

ICCVAM Test Method Evaluation Report
Identifying Chemical Eye Hazards with Fewer Animals

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

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

**National Institute of Environmental Health Sciences
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Table of Contents

List of Tables	iv
List of Figures	v
List of Abbreviations and Acronyms	vi
Acknowledgements	vii
Interagency Coordinating Committee on the Validation of Alternative Methods: Agency Representatives	viii
Preface	xi
Executive Summary	xiii
1.0 Introduction	1
2.0 ICCVAM Recommendations: Identifying Chemical Eye Hazards with Fewer Animals	2
2.1 Introduction and Applicable Regulatory Requirements.....	2
2.2 ICCVAM Recommendations.....	2
3.0 Analysis Supporting the Use of Fewer Animals for Evaluating Eye Hazards	3
3.1 Current Testing Procedures (16 CFR 1500.42).....	3
3.2 Optimization of the Number of Positive Animals Required to Identify a Substance as an Irritant.....	5
3.3 Comparison of Three Strategies for Reducing Animal Use.....	6
3.4 Previous Proposals to Reduce the Number of Animals Used for Eye Safety Testing.....	10
3.5 Animal Welfare Considerations.....	12
4.0 ICCVAM Consideration of Public and SACATM Comments	13
4.1 Public Comments in Response to 75 FR 26757 (May 12, 2010).....	13
4.2 Public and SACATM Comments: SACATM Meeting on June 17–18, 2010.....	13
4.3 Public and SACATM Comments: SACATM Meeting on June 16–17, 2011.....	14
4.4 Public Comments in Response to 76 FR 50220 (August 12, 2011).....	14
5.0 References	16
Appendix A ICCVAM Evaluation Timeline	A-1
Appendix B Federal Register Notices and Public Comments	B-1
Appendix C Ocular Toxicity Regulations and Guidelines	C-1

List of Tables

Table 3-1	Current Testing Procedures (16 CFR 1500.42).....	3
Table 3-2	Scores for Grading Severity of Eye Lesions	4
Table 3-3	Number of Positive Animals and Sequential Tests Required for Assignment of an Irritant Classification According to Current Testing Procedures (16 CFR 1500.42).....	5
Table 3-4	Probability of Observing 0 to 6 Positive Animals in a Sample of $n = 3$ or $n = 6$ for Various Population Positive Response Rates (p) Assuming a Binomial Model.....	6
Table 3-5	Percentage of Substances Classified as Eye Irritants Based on Various Population Positive Response Rates (p) for the Three Strategies	7
Table 3-6	Probability That Strategy 1 Will Result in a Negative Classification for $p = 20\%$	8
Table 3-7	Goodness of Fit for a Database of 481 Test Results Using a Mixture of Three Binomial Distributions.....	9
Table 3-8	Percentage of Substances Classified as Eye Irritants Based on Estimated Underlying Positive Response Rates for Three Strategies: Three Binomial Distributions.....	10
Table 3-9	Percentage of Substances That Would be Over- and Underpredicted for the Three Strategies.....	10
Table 3-10	Lack of Fit Using the Springer et al. (1993) Model on the NICEATM Database	11
Table 4-1	Opportunities for Public Comment	13

List of Figures

Figure 3-1	Strategy 2 Provides Eye Hazard Classification the Same as or Greater Than Current Testing Procedures (16 CFR 1500.42)	7
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List of Abbreviations and Acronyms

CFR	U.S. Code of Federal Regulations
CPSC	U.S. Consumer Product Safety Commission
EPA	U.S. Environmental Protection Agency
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FR	<i>Federal Register</i>
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
JaCVAM	Japanese Center for the Validation of Alternative Methods
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OTWG	ICCVAM Interagency Ocular Toxicity Working Group
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
U.S.C.	United States Code

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Preface

Each year, an estimated 2 million eye injuries occur in the United States. Of these, more than 40,000 cause permanent visual impairment (McGwin et al. 2006a). Chemicals and compounds are the third most common cause of eye injuries, with household cleaning products comprising the second leading type of product associated with consumer eye injuries (McGwin et al. 2006b). To warn consumers and workers of the potential for chemicals and products to cause eye injuries, eye safety testing is performed to determine if substances may cause temporary or permanent eye damage. Test results are then used for hazard classification of chemicals and products using appropriate national and/or international hazard classification systems.

