequately demonstrate that an *in vitro* testing strategy using the BCOP, CM, and EO can identify all four required EPA hazard categories for ocular irritation/corrosion.

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

Therefore, definitive recommendations on the usefulness and limitations of an *in vitro* testing strategy cannot be made at this time.

Test Method Protocols

The detailed test method protocols appended to the AMCP BRD submission use a variety of endpoints to predict ocular irritation potential. While they have not been demonstrated to be adequately validated for use in a testing strategy for AMCPs, 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-IV). 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.

Proposed Future Studies

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

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.

Appendix B
Peer Review Panel Member Biosketches

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Panel Member Biosketches

Hongshik Ahn, Ph.D.

Dr. Ahn received a Ph.D. in statistics with a minor in computer sciences from the University of Wisconsin–Madison. He is a Professor in the Department of Applied Mathematics and Statistics at Stony Brook University in New York. He has been a Visiting Scientist at the National Center for Toxicological Research at the U.S. Food and Drug Administration (FDA) since 1997 and a Senior Biostatistician for the General Clinical Research Center at Stony Brook since 2005. His research interests include tree-structured regression and classification, survival analysis, bioinformatics, generalized linear model, animal carcinogenicity studies, toxicology, and risk assessment. Dr. Ahn is Associate Editor for *Communications in Statistics* and a member of the International Biometric Society (Eastern North American Region) and the American Statistical Association. He is a referee for 18 statistical journals including *Journal of the American Statistical Association*, *Biometrics*, *Statistics in Medicine*, and *Risk Assessment*. In 2005, Dr. Ahn participated in the National Institutes of Health (NIH) Biostatistical Methods and Research Design Study Section. He has published three book chapters, 48 peer-reviewed publications, 21 proceedings, and has received 11 special invitations to serve as conference session chair or invited speaker.

Paul T. Bailey, Ph.D.

Dr. Bailey received his Ph.D. in psychopharmacology from Howard University. He is currently a consultant for Bailey & Associates Consulting in Neshanic Station, New Jersey. Dr. Bailey also has served as a toxicology consultant with expertise in clinical research; quality assurance (Good Laboratory Practice [GLP] and Good Clinical Practice); chemical exposure and health hazard and/or risk assessment; product liability; technical expertise; regulatory toxicology related to chemicals, petroleum products, cosmetics, personal health care, medical device, and household product industries; strategic planning and management of product safety evaluation and toxicological research programs that are needed to meet industry and regulatory requirements. Dr. Bailey is a former Senior Research Associate at Mobil Oil Corporation with expertise in the development and use of *in vitro* methods to assess the potential eye and skin irritation or sensitization potential of petroleum products and in the validation of alternative methods. At Proctor & Gamble, he was a Divisional Toxicologist (Group Leader) and supervised the dermal toxicology laboratory that focused on development of protocols and in-house or contract laboratory testing to assess the toxicology of potential personal care products. Dr. Bailey has served on numerous government scientific advisory panels (Federal Insecticide, Fungicide and Rodenticide Act [FIFRA]), National Institute of Environmental Health Sciences, National Toxicology Program

[NTP]) and trade organizations (e.g., The Cosmetic, Toiletry and Fragrance Association, Chemical Manufacturer's Association). He was a member of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Immunotoxicology Working Group and served on the Editorial Board of the *Journal of the Dermal Clinical Evaluation Society*. Dr. Bailey has contributed to 45 publications or meeting abstracts.

Richard Dubielzig, D.V.M.

Dr. Dubielzig received his Ph.D. from the University of Minnesota. Dr. Dubielzig is currently Professor of Pathology in the School of Veterinary Medicine at the University of Wisconsin–Madison. His primary research interests are comparative dental pathology and comparative ophthalmic pathology. Dr. Dubielzig is an honorary Diplomate of the American College of Veterinary Ophthalmologists (ACVO). He has trained over 40 postdoctoral residency or clinical instructor candidates in pathology or ophthalmology. Dr. Dubielzig is a member of the Central Committee of the Comparative Ophthalmic Research Laboratories, a collaborative research team that provides clinical, pathology, and basic science support to industry in the development of ocular compounds and evaluation of ocular toxicity. Dr. Dubielzig is a member of numerous professional and scientific organizations including the American Veterinary Medical Association, the American College of Veterinary Pathologists, the Association for Research in Vision and Ophthalmology, the Society of Toxicologic Pathologists, the International Society of Ocular Toxicology, and the International Society of Veterinary Ophthalmology. Dr. Dubielzig has authored or coauthored over 198 articles in peer-reviewed journals, 17 book chapters, and 259 abstracts. He has been invited to give 119 lectures.

Henry F. Edelhauser, Ph.D.

Dr. Edelhauser obtained his Ph.D. in physiology from Michigan State University. He is a Professor of Ophthalmology, Director of Ophthalmic Research, and Adjunct Professor of Biology at Emory University. Dr. Edelhauser is the Program Director of the National Eye Institute Research Training Grant “Multidisciplinary Training in Vision Research” at Emory University. His major research interests include physiological mechanisms of corneal transparency; role of sulfhydryls on corneal endothelial function; corneal permeability and cellular toxicity of intraocular irrigating solutions, drugs, and enzymes; the physiological effects of vitrectomy on ocular tissues; dynamics of intraocular fluids, ocular toxicology, corneal extracellular matrix, corneal endothelial physiology; corneal effects of eicosanoids and other lipid mediators; sclera permeability; and cellular mechanisms of ocular inflammation. He has served as chair of the Cornea Section of the Association for Research in Vision and Ophthalmology; chaired or participated in several National Eye Institute or

other NIH Study Sections, workshops, and Special Emphasis Panels; and serves on various editorial boards for eye research journals. Dr. Edelhauser has authored or coauthored 292 publications in peer-reviewed journals, contributed to 51 books or book chapters and four audiotapes, and given 29 lectures or invited talks as a visiting professor. In 2005, Dr. Edelhauser was an active participant on the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM Expert Panel to review the current validation status of four *in vitro* test methods for identifying ocular corrosives and severe irritants.

Mark Evans, D.V.M., Ph.D., DACVP

Dr. Evans received his D.V.M. and Ph.D. degrees from Michigan State University. He is the Pathology Lead for Ophthalmology Therapeutic Area in Drug Safety Research and Development at Pfizer Global Research and Development in La Jolla, California. Dr. Evans is on the Adjunct Clinical Faculty in the Department of Pathology, College of Veterinary Medicine at Michigan State University and serves as the point of contact for the Michigan State University/Pfizer cosponsored residency program. He is chair of the Corporate Partners Subcommittee of the American College of Veterinary Pathologists. He has 27 journal publications and 38 abstracts. He is a Diplomate of the American College of Veterinary Pathologists, the Society of Toxicologic Pathologists, the United States and Canadian Academy of Pathology, and the American Veterinary Medical Association.

A. Wallace Hayes, Ph.D., DABT, FATS, ERT

Dr. Hayes received his Ph.D. in biochemistry from Auburn University. He is a Principal Advisor for Spherix Incorporated in Bethesda, Maryland. Dr. Hayes is also a Research Professor in the Department of Pharmacology and Toxicology at the Medical College of Virginia in Richmond and an Adjunct Professor in the School of Veterinary Medicine at the Virginia Polytechnic Institute in Blacksburg, Virginia; the Department of Physiology and Pharmacology at Wake Forest University in Winston-Salem, North Carolina; and the Department of Pharmacology and Toxicology at the University of Louisville School of Medicine. Dr. Hayes is a Diplomate of the American Board of Toxicology, a registered regulatory toxicologist (ERT) for EUROTOX, and a Fellow of the American Toxicological Society in addition to being a member of a number of professional specialty boards. He holds a variety of editorial posts for journals including *Cutaneous and Ocular Toxicology*, *Toxicology and Applied Pharmacology*, *Regulatory Toxicology and Pharmacology*, and *Food and Chemical Toxicology*. Dr. Hayes has served on many advisory and expert panels for U.S. and international regulatory interests, including NICEATM-ICCVAM, and for risk assessment, health and safety, or toxicological interests. He has served on various task groups

and scientific advisory boards. He is a reviewer for 28 journals. He is a course director for Principles of Toxicology at the Harvard School of Public Health. Dr. Hayes has authored or coauthored 200 publications in peer-reviewed journals, 11 books, 73 invited presentations, nearly 100 invited seminars, and 152 abstracts presented at scientific meetings. Dr. Hayes is a member of numerous professional societies including the Society of Toxicology, the International Society of Regulatory Toxicology and Pharmacology, the American Society of Pharmacology and Experimental Therapeutics, the American College of Toxicology, and the American Society of Quality Control.

James V. Jester, Ph.D.

Dr. Jester received his Ph.D. in the Department of Pathology at the University of Southern California Medical Center in Los Angeles. Dr. Jester is a Professor of Ophthalmology and Biomedical Engineering at the University of California, Irvine, where he is the Jack H. Skirball Endowed Chair. Dr. Jester is a recognized international leader in the cell biology of corneal wound healing, a research field on which he has had a major impact. Dr. Jester is a member of numerous review boards for ocular pathology and eye irritation. He is an *ad hoc* reviewer for the National Eye Institute (NEI) VISA 1 (Vision Sciences A) and Small Business Innovation Research Study Sections and a reviewer on the Anterior Eye Disease Study Panel of the NEI. He has participated in numerous ocular workshops and symposia including the ICCVAM Ocular Symposia on Ocular Mechanisms held at the NIH in Bethesda, Maryland, in 2005 and the European Cosmetic, Toiletry and Perfumery Association workshop on Eye Irritation Alternatives held in Brussels in 2008. Dr. Jester participates on the editorial boards of eight ocular journals including *Investigative Ophthalmology & Visual Science*, *Experimental Eye Research*, *Cutaneous and Ocular Toxicology*, *Cornea*, and *Current Eye Research*. He also serves on various program-planning committees for ocular research and biology. Dr. Jester is a member of the American Association for the Advancement of Science, the New York Academy of Science, the Association for Research in Vision and Ophthalmology, the American Society for Cell Biology, the International Congress on Eye Research, and the International Society for Ocular Cell Biology. Dr. Jester has published 202 peer-reviewed manuscripts, 14 nonrefereed publications, 223 abstracts, and 45 invited presentations.