Eye safety testing procedures vary among U.S. agencies. Current testing procedures specified in the U.S. Code of Federal Regulations (16 CFR 1500.42) require 6 animals per test and may require up to three sequential tests for each substance, thereby requiring 6, 12, or 18 animals to reach a hazard decision (CPSC 2010). The requirement for second and third sequential tests is based on the number of positive responses in the previous test.

Based on previous initiatives in the United States to reduce the number of animals used for eye safety testing, some U.S. and international test guidelines for eye irritation/corrosion testing have been modified. The maximum number of animals currently used is typically 3 (OECD 2002; EPA 1998). U.S. agencies will accept data generated in accordance with test guidelines by the Organisation for Economic Co-operation and Development (OECD) that require only 3 animals per test. However, current testing procedures (16 CFR 1500.42) do not provide criteria to classify results from 3-animal tests. Therefore, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), conducted an analysis (Haseman et al. 2011) to determine classification criteria based on results from a 3-animal test that would maintain hazard classification equivalent to that provided by current testing procedures.

ICCVAM is charged by law with reviewing and evaluating alternative methods and approaches that can reduce animal use in testing. This test method evaluation report provides ICCVAM's recommendations for using fewer animals to identify chemical eye hazards while maintaining hazard classification equivalent to that provided by current testing procedures (16 CFR 1500.42). The process for developing these recommendations began with a critical review of the analysis (Haseman et al. 2011) and existing data by the ICCVAM Interagency Ocular Toxicity Working Group (OTWG). As part of ICCVAM's ongoing international collaborations, scientists from the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM) served as liaisons to the OTWG.

The analysis (Haseman et al. 2011) was provided to the Scientific Advisory Committee on the Validation of Alternative Toxicological Methods (SACATM) for comment. The public was also given the opportunity to comment. The OTWG then developed draft proposed ICCVAM recommendations regarding classification criteria based on results from a 3-animal test that would maintain hazard classification equivalent to that provided by current testing procedures (16 CFR 1500.42). The draft ICCVAM recommendations and the supporting analysis (Haseman et al. 2011) were made available on the NICEATM-ICCVAM website (<http://iccvam.niehs.nih.gov>) for comment by the broad stakeholder community. ICCVAM considered all public and SACATM comments before finalizing these recommendations. This ICCVAM test method evaluation report presents the recommendations and supporting analysis.

As required by the ICCVAM Authorization Act (42 U.S.C. 2851-3), ICCVAM will forward the recommendations to U.S. Federal agencies for consideration. Federal agencies are required to respond to ICCVAM within 180 days after receiving the ICCVAM recommendations. This report is available

to the public on the NICEATM–ICCVAM website (<http://iccvam.niehs.nih.gov>), and agency responses will be made available on the website as they are received.

We gratefully acknowledge the many individuals who contributed to the preparation, review, and revision of this report. We thank the OTWG for assuring a meaningful and comprehensive review. We especially thank Dr. Jill Merrill (U.S. Food and Drug Administration Center for Drug Evaluation and Research) for serving as Chair of the OTWG. Integrated Laboratory Systems, Inc., the NICEATM support contractor, provided excellent technical support, for which we thank Drs. David Allen, Elizabeth Lipscomb, Lori Rinckel, and Mr. James Truax. Finally, we thank the OTWG liaisons from our partner organizations in the International Cooperation on Alternative Test Methods for their participation in this review. Drs. João Barroso and Valerie Zuang were the liaisons from EURL ECVAM, and Dr. Hajime Kojima was the liaison from JaCVAM.

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Executive Summary

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), recently conducted an analysis to determine classification criteria that would identify chemical eye hazards with fewer animals (Haseman et al. 2011). NICEATM–ICCVAM analyzed results from 3-animal tests that would maintain eye hazard classification equivalent to that provided by current testing procedures specified in the U.S. Code of Federal Regulations (16 CFR 1500.42). Current testing procedures require 6 animals per test and may require up to three sequential tests for each substance, thereby requiring 6, 12, or 18 animals to reach a hazard decision (CPSC 2010).

In 2002, the Organisation for Economic Co-operation and Development (OECD) Test Guidelines Programme adopted U.S. proposed revisions to Test Guideline 405: Acute Eye Irritation/Corrosion (OECD 2002) to reduce the maximum number of animals required for eye hazard classification from 6 to 3. The Animal Welfare Act (7 U.S.C. 2131 et seq.) requires that only the minimum number of animals necessary to obtain scientifically valid results be used for testing. The Public Health Service Policy on Humane Care and Use of Laboratory Animals requires that a rationale for the appropriateness of the number of animals be provided to and approved by the Institutional Animal Care and Use Committee (OLAW 2002). In light of these policies and regulations, most *in vivo* eye safety testing would be expected to adhere to the 3-animal procedure described in the OECD and U.S. Environmental Protection Agency test guidelines (EPA 1998; OECD 2002). However, current testing procedures (16 CFR 1500.42) do not provide criteria to classify results obtained from a 3-animal test. Therefore, NICEATM–ICCVAM conducted an analysis to determine classification criteria based on results from a 3-animal test that would maintain hazard classification equivalent to that provided by current testing procedures. This analysis (Haseman et al. 2011) forms the basis for the ICCVAM recommendations described herein.

ICCVAM Recommendations

ICCVAM recommends that alternative *in vitro* test methods should always be considered and used where appropriate for eye safety testing. While currently approved *in vitro* test methods can identify some eye hazards (OECD 2009a, OECD 2009b), they are not sufficiently validated and accepted to completely replace all animal testing. When eye safety testing for those regulatory authorities still requiring the use of animals is necessary, testing should be conducted using the minimum number of animals in the most humane manner possible consistent with testing objectives.

ICCVAM concludes that using a classification criterion of one or more positive animals in a 3-animal test to identify chemicals and products that are eye hazards will maintain hazard classification equivalent to that provided by current testing procedures (16 CFR 1500.42 [CPSC 2010]), while using up to 50% to 83% fewer animals. ICCVAM therefore recommends consideration of the use of this classification criterion together with eye safety testing procedures that use a maximum of 3 animals per test substance. Consistent with ICCVAM's duty to foster national and international harmonization (42 U.S.C. 285l-3), this recommendation also harmonizes the number of animals used for eye safety testing across U.S. regulatory agencies and international test guidelines.

Analysis Supporting the Use of Fewer Animals for Evaluating Eye Hazards

The percentage of substances that would be classified as eye irritants was calculated for each of the three different classification criteria:

- Strategy 1: current testing procedures (16 CFR 1500.42)
- Strategy 2: at least one positive animal in a 3-animal test ($\geq 1/3$)
- Strategy 3: at least two positive animals in a 3-animal test ($\geq 2/3$)

In order to compare the frequency with which each strategy would identify substances as eye irritants, NICEATM–ICCVAM examined a number of different underlying population positive response rates. (The population positive response rate is the overall likelihood that an animal will show a positive response for a given substance.) In a separate approach, a NICEATM database of 481 rabbit eye test studies was analyzed using a mixture of three binomial distributions to estimate rates of over- and underprediction for each criterion.

In each instance, a classification criterion of at least one positive animal in a 3-animal test ($\geq 1/3$) more closely matched the expected outcome based on current testing procedures (16 CFR 1500.42) than did a criterion of at least two positive animals in a 3-animal test ($\geq 2/3$), which identified far fewer irritants. These results showed that using a classification criterion of at least one positive animal in a 3-animal test ($\geq 1/3$) to identify eye hazards will provide eye hazard classification the same as or greater than current testing procedures, while using up to 50% to 83% fewer animals.

ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process incorporates a high level of transparency. This process is designed to provide numerous opportunities for stakeholder involvement, including written comments and oral comments at the public meetings of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). In finalizing this test method evaluation report and the supporting analysis (Haseman et al. 2011), ICCVAM considered comments provided by SACATM and the public.

Four different opportunities for public comments were provided during the ICCVAM evaluation process. Three public comments, which supported using fewer animals to identify chemical eye hazards, were received (**Section 4.0**). SACATM members and two *ad hoc* experts agreed that the proposed 33% positive response rate provides appropriate criteria for eye safety testing compared to current testing procedures (16 CFR 1500.42).