Tadashi Kosaka, D.V.M., Ph.D.

Dr. Kosaka received his D.V.M. and Ph.D. degrees from the School of Veterinary Medicine at the Nippon Veterinary and Animal Science University. He is Associate Director and Chief of the Laboratory of Immunotoxicology and Acute Toxicology in the Toxicology Division in The Institute of Environmental Toxicology in Ibaraki, Japan. His research, which covers the

areas of immunotoxicology and acute toxicology, is represented in 24 publications in peer-reviewed journals. Dr. Kosaka is a member of the Japanese Association for Laboratory Animal Science, the Japanese Society of Toxicology, the Japanese Society of Immunotoxicology, and the Japanese Society of Alternatives to Animal Experiments.

Alison McLaughlin, MSc., DABT

Ms. McLaughlin received her Master's Degree in biology from Queen's University in Kingston, Ontario, Canada. A Diplomate of the American Board of Toxicology (2004), Ms. McLaughlin is a Senior Science Policy Analyst for the Environmental Impact Initiative in the Office of Science and Risk Management, Health Products and Food Branch of Health Canada in Ontario. Ms. McLaughlin was formerly a Toxicologist/Senior Evaluator and Acting Section Head in the New Substance Assessment and Control Bureau on Notifications for Food and Drug Products. In this capacity, she developed experience and interest in alternative test methods such as the hen's egg test – chorioallantoic membrane and the bovine corneal opacity and permeability test methods. Ms. McLaughlin served as an editor for the Parliament of Canada on the House of Commons Standing Committee on Environment and Sustainable Development to produce a year 2000 report on pesticides that included information on human health impacts, environmental impacts, and contaminants in the traditional diet of northern communities. Ms. McLaughlin has 17 publications, including results of several Canadian government-sponsored environmental impact studies.

J. Lynn Palmer, Ph.D.

Dr. Palmer received her Ph.D. in biometrics from the University of Texas Health Science Center, Houston. Dr. Palmer has a joint appointment as Associate Professor (Tenured) in the Department of Palliative Care and Rehabilitation Medicine–Research, Division of Cancer Medicine and Associate Professor of Biostatistics in the Department of Biostatistics and Applied Mathematics at the University of Texas M.D. Anderson Cancer Center. Dr. Palmer is a member of numerous professional and scientific organizations. These include the American Statistical Association, of which she served as a chair, a member of numerous committees, and as president of the local Houston chapter. She is also a member of the International Biometrics Society, the Royal Statistical Society, the International Society for Bayesian Analysis, the International Association of Hospice & Palliative Care, and the American Society of Clinical Oncology. Dr. Palmer has authored or coauthored 139 articles in peer-reviewed journals, plus seven additional publications (reviews, letters to editors, etc.) and five book chapters. Dr. Palmer has organized or chaired nine symposia or conferences and presented at 38 national and international scientific conferences.

Robert L. Peiffer, Jr., D.V.M., Ph.D., DACVO

Dr. Peiffer received a D.V.M. degree and a Ph.D. in comparative ophthalmology from the University of Minnesota, St. Paul. He is a Senior Investigator at the Merck Research Laboratories, Adjunct Professor of Ophthalmology at the Scheie Eye Institute at the University of Pennsylvania, Emeritus Professor of Ophthalmology and Pathology at the University of North Carolina in Chapel Hill, and Director of Bucks County Animal Ophthalmology. He has been a consultant in ophthalmology and comparative ophthalmic toxicology for several major pharmaceutical and eye care companies, medical schools, and zoological parks and animal preserves. Dr. Peiffer is on the review boards of 16 journals and is a contributing editor for several others. He has served on several committees for the National Academy of Sciences, the National Institutes of Health, a FIFRA Scientific Advisory Panel, and an ICCVAM Expert Panel (2005). Dr. Peiffer has published 152 articles in refereed journals, with three more in submission; 70 articles in nonrefereed journals; 9 book reviews; nearly 160 papers and presentations at scientific meetings; and numerous visiting professorships and lectureships in the U.S. and around the world. Dr. Peiffer is a member of the American Academy of Ophthalmology, the American Society of Veterinary Ophthalmology, the International Society of Ophthalmology, the International Society of Ocular Toxicology, and the International Society of Ophthalmic Pathology, among others.

Denise Rodeheaver, Ph.D., DABT

Dr. Rodeheaver received her Ph.D. in toxicology from the University of Georgia. She is currently Director of the Toxicology Department at Alcon Research, Ltd., in Fort Worth, Texas. Dr. Rodeheaver is responsible for the qualitative and quantitative achievements of Consumer Products Toxicology and *In Vitro* Toxicology, and oversight of Toxicology Compliance. Dr. Rodeheaver has experience in acute, subchronic, and chronic toxicity evaluations (e.g., ocular and systemic toxicity, genotoxicity, sensitization) conducted in-house or at contract research organizations. She is Diplomate of the American Board of Toxicology, a member of the Society of Toxicology, and Sigma Xi. Dr. Rodeheaver is currently a board member for the International Society of Ocular Toxicology. Dr. Rodeheaver has 13 publications in peer-reviewed journals, 13 abstracts or posters presented at scientific meetings, and 18 presentations at scientific meetings including the International Society of Ocular Toxicology Congress and the Association for Research in Vision and Ophthalmology annual meeting.

Donald C. Sawyer, D.V.M., Ph.D., DACVA, HDABVP

Dr. Sawyer received a Doctorate in Veterinary Medicine from Michigan State University and a Ph.D. in anesthesia and surgery at the Surgery Laboratory Advanced Degree Program at

Colorado State University. He is a member of the Scientific Advisory Board and a Manager of Veterinary Development for Minrad International. Dr. Sawyer was a Captain in the U.S. Air Force serving as a support surgeon at the School of Aerospace Medicine. Dr. Sawyer is Professor Emeritus in the College of Veterinary Medicine at Michigan State University. He served on the faculty of Michigan State University as Professor of Anesthesia, Coordinator of Lifelong Education and Alumni Affairs, and researcher on anesthesiology and pain assessment in cats and dogs. He is a founding member of the American College of Veterinary Anesthesiologists and cofounder of the American Board of Veterinary Practitioners. Dr. Sawyer is a council member and Secretary/Treasurer of the World Congress of Veterinary Anaesthesiology. He has been elected to two six year terms as a member of the American Veterinary Medical Association Council on Biologic and Therapeutic Agents and served as chair for 3 years. Dr. Sawyer has published nine books/monographs, two textbooks, 22 chapters, 68 scientific articles, and 94 abstracts/proceedings. He has had 210 invited papers and presentations.

Kirk Tarlo, Ph.D., DABT

Dr. Tarlo received a Ph.D. from the Rackham Graduate School at the University of Michigan. He is Scientific Director, Comparative Biology and Safety Sciences, at Amgen, Inc., in Thousand Oaks, California. Dr. Tarlo is former Scientific Director, Toxicology, at Allergan, Inc., in Irvine, California. His research interests include toxicology, *in vitro* cytotoxicity, safety evaluation, genetic toxicology, and regulatory issues relating to investigational new drugs and new drug applications. Dr. Tarlo has 11 publications in refereed journals and has given 18 presentations at professional/scientific meetings. He is a Diplomate of the American Board of Toxicology and a member of the Environmental Mutagen Society, the Society of Toxicology, and the Southern California Society of Toxicology.

Daryl Thake, D.V.M., DACVP

Dr. Thake received a D.V.M. from Iowa State University in Ames, Iowa. He is board certified by the American College of Veterinary Pathologists. Dr. Thake is the president and owner of Midwest ToxPath Science, Inc., and was a principal and co-owner of Seventh Wave Pathology and Biotechnical Solutions in Chesterfield, Missouri. Dr. Thake held numerous leadership roles in toxicology and pathology at Pharmacia and its legacy companies, Searle and Monsanto. He was a Senior Science Fellow and Global Head of Pathology Sciences at Pharmacia Corporation in St. Louis, Missouri, where he was responsible for the in-house and CRO pathology functions across five sites in the U.S. and Europe. As the Head of Carcinogenicity Assessment, Global Pathology Sciences, Dr. Thake developed experience in

pathology laboratory techniques including immunohistochemistry, *in situ* hybridization, laser capture microscopy, and imaging. As a consulting pathologist, his work involves gross and microscopic pathology evaluation of preclinical toxicology studies in support of drug discovery and development. He is also involved in the design and conduct of studies for management of toxicology issues in response to regulatory agency concerns with target products. He has been particularly involved in peer reviews to identify and resolve pathology issues and/or problems. Dr. Thake is a member of the Society of Toxicologic Pathologists, American College of Veterinary Pathologists, and the American Veterinary Medical Association. He serves on the editorial board of the *American Journal of Veterinary Pathology*. He is past chairman of the Scientific and Regulatory Policy Committee, Society of Toxicologic Pathologists, and past chairman and current member of the Government Policy Committee of the American College of Veterinary Pathologists. Dr. Thake has 23 publications in peer-reviewed journals.

Scheffer Chuei-Goong Tseng, M.D., Ph.D.