1.0 Introduction

In 2002, the Organisation for Economic Co-operation and Development (OECD) Test Guidelines Programme adopted U.S. proposed revisions to Test Guideline 405: Acute Eye Irritation/Corrosion (OECD 2002) to reduce the maximum number of animals required for eye hazard classification from 6 to 3. The Animal Welfare Act (7 U.S.C. 2131 et seq.) requires that only the minimum number of animals necessary to obtain scientifically valid results be used for testing. The Public Health Service Policy on Humane Care and Use of Laboratory Animals requires that a rationale for the appropriateness of the number of animals be provided to and approved by the Institutional Animal Care and Use Committee (OLAW 2002). In light of these policies and regulations, most *in vivo* eye safety testing would be expected to adhere to the 3-animal procedure described in the OECD and U.S. Environmental Protection Agency test guidelines (EPA 1998; OECD 2002). However, current testing procedures specified in the U.S. Code of Federal Regulations (16 CFR 1500.42) do not provide criteria to classify results obtained from a 3-animal test (CPSC 2010). Therefore, the National Toxicology Program Interagency Committee for the Evaluation of Alternative Toxicological Methods (NICEATM), in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), conducted an analysis to determine classification criteria based on results from a 3-animal test that would maintain hazard classification equivalent to that provided by current testing procedures. This analysis (Haseman et al. 2011) forms the basis for the ICCVAM recommendations described herein.

In accordance with the ICCVAM Authorization Act of 2000 (42 U.S.C. 285l-3), ICCVAM coordinates the technical evaluation of new, revised, and alternative test methods with regulatory applicability. The ICCVAM Interagency Ocular Toxicity Working Group (OTWG) worked with NICEATM in conducting the analysis. The European Union Reference Laboratory for Alternatives to Animal Testing and the Japanese Center for the Validation of Alternative Methods designated liaison members to the OTWG.

ICCVAM provided the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) with the analysis to determine classification criteria based on results from a 3-animal test that would maintain hazard classification equivalent to that provided by current testing procedures (16 CFR 1500.42) for discussion at their meeting on June 17–18, 2010. Public stakeholders were given the opportunity to comment at the meeting. A second opportunity for SACATM and public comments was provided at the SACATM meeting on June 16–17, 2011. On August 12, 2011, ICCVAM announced the availability of the draft ICCVAM recommendations. The draft ICCVAM recommendations and the supporting analysis (Haseman et al. 2011) were posted on the NICEATM-ICCVAM website (<http://iccvam.niehs.nih.gov/>). A detailed timeline of the ICCVAM evaluation for identifying chemical eye hazards with fewer animals is included with this report (**Appendix A**).

ICCVAM considered all public and SACATM comments (**Appendix B**) before finalizing its recommendations. The recommendations and the supporting analysis (Haseman et al. 2011) are presented in this ICCVAM test method evaluation report. As required by the ICCVAM Authorization Act (42 U.S.C. 285l-3), ICCVAM will forward the recommendations to U.S. Federal agencies for consideration. Federal agencies are required to respond to ICCVAM within 180 days after receiving the ICCVAM recommendations. This report is available to the public on the NICEATM-ICCVAM website, and agency responses will be made available on the website as they are received.

2.0 ICCVAM Recommendations: Identifying Chemical Eye Hazards with Fewer Animals

2.1 Introduction and Applicable Regulatory Requirements

Relevant U.S. and international ocular toxicity regulations and test guidelines are summarized in **Appendix C**. Eye safety testing procedures vary among U.S. agencies. Current testing procedures specified in 16 CFR 1500.42 provide criteria and procedures for identifying eye hazards based on rabbit eye test results (CPSC 2010). However, current testing procedures do not provide criteria to classify results from a 3-animal test. Therefore, NICEATM–ICCVAM conducted an analysis to determine classification criteria based on results from a 3-animal test that would maintain hazard classification equivalent to that provided by current testing procedures (Haseman et al. 2011).

In the analysis (Haseman et al. 2011), the frequency with which current testing procedures (16 CFR 1500.42) identify substances as eye irritants was compared with the frequency with which a classification criterion of either at least one or two positive animals in a 3-animal test would identify these substances. A number of different underlying population positive response rates for identifying substances as eye irritants were examined. A NICEATM database of 481 rabbit eye test studies using 6 animals per test was also used to estimate over- and underprediction rates for each criterion using a mixture of three binomial distributions. In each instance, a classification criterion of at least one positive animal in a 3-animal test more closely matched the expected outcome based on current testing procedures, while a criterion of at least two positive animals in a 3-animal test identified far fewer irritants. These results showed that using a classification criterion of at least one positive animal in a 3-animal test to identify eye hazards will provide the same as or greater than level of eye hazard classification as current testing procedures, while using up to 50% to 83% fewer animals. ICCVAM developed the following recommendations based on the results of this analysis (Haseman et al. 2011).

2.2 ICCVAM Recommendations

ICCVAM recommends that alternative *in vitro* test methods should always be considered and used where appropriate for eye safety testing. While currently approved *in vitro* test methods can identify some eye hazards (OECD 2009a, 2009b), they are not sufficiently validated and accepted to completely replace all animal testing. When eye safety testing for those regulatory authorities still requiring the use of animals is necessary, testing should be conducted using the minimum number of animals in the most humane manner possible consistent with testing objectives.

ICCVAM concludes that using a classification criterion of one or more positive animals in a 3-animal test to identify chemicals and products that are eye hazards will maintain hazard classification equivalent to that provided by current testing procedures (16 CFR 1500.42 [CPSC 2010]), while using up to 50% to 83% fewer animals. ICCVAM therefore recommends consideration of the use of this classification criterion together with eye safety testing procedures that use a maximum of 3 animals per test substance. Consistent with ICCVAM's duty to foster national and international harmonization (42 U.S.C. 285l-3), this recommendation also harmonizes the number of animals used for eye safety testing across U.S. regulatory agencies and international test guidelines.

3.0 Analysis Supporting the Use of Fewer Animals for Evaluating Eye Hazards

3.1 Current Testing Procedures (16 CFR 1500.42)

Current testing procedures specified in 16 CFR 1500.42 provide information on conducting the rabbit eye test, a description of positive responses for individual animals, and a testing strategy for determining the overall results of the test (CPSC 2010) (**Table 3-1**). Testing is conducted using an initial group of 6 albino rabbits, and 0.1 mL or 0.1 gram of the test substance is placed in the conjunctival sac of one eye with the contralateral eye serving as a negative or solvent control. The eyes are examined 24, 48, and 72 hours after test substance administration. Severity scores are recorded for the following eye injuries: corneal ulceration/opacity, iritis, conjunctival swelling, and conjunctival redness (**Table 3-2**). Positive responses for individual animals are based on meeting or exceeding the minimum severity criteria for any of the types of eye injuries at any of the three time points (**Table 3-2**). Significant corneal ulceration can be used as a humane endpoint to terminate a study (OECD 2002). The number of animals exhibiting a positive response in each test group determines whether the hazard test result is positive, negative, or if a second or third test is required (**Table 3-1**).

Table 3-1 Current Testing Procedures (16 CFR 1500.42)

<p>Positive Responses for Individual Animals^a</p>	<p>Corneal ulceration^b or corneal opacity^c ≥ 1 Iritis^d ≥ 1 Conjunctival swelling^e ≥ 2 Conjunctival redness^e ≥ 2</p>
<p>Testing Strategy – Positive, Negative, or Repeat Test</p>	<p><u>First Test: Test 6 animals</u></p> <ul style="list-style-type: none"> • If $\geq 4/6$ animals are positive, the test is positive. • If ≤ 1 animal is positive, the test is negative. • If $2/6$ or $3/6$ animals are positive, a second test is conducted using a different group of 6 animals. <p><u>Second Test: Test 6 animals</u></p> <ul style="list-style-type: none"> • If $\geq 3/6$ animals are positive, the test is positive. • If $0/6$ are positive, the test is negative. • If $1/6$ or $2/6$ is positive, a third test is conducted using a different group of 6 animals. <p><u>Third Test: Test 6 animals</u></p> <ul style="list-style-type: none"> • If $\geq 1/6$ animals are positive, the test is positive. • If $0/6$ are positive, the test is negative.

^a Based on meeting or exceeding the minimum severity criteria for any of the types of eye injuries at 24, 48, and 72 hours, as outlined in the *Illustrated Guide for Grading Eye Irritation Caused by Hazardous Substances*, referenced in 16 CFR 1500.42 (see **Table 3-2**).

^b Ulceration of the cornea (other than a fine stippling)

^c Opacity of the cornea (other than a slight dulling of the normal luster)

^d Inflammation of the iris (other than a slight deepening of the folds [or rugae] or a slight circumcorneal injection of the blood vessels)

^e Obvious conjunctival swelling with partial eversion of the lids or conjunctival redness with diffuse crimson red; individual vessels not easily discernible

Table 3-2 Scores for Grading Severity of Eye Lesions

Lesion ^a	Score ^b
Cornea	
No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Complete corneal opacity, iris not discernible	4
Iris	
Normal	0
Markedly deepened folds, congestion, swelling, moderate circumcorneal injection (any one of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any one or all of these)	2
Conjunctiva	
A. Redness (refers to palpebral and bulbar conjunctiva only)	
Normal	0
Some vessels definitely injected above normal	1
Diffuse, crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
B. Chemosis	
Normal	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of the lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4

Table is adapted from the *Illustrated Guide for Grading Eye Irritation Caused by Hazardous Substances*, referenced in 16 CFR 1500.42.