Dr. Tseng received his M.D. degree from the National Taiwan University Medical School and his Ph.D. degree in experimental pathology from the Department of Pathology, University of California, San Francisco, Medical Center. He was board certified by the American Board of Ophthalmology. Dr. Tseng is Director of the Ocular Surface Center; Research Director of the Ocular Surface Research & Education Foundation; Medical Director and Consultant for Bio-Tissue, Inc.; Director of Research and Development of TissueTech, Inc.; and a Board Director for MedNet, Inc. He is an adjunct investigator in the Division of Medical Engineering at the National Health Research Institute in Taiwan and has served on various NIH committees as an *ad hoc* member. His research interests include ocular surface biochemistry and biology, reconstruction and surgical procedures for limbal epithelial stem cell transplantation for total limbal deficiency. Dr. Tseng has published 30 books, 193 peer-reviewed journal manuscripts, and a large body of other works, publications, abstracts, and presentations. Dr. Tseng also has six invention disclosures and holds 12 U.S. or Taiwanese patents or provisional patents. He serves as a reviewer for 28 journals including *Ophthalmology*, *American Journal of Ophthalmology*, *The Lancet*, *New England Journal of Medicine*, *Journal of Refractive Surgery*, and *Gene*. He serves on the editorial board of six journals including *Ocular Surface*, *Cornea*, and *Investigative Ophthalmology Visual Sciences*. Dr. Tseng is a member of 19 professional societies including the American Medical Association, Association for Research in Vision and Ophthalmology, and the American Academy of Ophthalmology.

Jan van der Valk, Ph.D.

Dr. van der Valk received a Ph.D. from the Australian National University in Canberra. He is a Senior Scientist at the Netherlands Centre for Alternatives to Animal Use in the Department for Animals, Science & Society of the Faculty of Veterinary Medicine at Utrecht University. Dr. van der Valk is the Dutch representative on the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC). He has served on several other committees involved in evaluation and review of alternative toxicological methods including the ESAC Shadow Review Panel (chair) of the Joint ICCVAM/ECVAM validation study on organotypic assays, INVITTOX (2004, 2006), the Congress on Alternatives held at the University of Linz, Austria (2006, 2008), and the European Society of Toxicology In Vitro (ESTIV; 2008). Dr. van der Valk also serves as Secretary of ESTIV and of INVITROM (Dutch-Belgian Society for *In Vitro* Methods). Dr. van der Valk was a board member of ecopa (European consensus-platform for alternatives).

Phillipe A. Vanparrys, Ph.D

Dr. Vanparrys received his Ph.D. with Greatest Distinction from the Catholic University of Louvain in Belgium. He is the Managing Director of the Centre for Advanced Research & Development on Alternative Methods (CARDAM) in Mol, Belgium. He was formerly a Senior Research Fellow and Head of Genetic and *In Vitro* Toxicology at Johnson and Johnson Pharmaceutical Research & Development (J&J) in Beerse, Belgium. Dr. Vanparrys was a representative for J&J (Beerse) on the J&J Research & Development Committee for *In Vitro* Alternatives. He was also an Industrial Representative in the Belgian Platform for Alternative Methods and serves as a representative for the pharmaceutical industry in the Structure Working Group and Technical Working Group of the Foundation for Alternatives to Animal Testing. Dr. Vanparrys also serves as a nominated test method expert on the Genotoxicity/Mutagenicity and the Eye Irritation subgroups for ECVAM to establish timetables for phasing out animal testing as required by the 7th Amendment to the Cosmetics Directive (2003/15/EC). Dr. Vanparrys serves as Chairman of the Expert Group on Cell Transformation testing and as a member of the Expert group on *in vitro* micronucleus testing and the Carcinogenicity Taskforce at ECVAM. He is the Belgian representative in the Organisation for Economic Co-operation and Development Task Force on the application of GLP principles to *in vivo* studies. He also served on an ICCVAM Expert Panel for Ocular Corrosives. Dr. Vanparrys holds numerous professional memberships including the European and Belgian Environmental Mutagen Societies, member of and auditor for the Belgian and European Toxicology Societies, the European Society of Toxicology In Vitro, the Environmental Mutagen Society, and the *In Vitro* Testing Industrial Platform. Dr. Vanparrys has 44 publications, with three in preparation, and three international reports. He has also

contributed to several hundred confidential internal reports, reviews, and expert reports for Janssen Research Foundation and J&J Research and Development.

Maria Pilar Vinardell, Ph.D.

Dr. Vinardell is currently Director of the Department of Physiology and Professor of Physiology and Physiopathology in the Faculty of Pharmacy at the University of Barcelona. Dr. Vinardell teaches *in vitro* toxicology courses in various Latin American countries including Argentina, Cuba, Chile, and Brazil. A registered toxicologist (Spain and EUROTOX), Dr. Vinardell is responsible for the research group “Interaction of surfactants and cell membranes.” She was responsible for and has conducted more than 500 *in vitro* and *in vivo* studies on preclinical toxicology for cosmetic, pharmaceutical, veterinary, and chemical industries since 1978. These studies include skin and eye irritation, acute toxicity, subacute toxicity, subchronic toxicity, sensitization, pyrogens, intramuscular irritation, assessment of analgesic and anti-inflammatory activities, histology, and interleukin determinations. Dr. Vinardell has experience in writing standard operating procedures for risk assessment. She is actively involved in research in alternatives to eye and skin irritation and to the rabbit pyrogen test. She has collaborated with and provided draft scientific reports to ECVAM and other research centers. Dr. Vinardell is a peer reviewer for 17 journals and has provided public comment and submitted material on several ICCVAM-related activities. She has given over 100 presentations or invited lectures at national and international congresses. Dr. Vinardell has 90 publications in peer-reviewed journals, 12 review articles, 6 book or educational publications, and 12 books by invitation.

Sherry Ward, Ph.D., MBA

Dr. Ward received her Ph.D. in biochemistry from Michigan State University, an MBA from the University of Maryland University College (UMUC), and an executive M.S. in Technology Management from UMUC. She currently consults for BioTred Solutions in New Market, Maryland. Dr. Ward has expertise in *in vitro* toxicology, scientific/technical/business writing and communication, research and project management, grant proposal review, and grant writing. She also has experience in market research, commercialization, and strategy development and is a contributing editor to AltTox. Dr. Ward is an adjunct faculty member in Biotechnology & Project Management at UMUC. She has animal welfare experience. As a Staff Scientist and *In Vitro* Toxicology Laboratory Manager at the Gillette Company, she developed, characterized, and drafted patent applications for the first human conjunctival epithelial cell lines and gained experience in bioassay development and validation. Dr. Ward has served on numerous scientific panels and committees and was a panel member and presenter at the ICCVAM symposia on mechanisms of ocular injury and recovery and minimizing pain and distress in ocular toxicity testing held at NIH in 2005. She has been

actively involved with trade organizations and served on the European Cosmetic, Toiletry and Perfumery Association Eye Irritation Task Force and the International Life Sciences Institute--Health and Environmental Sciences Institute Alternatives to Animals Task Force. Dr. Ward's experience in models of eye irritation and mechanisms of injury is reflected in 19 publications in peer-reviewed journals, four unpublished validation or prevalidation documents related to ICCVAM activities, 17 presentations, 28 abstracts, and a patent. She is a member of the Hopkins Medical and Surgical Association and the Washington Academy of Sciences.

Daniel M. Wilson, Ph.D., DABT

Dr. Wilson received his Ph.D. in biochemistry/toxicology from Michigan State University. He is currently a Mammalian Toxicology Consultant in Toxicology for Environmental Research and Consulting at the Dow Chemical Company in Midland, Michigan. Dr. Wilson is a board-certified toxicologist with expertise in mammalian toxicology, genetic toxicology, genetic polymorphisms, *in vitro* alternatives, biochemistry, nutritional biochemistry, FDA-regulated food-contact toxicology, and medical device toxicology. He has technical experience in risk assessment for Dow operations and products, for risks associated with intermediates used for contract pharmaceutical formulations, and for characterization of health risks to workers and consumers. Dr. Wilson also has responsibility for the identification and facilitation of testing for particular products and assesses data requirements for setting appropriate occupational exposure and manufacturing limits. Dr. Wilson provides expert business assistance in the area of environmental health and safety to Dow businesses, toxicological review of the chemistry and products within the business, and international registration activity. He participates in trade associations relevant to business activities and is an active member of the Animal Welfare Opportunity Team. Dr. Wilson has published 18 articles in peer-reviewed journals, 1 book chapter, and 30 abstracts. He is a Diplomate of the American Board of Toxicology. Dr. Wilson is a member of the Society of Toxicology and past president and Secretary of the Midwest Regional Chapter. He was a member of the 2006 NICEATM-ICCVAM Expert Review Panel for Alternatives to Acute Toxicity Testing and served on several animal welfare, ISO standardization, biosafety, and radiation safety committees.

Fu-Shin Yu, Ph.D.

Dr. Yu received his Ph.D. from Wayne State University. Dr. Yu is currently Professor and Director of Research at the Kreske Eye Institute in the Department of Ophthalmology, Department of Anatomy and Cell Biology at the Wayne State University School of Medicine. He was an Associate Professor at the Schepens Eye Institute at Harvard University. Dr. Yu is

a member of the Association for Research in Vision and Ophthalmology. He serves as a reviewer for four ocular research journals and for 10 other journals or organizations (e.g., the Wellcome Trust). Dr. Yu currently receives funding for studies on the molecular regulation of corneal wound healing, modulation of epithelial barrier function during corneal infection, and mechanisms of flagellin-induced protection against bacterial keratitis. Dr. Yu has published 59 articles in peer-reviewed journals and three book chapters or review articles; he was an invited speaker or presenter at 17 seminars or ocular research meetings. A participant on state and local boards and committees, Dr. Yu is also an editorial board member of the *Journal of Toxicology–Cutaneous and Ocular Toxicology* and a member of the National Scientific Advisory Council and the American Federation for Aging Research.

Appendix C
Questions for the Peer Review Panel

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Appendix C1
General Instructions for the Peer Review Panel

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The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in conjunction with National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) is evaluating the validation status of several *in vitro* ocular toxicity test methods and testing strategies, along with modifications to the Draize rabbit eye test intended to minimize pain and distress. ICCVAM and NICEATM have developed draft Background Review Documents (BRDs) that provide a comprehensive compilation of all available relevant data, information, and analyses for each of these topics. Based on the information in the draft BRDs, ICCVAM has developed draft proposed recommendations on the potential usefulness, limitations, standardized protocols, and future studies for each test method or topic. The next step in the ICCVAM evaluation process is the independent scientific peer review of these documents, including consideration of the extent that the draft ICCVAM recommendations are supported by the information in the draft BRDs.

For each test method or topic, the Panel is asked to review and address three different aspects. ICCVAM has developed a series of questions for each of these three aspects to assist in your review. You are first asked to review the information in the draft ICCVAM BRDs for completeness, and to identify any errors or omissions of existing relevant data or information that should be included. If you are aware of any related publications or studies for which the data are publicly available, please let NICEATM know as soon as possible so that this information can be obtained and provided to the entire panel for their consideration and to the public for comment.

You are next asked to evaluate the information in the draft BRDs to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM 2003) have been appropriately addressed for each proposed test method, testing strategy, and test method protocol modification. Adequate validation¹⁹ is a prerequisite for a test method to be considered for use in regulatory decision-making by U.S. Federal agencies. The validation process characterizes the usefulness and limitations of a test method for a specific intended use. The extent and nature of the data necessary to support a specific proposed use for a test method will vary, depending on the purpose of the proposed test method. The acceptability of performance in terms of sensitivity, specificity, and reliability should be considered in the context of the intended purpose of the test method and the established validation and acceptance criteria. The overall criteria for acceptance is that the specified use of the proposed test method or approach will provide for equivalent or

¹⁹ Validation is the process by which the reliability and accuracy of a test method are established for a specific purpose (ICCVAM 2003).

better protection than the test method or approach for which it is proposed to substitute or replace.

Lastly, you are asked to consider the ICCVAM draft recommendations proposed for each topic and comment on whether the recommendations are supported by the information provided in the draft BRD. Draft recommendations are provided for one or more of the following for each topic that will be reviewed: 1) proposed test method usefulness and limitations; 2) proposed standardized protocols; 3) proposed test method performance standards; and 4) proposed additional studies to further characterize the usefulness and limitations of the proposed test method or approach.

The questions relating to the draft BRD that must be addressed for each topic are provided in **Sections I** and **II** of this guidance, while **Section III** contains questions relating to the draft ICCVAM test method recommendations. The ICCVAM Ocular Toxicity Working Group (OTWG) prepared the questions to ensure that the Panel review provides adequate information to facilitate U.S. Federal agency decisions on the regulatory acceptability of each proposed test method and approach. The questions are also intended to obtain guidance from the Panel that will be helpful to federal agencies and other organizations involved in conducting or supporting further development, standardization, and/or validation studies.

Appendix C2
Questions for the Peer Review Panel:
Use of Topical Anesthetics and Systemic Analgesics to Minimize Pain and
Distress in Ocular Toxicity Testing

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Following the Panel’s review of each of the three sets of questions below, the Panel is asked to address the overall question: Will the proposed modifications to the Draize eye test for the routine use of topical anesthetics and systemic analgesics help avoid or minimize pain and distress, and will their use continue to support accurate hazard classification determinations?

I. Review of the Draft BRD for Errors and Omissions

1. In the draft BRD, are there any errors that should be corrected, or omissions of existing relevant data, information, publications, or reports that should be included?

II. Use of Topical Anesthetics and Systemic Analgesics

1. Avoiding or Minimizing Pain and Distress Associated with Initial Application of Test Substances and Initial Chemically-Induced Ocular Injuries
 - a. Are the proposed topical anesthetics, doses, and time of administration the most appropriate to optimize local anesthesia of the cornea to avoid pain and distress from the topical administration of test substances? If not what suggestions do you have?
 - b. What is the duration of topical anesthesia that can be expected?
 - c. Based on previous studies provided in the draft BRD and current understanding of the proposed topical anesthetics on ocular physiology, please comment on: 1) the potential for topical anesthetics to alter the ocular injury response for the range of substances that might be tested; and 2) the potential effect of these changes on the outcome of the test with regard to current hazard classification categories (i.e. U.S. Environmental Protection Agency [EPA], United Nations Globally Harmonized System of Classification and Labeling of Chemicals [GHS], European Union [EU]), if any?
 - d. Are there any testing situations where it would not be advisable to administer pretreatment topical anesthetics because of the potential to interfere with the outcome of the test in terms of current hazard classification categories (i.e. EPA, GHS, EU)?
 - e. Is the proposed pretreatment with systemic analgesics in terms of the selected analgesic, dose, and time of administration the most appropriate to optimize analgesia in order to avoid or minimize pain and distress resulting from initial injuries resulting from topical administration of test substances? If not, what suggestions do you have?

- f. Do you agree that opioids are the most appropriate class of analgesics for this type of testing and that nonsteroidal anti-inflammatory drugs should be avoided?
- g. Are there other systemic analgesics that might have greater efficacy in relieving ophthalmic pain associated with chemically-induced injuries?
- h. What is the duration of ophthalmic analgesia that can be expected?
- i. Are there specific pain-related chemically-induced ocular injuries to the eye that the proposed analgesic may not adequately address? If so, are there other topical or systemic agents that should be considered for treating this pain, and is there any known or suspected potential for delaying recovery from the injury?
- j. Please comment on the potential effectiveness and use of transdermal patches to deliver pretreatment analgesia, and the optimal time for application in order to achieve optimal tissue levels prior to the test substance application?
- k. Based on current understanding of the proposed systemic analgesics on ocular physiology, please comment on: 1) the potential for systemic analgesics to alter the ocular injury response for the range of substances that might be tested; and 2) the potential effect of these changes on the outcome of the test with regard to current hazard classification categories (i.e. EPA, GHS, EU), if any?
- l. Are there any testing situations where it would not be advisable to administer pretreatment analgesics because of the potential to interfere with the outcome of the test in terms of current hazard classification categories (i.e. EPA, GHS, EU)?
- m. What specific observations, to be recorded immediately after test substance application, should be made in order to assess the effectiveness of the pretreatment topical anesthesia and systemic analgesia?
- n. How often should subsequent pain assessments be made and recorded? Are there any available pain scoring systems that could be used for this purpose?
- o. Are the database and/or information available on these anesthetics/analgesics sufficient to warrant their inclusion in Draize eye tests for any of the types of chemicals and products that are typically tested for ocular irritation potential? If not, what are the relevant chemical classes/properties that should be tested

with caution, or not evaluated with these modifications? What chemicals or products should be evaluated to fill this data gap?

- p. The NICEATM evaluation of the effect of topical anesthetics on reversibility of ocular lesions (see Appendix A of the BRD) is based on studies that used tetracaine as the topical anesthetic. Is there any reason that these results should not also be applied to other similar topical anesthetics (e.g., proparacaine)?
2. Avoiding or Minimizing Pain and Distress Associated with Post-Application Chemically-Induced Ocular Injuries
- a. Are the post-application ocular lesions that would be expected to cause ophthalmic pain and therefore serve as the basis for administering subsequent systemic analgesics adequately described? Are there other lesions that should be added, or modifications made to the existing lesions?
 - b. Are the post-application clinical signs of pain and distress that should serve as the basis for administering subsequent systemic analgesics adequately described? Are there other clinical signs that should also be added?
 - c. Is the proposed post-application treatment with systemic analgesics in terms of the selected analgesic, dose, and time of administration the most appropriate in order to avoid or minimize pain and distress from injuries resulting from topical administration of test substances? If not what suggestions do you have?
 - d. Are there other systemic analgesics that might have greater efficacy in relieving ophthalmic pain associated with chemically-induced injuries?
 - e. What is the duration of ophthalmic analgesia that can be expected?
 - f. Are there specific pain-related chemically-induced ocular injuries to the eye that the proposed analgesic may not adequately address? If so, are there other topical or systemic agents that should be considered for treating this pain, and is there any known or suspected potential for delaying recovery from the injury?
 - g. Please comment on the potential effectiveness and use of transdermal patches to deliver post-application analgesia, and the optimal time for application in order to achieve optimal tissue levels to address pain from injuries if a pre-application injection of analgesic was used?

- h. Based on current understanding of the proposed systemic analgesics on ocular physiology, please comment on: 1) the potential for use of systemic analgesics beyond the initial pre-application treatment to alter the ocular injury response for the range of substances that might be tested; and 2) the potential effect of these changes on the outcome of the test with regard to current hazard classification categories (i.e. EPA, GHS, EU), if any?
 - i. Are there any testing situations where it would not be advisable to administer post-application analgesics because of the potential to interfere with the outcome of the test in terms of current hazard classification categories (i.e. EPA, GHS, EU)?
 - j. Since it is possible for a corneal abrasion to get infected, and since one rabbit with a severe effect can drive the regulatory classification of a test substance, should measures be taken to prevent secondary infections and avoid a potential overclassification? If so, what measures might provide the least interference with healing and time to recovery?
 3. Consideration of All Available Data and Relevant Information
 - a. Based on the draft BRD, have all the relevant data identified in published or unpublished studies been adequately considered? Are there other comparative data that were not considered in the draft BRD, but are available for consideration? If yes, please explain how to obtain such data.

III. Draft ICCVAM Test Method Recommendations: Use of Topical Anesthetics, Systemic Analgesic, and Humane Endpoints to Minimize Pain and Distress

1. Test Method Usefulness and Recommendations
 - a. Do you agree that the available data and information support the ICCVAM draft recommendations on the routine use of topical anesthetics and systemic analgesics? Please explain your answer.
2. Test Method Protocol
 - a. Do you agree that the available data and information support the ICCVAM draft recommendations on the type and frequency of dosing for topical anesthetics and systemic analgesics? If not, what recommendations would you make? Please explain your answer.
 - b. Do you consider the available guidance on measuring fluorescein staining to be adequate for laboratories to obtain consistent results?

3. Future Studies

- a. Do you agree that the available data support the ICCVAM draft recommendations for use of anesthetics/analgesics in terms of the proposed future studies? If not, then what recommendations would you make? Please explain your answer.
- b. Are there gaps in our knowledge regarding the severity and duration of pain associated with the range and severity of ocular lesions in animals? If so, how might these be addressed?
- c. What additional research should be considered to support the development and validation of improved treatment strategies to avoid pain and distress from ocular injuries during testing without altering hazard classification outcome?

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Appendix C3
Questions for the Peer Review Panel:
Use of Humane Endpoints to Minimize Pain and Distress in
Ocular Toxicity Testing

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Following the Panel's review of each of the three sets of questions below, the Panel is asked to address the overall question: Will the proposed modifications to the Draize rabbit eye test for the routine use of topical anesthetics and systemic analgesics help avoid or minimize pain and distress, and will their use continue to support accurate hazard classification determinations?

I. Review of the Draft BRD for Errors and Omissions

1. In the draft BRD, are there any errors that should be corrected, or omissions of existing relevant data, information, publications, or reports that should be included?

II. Use of Humane Endpoints to Justify Early Termination of a Study

- a. Are each of the current and proposed humane endpoints expected to be sufficiently predictive of irreversible or severe effects (GHS Category 1, EPA Category I, EU R41), such that they should routinely be used as humane endpoints to terminate a study as soon as they are observed?
- b. How often should observation for and recording of the presence or absence of these lesions be recorded in order to ensure that termination decisions are made in a timely manner?
- c. Do you consider pannus to be an irreversible effect that, once it appears, should signal that a study be terminated?
- d. Do you consider fluorescein staining at each observation time point to be an appropriate and practical measure for determining severe ocular lesions? Can the area of fluorescein staining be monitored effectively such that one can accurately determine whether staining has not diminished over time? If yes, would you consider this to be adequate justification for terminating a study? At what point should the study be terminated (i.e., how long should one look for reversibility before the study is terminated)?
- e. Are there other observations or lesions that would suggest that the proposed endpoints might completely reverse, and therefore the proposed endpoints should not be used to terminate the study?
- f. Are there other objective biomarkers (e.g., extent and depth of corneal damage) that are or would be considered sufficiently predictive of severe or irreversible effects that they should be used as routine humane endpoints?

- g. Are there other potentially more sensitive biomarkers that are indicative of severe or irreversible effects that should be investigated for their usefulness as early endpoints?
- h. Are there other earlier biomarkers/criteria indicative that painful lesions can be expected to fully reverse to EPA Category II (< 21 days) or III lesions (< 7 days), and which could thus be used as a basis for early termination of studies and classification in these reversible injury categories?
- i. Are there additional data that are recommended for collection during future animal studies that might aid in identifying earlier more humane endpoints for ocular testing?
- j. Are the additional endpoints provided for termination of a Draize test adequate? Should any endpoints be added or omitted? Please explain your answer.
- k. Are the timeframes for consideration of termination of a study based on the use of these endpoints adequate to insure that reversal would not be expected? Please explain your answer.

III. Draft ICCVAM Test Method Recommendations: Use of Humane Endpoints to Minimize Pain and Distress

- 1. Test Method Usefulness and Recommendations
 - a. Do you agree that the available data and information support the ICCVAM draft recommendations on the routine use of humane endpoints? Please explain your answer.
- 2. Future Studies
 - a. What additional research should be considered to support the development and validation of improved treatment strategies to avoid pain and distress from ocular injuries during testing without altering hazard classification outcome?
 - b. What are the knowledge gaps regarding predictive early humane endpoints that should be addressed in research, development, and validation efforts?

Appendix C4
Questions for the Peer Review Panel:
The Hen's Egg Test - Chorioallantoic Membrane Test Method

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The overall question to consider is whether the validation status of the hen's egg test—chorioallantoic membrane (HET-CAM) test method has been adequately characterized for its intended purpose, and is it sufficiently accurate and reliable to be used for the identification of nonsevere irritants in place of the traditional rabbit eye test procedure.

I. Review of the Draft HET-CAM Test Method BRD for Errors and Omissions

1. In the draft HET-CAM BRD, are there any errors that should be corrected, or omissions of existing relevant data or information that should be included?

II. Draft HET-CAM BRD

1. Test Method Protocol

- a. Is the protocol sufficiently detailed that it can be expected to be conducted reproducibly in other laboratories?
- b. Are critical aspects of the test method protocol, as outlined in the ICCVAM Submission Guidelines, adequately justified and described in the BRD (e.g., the decision criteria [and their rationale] used to classify the response as positive or negative; the basis for proposed positive and negative controls; the procedure for dose selection)? Please explain your answer.

2. Substances Used for the Validation Studies

- a. Do you consider the database for the HET-CAM test method representative of a sufficient range of chemical classes and physicochemical properties and that it would be applicable to any of the types of chemicals and products that are typically tested for ocular irritation potential? If not, what are the relevant chemical classes/properties (other than those that are identified as limitations in the previous ICCVAM BRD) that should be tested with caution, or not evaluated using this test method? What chemicals or products should be evaluated to fill this data gap? Please explain your answer.

3. Test Method Accuracy

- a. The current accuracy analysis is based on overall concordance with the traditional Draize rabbit eye test. Is this data adequate for assessing the accuracy of the test method? Please explain your answer.
- b. Has the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of the HET-CAM test method been adequately evaluated and compared to the traditional rabbit test (refer also to

Table 6-1 of the draft HET-CAM BRD)? If not, what other analyses should be performed? Please explain your answer.

4. Test Method Reliability (Intra- and Inter-laboratory Reproducibility)
 - a. Has the intralaboratory reproducibility of the HET-CAM test method been adequately evaluated and compared to the traditional rabbit eye test? If not, what other analyses should be performed? Are any limitations apparent based on this intralaboratory reproducibility assessment? Please explain your answer.
 - b. Has the interlaboratory reproducibility of the HET-CAM test method been adequately evaluated and compared to the traditional rabbit test (refer also to Tables 7-2 and 7-3 of the draft HET-CAM BRD)? If not, what other analyses should be performed? Are any limitations apparent based on this interlaboratory reproducibility assessment? Please explain your answer.
 - c. The draft HET-CAM BRD analyzes data from validation studies that used coded substances, as well as studies that were not coded. Does the lack of coding of test substances adversely impact or bias the current evaluation? Please explain your answer.
5. Data Quality
 - a. Not all of the studies evaluated in the draft HET-CAM BRD were conducted in accordance with Good Laboratory Practices (GLP) guidelines although they were reportedly done in laboratories that conduct GLP studies. Please discuss what impact this might have on the evaluation of the ocular test methods.
 - b. The original records for these studies are available upon request, but have not yet been obtained. As a result, an independent audit has not been conducted to confirm that the reported data is the same as the data recorded in laboratory notebooks. Should any recommendations from ICCVAM be contingent upon the completion of such an audit and findings that there were no significant errors in data transcription? Please explain your answer.
6. Consideration of All Available Data and Relevant Information
 - a. Based on available information contained in the draft HET-CAM BRD, have all the relevant data identified in published or unpublished studies that employ this test method been adequately considered? Are there other comparative test method data that were not considered in the draft BRD, but

are available for consideration? If yes, please explain how to obtain such data.

III. Draft ICCVAM Test Method Recommendations on the HET-CAM Test Method to Identify Nonsevere Irritants

1. Test Method Usefulness and Recommendations

- a. Do you agree that the available data and test method performance (accuracy and reliability) support the ICCVAM draft recommendations for the HET-CAM test method in terms of the proposed test method usefulness and limitations? Please explain your answer.
- b. Do you consider it necessary to conduct additional validation studies on which to base expanding the applicability domain of HET-CAM beyond cosmetic and personal care formulations that are oil/water emulsions or surfactant containing formulations?
- c. When evaluating the HET-CAM for its ability to distinguish substances as not labeled as irritants from all other irritant classes, the false negative rate for the EU and GHS systems is 0% (0/26 or 0/31) and therefore the HET-CAM is recommended for such testing purposes. By comparison, the false negative rate was 9% (4/45) for the EPA system. 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. For one of the substances, one out of the six rabbits tested had a conjunctival redness score of two that required 14 days to resolve. Four of the remaining five rabbits in this study had conjunctival redness scores of two that resolved within three days; the last rabbit did not have this lesion. Do you agree that the severity and number of ocular lesions noted *in vivo* do not present a significant risk to the user and as such HET-CAM could be considered useful as a screening for EPA Category IV substances?
- d. The validation database does not include any substances currently regulated by EPA. Should additional testing be required before a recommendation on the usefulness of HET-CAM for identifying Category IV substances is made?

2. Test Method Protocol

- a. Do you agree that the available data support the ICCVAM draft recommendations for the HET-CAM test method procedure in terms of the

proposed test method standardized protocols? If not, what recommendations would you make? Please explain your answer.

3. Future Studies

- a. Do you agree that the available data support the ICCVAM draft recommendations for the HET-CAM test method in terms of the proposed future studies? If not, then what recommendations would you make? Please explain your answer.

4. Performance Standards

- a. Does the panel agree that the results described above do not warrant the development of performance standards for the HET-CAM test method at this time? Please explain your answer.

Appendix C5
Questions for the Peer Review Panel:
The Isolated Chicken Eye Test Method

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The overall question to consider is whether the validation status of the isolated chicken eye (ICE) test method has been adequately characterized for its intended purpose, and is it sufficiently accurate and reliable to be used for the identification of nonsevere ocular irritants (i.e., those that induce reversible ocular damage) and substances not labeled as irritants (i.e., EPA Category IV, EU Not Labeled, GHS Not Classified) in place of the traditional rabbit eye test procedure.

I. Review of the Draft ICE Test Method BRD for Errors and Omissions

1. In the draft ICE BRD, are there any errors that should be corrected, or omissions of existing relevant data or information that should be included?

II. Draft ICE BRD

1. Test Method Protocol
 - a. Is the protocol sufficiently detailed that it can be expected to be conducted reproducibly in other laboratories?
 - b. Have critical aspects of the test method protocol for ICE been adequately justified and described in the BRD for the identification of nonsevere ocular irritants (i.e., those that induce reversible ocular damage) and substances not labeled as irritants (i.e., EPA Category IV, EU Not Labeled, GHS Not Classified)? Please explain your answers.
2. Substances Used for the Validation Studies
 - a. Do you consider the database for the ICE test method representative of a sufficient range of chemical classes and physicochemical properties and that it would be applicable to any of the types of chemicals and products that are typically tested for ocular irritation potential? If not, what are the relevant chemical classes/properties (other than those that are identified as limitations in the previous ICCVAM BRD) that should be tested with caution, or not evaluated using these test method? What chemicals or products should be evaluated to fill this data gap? Please explain your answer.
3. Test Method Accuracy
 - a. The current accuracy analysis is based on overall concordance with the traditional Draize rabbit eye test. Are these data adequate for assessing the accuracy of the test method? Please explain your answer.
 - b. Has the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of the ICE test method been adequately

evaluated and compared to the traditional rabbit test (refer also to Table 6-1 to Table 6-15 of the draft ICE BRD)? If not, what other analyses should be performed? Please explain your answer.

4. Test Method Reliability (Intra- and Interlaboratory Reproducibility)
 - a. Has the intralaboratory reproducibility of the ICE test method been adequately evaluated and compared to the traditional rabbit eye test (refer to ICCVAM ICE BRD [ICCVAM 2006b], Section 7.2)? If not, what other analyses should be performed? Are any limitations apparent based on this intralaboratory reproducibility assessment? Please explain your answer.
 - b. Has the interlaboratory reproducibility of the ICE test method been adequately evaluated and compared to the traditional rabbit eye test (refer also to Table 7-1 to Table 7-6 of the draft ICE BRD)? If not, what other analyses should be performed? Are any limitations apparent based on this interlaboratory reproducibility assessment? Please explain your answer.
 - c. The draft ICE BRD analyzes data from validation studies that used coded substances (Balls et al. 1995), as well as studies that were not coded (Prinsen and Koëter 1993; Prinsen 1996, 2005). Does the lack of coding of test substances adversely impact or bias the current evaluation? Please explain your answer.
5. Data Quality
 - a. Ideally, all data supporting the validity of a test method should be obtained and reported in accordance with GLP guidelines and with the use of coded chemicals (OECD 1998; EPA 2003b, 2003c; FDA 2003). The data quality was evaluated by a review of the methods section in literature references and the submitted reports. The data quality presented in the reviewed literature references can only be evaluated to the extent such information was provided in the published reports. Based on the available information, all ICE test method studies evaluated were conducted according to GLP guidelines. Please discuss if you agree with this assessment and what impact this might have on the evaluation of the ocular test methods.
 - b. The original records for these studies are available upon request, but have not yet been obtained. As a result, an independent audit has not been conducted to confirm that the reported data are the same as the data recorded in laboratory notebooks. Should any recommendations from ICCVAM be

contingent upon the completion of such an audit and findings that there were no significant errors in data transcription? Please explain your answer.

6. Consideration of All Available Data and Relevant Information

- a. Based on available information contained in the draft ICE BRD, have all the relevant data identified in published or unpublished studies that employ this test method been adequately considered? Are there other comparative test method data that were not considered in the draft BRD, but are available for consideration? If yes, please explain how to obtain such data.

III. Draft ICCVAM Test Method Recommendations on the ICE Test Method to Identify Nonsevere Ocular Irritants (i.e., Those That Induce Reversible Ocular Damage) and Substances Not Labeled as Irritants (i.e., EPA Category IV, EU Not Labeled, GHS Not Classified)

1. Test Method Usefulness and Recommendations

- a. Do you agree that the available data and test method performance (accuracy and reliability) support the ICCVAM draft recommendations for the ICE test method in terms of the proposed test method usefulness and limitations? Please explain your answer.
- b. When evaluating the ICE for its ability to distinguish substances as not labeled as irritants from all other irritant classes, the false negative rate for the GHS system is 6% (4/62). However, among these false negatives is a Category 1 substance. Do you agree that this result should result in a recommendation that ICE not be used as a screening test to identify GHS Not Labeled substances?

2. Test Method Protocol

- a. Do you agree that the available data support the ICCVAM draft recommendations for the ICE test method procedure in terms of the proposed test method standardized protocols? If not, what recommendations would you make? Please explain your answer.

3. Future Studies

- a. Do you agree that the available data support the ICCVAM draft recommendations for the ICE test method in terms of the proposed future studies? If not, then what recommendations would you make? Please explain your answer.

4. Performance Standards

Does the panel agree that the results described above do not warrant the development of performance standards for the ICE test method at this time? Please explain your answer.

Appendix C6
Questions for the Peer Review Panel:
The Isolated Rabbit Eye Test Method

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The overall question to consider is whether the validation status of the isolated rabbit eye (IRE) test method has been adequately characterized for its intended purpose, and is it sufficiently accurate and reliable to be used for the identification of nonsevere ocular irritants (i.e., those that induce reversible ocular damage) and substances not labeled as irritants (i.e., EPA Category IV, EU Not Labeled, GHS Not Classified) in place of the traditional rabbit eye test procedure.

I. Review of the Draft IRE Test Method BRD for Errors and Omissions

1. In the draft IRE BRD, are there any errors that should be corrected, or omissions of existing relevant data or information that should be included?

II. Draft ICCVAM Test Method Recommendations on the IRE Test Method to Identify Nonsevere Ocular Irritants (i.e., Those That Induce Reversible Ocular Damage) and Substances Not Labeled as Irritants (i.e., EPA Category IV, EU Not Labeled, GHS Not Classified)

1. Test Method Usefulness and Recommendations
 - a. Do you agree that 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 test method accuracy and reliability when all these endpoints are evaluated in a single study?
 - b. Do you agree that additional optimization and validation studies are needed to further evaluate the relevance and reliability of the IRE test method, and in turn develop more definitive recommendations?
2. Test Method Protocol
 - a. Do you agree that the available data support the ICCVAM draft recommendations for the IRE test method procedure in terms of the proposed test method standardized protocols? If not, what recommendations would you make? Please explain your answer.
3. Future Studies
 - a. Do you agree that the available data support the ICCVAM draft recommendations for the IRE test method in terms of the proposed future studies? If not, then what recommendations would you make? Please explain your answer.

4. Performance Standards

- a. Does the panel agree that the results described above do not warrant the development of performance standards for the IRE test method at this time? Please explain your answer.

Appendix C7
Questions for the Peer Review Panel:
The Bovine Corneal Opacity and Permeability Test Method

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The overall question to consider is whether the validation status of the bovine corneal opacity and permeability (BCOP) test method has been adequately characterized for its intended purpose, and is it sufficiently accurate and reliable to be used for the identification of nonsevere irritants in place of the traditional rabbit eye test procedure.

I. Review of the Draft BCOP Test Method BRD for Errors and Omissions

1. In the draft BCOP BRD, are there any errors that should be corrected, or omissions of existing relevant data or information that should be included?

II. Draft BCOP BRD

1. Test Method Protocol
 - a. Is the protocol sufficiently detailed that it can be expected to be conducted reproducibly in other laboratories?
 - b. Are critical aspects of the test method protocol, as outlined in the ICCVAM Submission Guidelines, adequately justified and described in the BRD (e.g., the decision criteria [and their rationale] used to classify the response as positive or negative; the basis for proposed positive and negative controls; the procedure for dose selection)? Please explain your answers.
2. Substances Used for the Validation Studies
 - a. Do you consider the database for the BCOP test method representative of a sufficient range of chemical classes and physicochemical properties and that it would be applicable to any of the types of chemicals and products that are typically tested for ocular irritation potential? If not, what are the relevant chemical classes/properties (other than those that are identified as limitations in the previous ICCVAM BRD) that should be tested with caution, or not evaluated using these test method? What chemicals or products should be evaluated to fill this data gap? Please explain your answer.
3. Test Method Accuracy
 - a. The current accuracy analysis is based on overall concordance with the traditional Draize rabbit eye test. Are these data adequate for assessing the accuracy of the test method? Please explain your answer.
 - b. Has the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of the BCOP test method been adequately evaluated and compared to the traditional rabbit test (refer also to Tables 6-1,

6-3, 6-8, and 6-13 of the draft BCOP BRD)? If not, what other analyses should be performed? Please explain your answer.

4. Test Method Reliability (Intra- and Inter-laboratory Reproducibility)
 - a. Has the intralaboratory reproducibility of the BCOP test method been adequately evaluated and compared to the traditional rabbit eye test (refer to the ICCVAM BCOP BRD [ICCVAM 2006c], Section 7.2)? If not, what other analyses should be performed? Are any limitations apparent based on this intralaboratory reproducibility assessment? Please explain your answer.
 - b. Has the interlaboratory reproducibility of the BCOP test method been adequately evaluated and compared to the traditional rabbit test (refer also to Tables 7-2, 7-4, and 7-6 of the draft BCOP BRD)? If not, what other analyses should be performed? Are any limitations apparent based on this interlaboratory reproducibility assessment? Please explain your answer.
 - c. The draft BCOP BRD analyzes data from validation studies that used coded substances, as well as studies that were not coded. Does the lack of coding of test substances adversely impact or bias the current evaluation? Please explain your answer.
5. Data Quality
 - a. Not all of the studies evaluated in the draft BCOP BRD were conducted in accordance with GLP; it could not be ascertained as to whether all of the *in vitro* data contained in the BRD for *in vitro* testing strategies for ocular hazard categorization of antimicrobial cleaning products (AMCPs) were generated under full GLP compliance, but where possible, that information is contained in the spreadsheets that form the database from which this BRD was generated. Please discuss what impact this might have on the evaluation of the ocular test methods.
 - b. The original records for all studies are available upon request, but have not yet been obtained. As a result, an independent audit has not been conducted to confirm that the reported data is the same as the data recorded in laboratory notebooks. Should any recommendations from ICCVAM be contingent upon the completion of such an audit and findings that there were no significant errors in data transcription? Please explain your answer.
6. Consideration of All Available Data and Relevant Information

- a. Based on available information contained in the draft BCOP BRD, have all the relevant data identified in published or unpublished studies that employ this test method been adequately considered? Are there other comparative test method data that were not considered in the draft BRD, but are available for consideration? If yes, please explain how to obtain such data.

III. Draft ICCVAM Test Method Recommendations on the BCOP Test Method to Identify Nonsevere Irritants

1. Test Method Usefulness and Recommendations

- a. Do you agree that the available data and test method performance (accuracy and reliability) support the ICCVAM draft recommendations for the BCOP test method in terms of the proposed test method usefulness and limitations? Please explain your answer.

2. Test Method Protocol

- a. Do you agree that the available data support the ICCVAM draft recommendations for the BCOP test method procedure in terms of the proposed test method standardized protocols? If not, what recommendations would you make? Please explain your answer.
- b. When evaluating the BCOP for its ability to distinguish substances as not labeled as irritants from all other irritant classes, the false negative rate for the EU and GHS systems is 0% (0/54 or 0/97) and therefore the BCOP is recommended for such testing purposes. 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 data. For 38% (3/8) of these substances, the categorization was based on at least one rabbit with a corneal opacity score of one that was not resolved until day three of the study. Another substance was categorized based on all six rabbits with a conjunctival redness score of three that was not resolved until day seven of the study. Do you agree that the severity and number of ocular lesions noted *in vivo* present a significant risk to the user and as such BCOP should not be recommended as a screening for EPA Category IV substances?
- c. Do you consider differing recommendations among hazard classification systems on test method usefulness to be appropriate and justified?

3. Future Studies

- a. Do you agree that the available data support the ICCVAM draft recommendations for the BCOP test method in terms of the proposed future studies? If not, then what recommendations would you make? Please explain your answer.
4. Performance Standards
 - a. Does the panel agree that the results described above do not warrant the development of performance standards for the BCOP test method at this time? Please explain your answer.

Appendix C8
Questions for the Peer Review Panel:
The Low Volume Eye Test

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The overall question to consider is whether the validation status of the low volume eye test (LVET) has been adequately characterized for its intended purpose, and is it sufficiently accurate and reliable to be used for the identification of all ocular hazard categories in place of the traditional rabbit eye test procedure, and as such should be considered an adequate reference test method against which to compare the performance of an *in vitro* alternative test method.

I. Review of the Draft LVET Summary Review Document for Errors and Omissions

1. In the draft LVET Summary Review Document (SRD), are there any errors that should be corrected, or omissions of existing relevant data or information that should be included?

II. Draft LVET SRD

1. Test Method Protocol
 - a. Is the protocol for the LVET test method adequate for its intended use? Please explain your answer.
 - b. The LVET has primarily been used to test surfactants and surfactant-containing products. Is this limited database adequate to determine its validity for use as an *in vivo* reference test in general or should such consideration only be relevant to this limited applicability domain? Please explain your answer.
 - c. Should there be concern that direct application of the test substance to the cornea is causing additional pain and distress relative to the Draize eye test (where the substance is applied into the conjunctival sac)?
2. Substances Used for the Validation Studies
 - a. Do you consider the database for the LVET (which is restricted primarily to surfactants and surfactant containing materials) representative of a sufficient range of chemical classes and physicochemical properties that are applicable to the types of chemicals and products that are typically tested for ocular irritation potential? If not, what are the relevant chemical classes/properties that should be tested with caution, or not evaluated using these test method? What chemicals or products should be evaluated to fill this data gap? Please explain your answer.

- b. Are you aware of any other data available in the published literature that could expand the applicability domain of the database? Please explain your answer.

3. Test Method Accuracy

- a. The current accuracy analysis is based on overall concordance with the traditional Draize rabbit eye test. Is this data adequate for assessing the accuracy of the test method? Please explain your answer.
- b. Has the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of these test methods been adequately evaluated and compared to the traditional rabbit test (refer also to Tables 4-1 to 4-3 of the draft ICCVAM LVET SRD)? If not, what other analyses should be performed? Please explain your answer.
- c. Substances tested in humans for ethical reasons are limited to mild ocular irritants and nonirritants whereas accidental exposure data with more severe irritants is vague with respect to concentration of the test substance and to the volume of exposure. Thus, the LVET data is being compared to human data where the severity of the irritants may be limited and there is concern that the LVET has not been shown to be capable of detecting a severe irritant or corrosive test substance. Is this concern justified? Please explain your answer.
- d. It is difficult to compare LVET data with Draize test data because the LVET has been reported to underpredict relative to the Draize test and overpredict relative to human experience data. For example, a Draize EPA Category I test substance might be labeled as an EPA Category II or III when tested in the LVET. Is there a statistically meaningful way to compare these data? Please explain your answer.
- e. Are you aware of any instances where the Draize test failed to predict a severe irritant/corrosive response in the human?

4. Data Quality

- a. Not all of the studies evaluated in the draft ocular SRD were conducted in accordance with GLP guidelines. Please discuss what impact this might have on the evaluation of the ocular test methods.
- b. The original records for these studies are available upon request, but have not yet been obtained. As a result, an independent audit has not been conducted

to confirm that the reported data is the same as the data recorded in laboratory notebooks. Should any recommendations from ICCVAM be contingent upon the completion of such an audit and findings that there were no significant errors in data transcription? Please explain your answer.

5. Consideration of All Available Data and Relevant Information

- a. Based on the draft LVET SRD, have all the relevant data identified in published or unpublished studies that employ this test method been adequately considered? Are there other comparative test method data that were not considered in the draft AMCP BRD or in the BRD on the LVET prepared by the European Centre for the Validation of Alternative Methods (ECVAM), but are available for consideration? If yes, please explain how to obtain such data.

III. Draft ICCVAM Test Method Recommendations on the LVET

1. Test Method Usefulness and Recommendations

- a. Do you agree that the available data and test method performance support the ICCVAM draft recommendations for the ocular test methods in terms of the proposed test method usefulness and limitations? Please explain your answer.

2. Test Method Protocol

- a. Do you agree that the available data support the ICCVAM draft recommendations for the LVET test method in terms of the proposed test method standardized protocols? If not, what recommendations would you make? Please explain your answer.
- b. Should topical anesthetics and systemic analgesics be routinely used in future studies that might be conducted to evaluate the validity of the LVET?

3. Future Studies

- a. Do you agree that the available data support the ICCVAM draft recommendations for the LVET test method in terms of the proposed future studies? If not, then what recommendations would you make? Please explain your answer.

4. Performance Standards

- a. Do you agree that the results described above do not warrant the development of performance standards for the LVET at this time? Please explain your answer.

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Appendix C9
Questions for the Peer Review Panel:
***In Vitro* Testing Strategies for Ocular Hazard Categorization of**
Antimicrobial Cleaning Products

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The overall question to consider is whether the validation status of the AMCP testing strategy and the individual test methods used has been adequately characterized for its intended purpose, and is it sufficiently accurate and reliable to be used for the ocular hazard classification and labeling of test substances in place of the traditional rabbit eye test procedure.

I. Review of the Draft AMCP SRD for Errors and Omissions

1. In the draft AMCP SRD, are there any errors that should be corrected, or omissions of existing relevant data or information that should be included?

II. Draft AMCP SRD

1. AMCP Test Methods

Bovine Corneal Opacity and Permeability (BCOP) Test Method

- a. Is the protocol sufficiently detailed that it can be expected to be conducted reproducibly in other laboratories?
- b. Are critical aspects of the BCOP test method protocol, as outlined in the ICCVAM Submission Guidelines, been adequately justified and described in the BRD? Please explain your answers.
- c. Do you agree that the current database of histopathology results for BCOP does not justify its use and that additional data are needed before a recommendation for the use of histopathology in BCOP for hazard classification of AMCPs can be made? Please explain your answer.

Cytosensor Microphysiometer (CM) Test Method

- a. Is the protocol sufficiently detailed that it can be expected to be conducted reproducibly in other laboratories?
- b. Have critical aspects of the CM test method protocol, as outlined in the ICCVAM Submission Guidelines, been adequately justified and described in the BRD? Please explain your answers.
- c. The draft OTWG position is that the LVET predictivity for the Draize test and the lack of LVET data for substances that are known to cause moderate and severe irritation and ocular corrosion makes it inadequate to serve as a reference test method to support the validity of *in vitro* test methods. For this reason, the CM, for which only LVET reference data for AMCPs exists, was not considered adequate to support the proposed testing strategy. Do you agree with this assessment? Please explain your answer.

- d. However, additional data on 53 surfactant and surfactant-containing formulations were provided in a BRD prepared by ECVAM where there were data from the traditional Draize rabbit test available to assess the accuracy of the CM test method. These substances were not claimed as AMCPs, but they were surfactant-containing formulations with similar composition to many AMCPs. Based on the performance of the CM test method using these 53 substances, ICCVAM has proposed²⁰ that the CM test method can be used 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 EPA Category I, GHS Category 1, or EU Category R41; or as EPA Category IV, GHS Not Labeled, EU Not Classified in a tiered-testing strategy, as part of a weight-of-evidence approach. Do you consider these results with non-AMCP test substances suggestive that the CM test method could be useful in a testing strategy?
- e. If the Panel finds use of the CM for a restricted data set acceptable as proposed in the original AMCP strategy, is use of the CM test method as proposed in the testing strategy adequate for the classification and labeling of AMCPs for EPA registration? Please explain your answer.
- f. Molecular Devices Corporation in Sunnyvale, CA, has stopped production of the CM instrument, although the Transwell™ inserts and other test method-specific materials are expected to be available for some time to current CM users. Should this impact any recommendation on the usefulness of the CM test method in the testing strategy? Please explain your answer.
- g. Has the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) for CM been adequately evaluated and compared to the traditional rabbit test and/or the LVET? If not, what other analyses should be performed? Please explain your answer.

EpiOcular (EO) Test Method

- a. Is the protocol sufficiently detailed that it can be expected to be conducted reproducibly in other laboratories?

²⁰ This evaluation is currently undergoing separate peer review by an ECVAM Scientific Advisory Committee Peer Review Panel, which includes two members of the ICCVAM Ocular Peer Review Panel (Drs. Hayes and Wilson).

- b. Are critical aspects of the test method protocol, as outlined in the ICCVAM Submission Guidelines, adequately justified and described in the BRD? Please explain your answers.
 - c. The EO protocol included in the AMCP BRD (which uses a time-to-toxicity protocol) is different than the protocol included in a recent EO submission to ECVAM (which uses a threshold of relative viability at a single time point) and upon which a planned validation study is based. Do you consider one protocol to be more appropriate than the other for the hazard classification of AMCPs?
 - d. Has the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) for EO been adequately evaluated and compared to the traditional rabbit test for the types of substances included in the AMCP database (i.e., surfactants, solvents, bases, acids, oxidizers) (refer also to Table 6-1 of the draft AMCP SRD)? If not, what other analyses should be performed? Please explain your answer.
2. AMCP Testing Strategy
- a. Is the database supporting the AMCP testing strategy as originally proposed using the BCOP, CM, and EO adequate for the classification and labeling of AMCP for EPA registration? Please explain your answer.
 - b. Is the database supporting the proposed alternative strategy using the BCOP and EO test method data with paired Draize data adequate for determining its usefulness for classification and labeling of AMCP for EPA registration? Please explain your answer.
 - c. Are there other test methods that should be considered in a testing strategy that would be expected to improve classification and labeling of AMCP for EPA registration?
3. Substances Used for the Validation Studies
- a. The AMCP BRD included data for 228 substances tested in one or two of the three *in vitro* test methods proposed for use in the testing strategy. However, none of the substances had been tested in all three *in vitro* test methods. Therefore, there are no data available for the proposed substances with which to characterize the actual performance of a testing strategy that includes BCOP, CM, and EO. Do you agree that this limitation prevents any definitive recommendation on the proposed testing strategy?

- b. Of the 228 substances, 28 are EPA registered AMCPs, with eight additional materials being in-use dilutions of EPA registered antimicrobial concentrates. Do you consider this small proportion of the total database to be problematic with regard to any conclusions that may be reached on the usefulness of the proposed strategy for classification and labeling of AMCPs?
 - c. Do you consider the database for each test method used in the AMCP strategy representative of a sufficient range of chemical classes and physicochemical properties that it would be applicable to any of the types of chemicals and products that are typically tested for ocular irritation potential? If not, what are the relevant chemical classes/properties (other than those that are identified as limitations in the previous BRD for BCOP) that should be tested with caution, or not evaluated using these test method? What chemicals or products should be evaluated to fill this data gap? Please explain your answer.
4. Test Method Accuracy
- a. The current accuracy analysis is based on overall concordance with the traditional Draize rabbit eye test. Is this data adequate for assessing the accuracy of the test method? Please explain your answer.
 - b. Has the relevance (e.g., accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of these test methods been adequately evaluated and compared to the traditional rabbit test for the types of substances included in the AMCP database (i.e., surfactants, solvents, bases, acids, oxidizers) (refer also to Table 6-1 of the draft AMCP SRD)? If not, what other analyses should be performed? Please explain your answer.
5. Test Method Reliability (Intra- and Inter-laboratory Reproducibility)
- a. Has the intralaboratory reproducibility of these test methods been adequately evaluated and compared to the traditional rabbit eye test for the types of substances included in the AMCP database (i.e., surfactants, solvents, bases, acids, oxidizers) (refer to Section 7.0 of Appendix A in the ICCVAM SRD)? If not, what other analyses should be performed? Are any limitations apparent based on this intralaboratory reproducibility assessment? Please explain your answer.
 - b. Has the interlaboratory reproducibility of these test methods been adequately evaluated and compared to the traditional rabbit test for the types of substances included in the AMCP database (i.e., surfactants, solvents, bases,

acids oxidizers) (refer to Section 7.0 of Appendix A in the ICCVAM SRD)? If not, what other analyses should be performed? Are any limitations apparent based on this interlaboratory reproducibility assessment? Please explain your answer.

- c. Does the lack of coding of test substances adversely impact or bias the current evaluation? Please explain your answer.

6. Data Quality

- a. Not all of the studies evaluated in the draft AMCP SRD were conducted in accordance with GLP guidelines although they were reportedly done in laboratories that conduct GLP studies. Please discuss what impact this might have on the evaluation of the ocular test methods.
- b. The original records for these studies are available upon request, but have not yet been obtained. As a result, an independent audit has not been conducted to confirm that the reported data is the same as the data recorded in laboratory notebooks. Should any recommendations from ICCVAM be contingent upon the completion of such an audit and findings that there were no significant errors in data transcription? Please explain your answer.

7. Consideration of All Available Data and Relevant Information

- a. Based on information in the draft AMCP SRD, have all the relevant data identified in published or unpublished studies that employ these test methods been adequately considered? Are there other comparative test method data that were not considered in the draft SRD, but are available for consideration? If yes, please explain how to obtain such data.

III. Draft ICCVAM Test Method Recommendations on the AMCP Testing Strategy

1. Test Method Usefulness and Recommendations

- a. Do you agree that the available data and test method performance (accuracy and reliability) support the ICCVAM draft recommendations for the ocular test methods and testing strategy in terms of the proposed test method usefulness and limitations? Please explain your answer.
- b. Using CM to identify ocular nonirritants among the database of 53 surfactant-containing substances, the false negative rate ranged from 0-2% (0/27 to 1/46) when compared to *in vivo* results. The one false negative when using the EPA classification system was Category III based on *in vivo* data. For this substance, six rabbits were included in the *in vivo* test. One rabbit

had no observable effects, three rabbits had conjunctival redness (score = 1) that cleared after one (n=1) or two days (n=2), and two rabbits had corneal opacity (score = 1) that cleared after one day. Do you consider the type and number of lesions observed in this study to be reason for concern regarding the use of CM to identify EPA Category IV substances?

- c. When using CM to distinguish substances as not labeled as irritants from all other irritant classes, the false negative rate for the EU and GHS systems is 0% (0/27 or 0/28) while EPA is 2% (1/46) and therefore the Cytosensor is recommended for such testing purposes. The Cytosensor validation database does not include any substances currently regulated by EPA. Should additional testing be required before a recommendation on the usefulness of Cell Function-Based Assays for identifying Category IV substances is made?
 - d. The false positive rates for CM ranged from 50-69% (3/6 to 18/26) when compared to *in vivo* results. Three substances were false positives when using the EPA classification system and were classified *in vitro* as Category II/III. 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 classified *in vitro* as R36 (n=17) or R41 (n=1). Do these high false positive rates raise concern regarding the usefulness of CM as a screening test for not labeled substances, even if the false negative rate is near 0%?
 - e. Do you agree that there are insufficient available data on which to base definitive recommendations on the proposed alternate testing strategy (i.e., BCOP and EO)?
 - f. Can a retrospective evaluation of results in more than one test method suffice as an adequate performance evaluation, even if the same substances were not tested in each method proposed in a strategy?
 - g. Do you agree 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?
2. Test Method Protocol
- a. Do you agree that the available data support the ICCVAM draft recommendations for the ocular test method procedures in terms of the

proposed test method standardized protocols? If not, what recommendations would you make? Please explain your answer.

3. Future Studies

- a. Do you agree that the available data support the ICCVAM draft recommendations for each of the ocular test methods in terms of the proposed future studies? If not, then what recommendations would you make? Please explain your answer.
- b. Given that testing in EO alone would provide the same performance as the BCOP/EO strategy based on the 28 substances evaluated, do you think that additional studies should focus on EO instead of the testing strategy?

4. Performance Standards

- a. Does the panel agree that the results described above do not warrant the development of performance standards for the AMCP testing strategy at this time? Please explain your answer.

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