^a Positive responses for individual animals are based on meeting or exceeding the minimum severity criteria for any of the types of eye injuries at any of the three time points.

^b Scores in **bold** indicate positive responses.

The United States proposed revisions to OECD Test Guideline 405: Acute Eye Irritation/Corrosion (OECD 1987) to reduce the maximum number of required animals by 50% from 6 to 3 (de Silva et al. 1997; OECD 1999; Springer et al. 1993). The revised Test Guideline 405 was adopted in 2002

(OECD 2002). In accordance with the OECD Mutual Acceptance of Data Treaty (OECD 1981), U.S. agencies accept for review test data generated in accordance with OECD test guidelines.

The Animal Welfare Act (7 U.S.C. 2131 et seq.) requires that only the minimum number of animals necessary to obtain scientifically valid results be used for testing. The Public Health Service Policy on Humane Care and Use of Laboratory Animals requires that a rationale for the appropriateness of the number of animals be provided to and approved by the Institutional Animal Care and Use Committee (OLAW 2002). In light of these policies and regulations, most *in vivo* eye safety testing would be expected to adhere to the 3-animal procedure described in the OECD and U.S. Environmental Protection Agency test guidelines (EPA 1998; OECD 2002). However, current testing procedures (16 CFR 1500.42) do not provide criteria to classify results from a 3-animal test. Therefore, NICEATM–ICCVAM conducted an analysis (Haseman et al. 2011) to determine classification criteria based on results from a 3-animal test that would maintain hazard classification equivalent to that provided by current testing procedures.

3.2 Optimization of the Number of Positive Animals Required to Identify a Substance as an Irritant

To determine the optimal number of positive animals required to identify a substance as an irritant, the minimum number of positive animals necessary to classify an irritant by current testing procedures (16 CFR 1500.42) was evaluated for each of the possible test outcomes. As indicated in **Table 3-3**, the weakest possible response that is considered positive by the current sequential testing strategy is 22% (2/6 + 1/6 + 1/6, or 4/18), while a response of 17% (1/6 or 3/18) is considered negative. Therefore, it could be argued that the threshold positive response rate for considering a substance as an irritant for current testing procedures should logically lie between 17% and 22%, perhaps 20%. However, this conclusion is complicated by the fact that an observed response rate of 28% (3/6 + 2/6 + 0/6, or 5/18) may occur and result in a chemical to not be classified as an irritant (**Table 3-3**). Ideally, a testing strategy should not produce inconsistent results, in which the percentage of positive animal responses that can result in an irritant classification overlaps with the percentage that do not result in an irritant classification.

Table 3-3 Number of Positive Animals and Sequential Tests Required for Assignment of an Irritant Classification According to Current Testing Procedures (16 CFR 1500.42)

Positive Test Criteria for Irritant Classification	Positive Animals					
	≥4/6	2/6 or 3/6	3/6	3/6	2/6	2/6
First Test Results	≥4/6	2/6 or 3/6	3/6	3/6	2/6	2/6
Results from Second Test (when required)	Second test not required	≥3/6	2/6	1/6	2/6	1/6
Results from Third Test (when required)	Third test not required	Third test not required	≥1/6	≥1/6	≥1/6	≥1/6
Minimum Number of Positive Animals for Irritant Classification	4/6 (67%)	5/12 (42%)	6/18 (33%)	5/18 (28%)	5/18 (28%)	4/18 (22%)
Maximum Number of Positive Animals for Not Classified as an Irritant	1/6 (17%)	3/12 (25%)	5/18 (28%)	4/18 (22%)	4/18 (22%)	3/18 (17%)

3.3 Comparison of Three Strategies for Reducing Animal Use

The percentage of substances that would be classified as eye hazards was calculated for each of three different decision strategies. The first strategy (Strategy 1) used current testing procedures (16 CFR 1500.42) to identify eye hazards. The second strategy (Strategy 2) used a minimum threshold of $\geq 1/3$ (33%) positive animals. The third strategy (Strategy 3) used a minimum threshold of $\geq 2/3$ (67%) positive animals.

The frequency with which each strategy would identify substances as eye irritants was calculated for a number of different underlying population positive response rates. This population positive response rate, denoted by p , is the overall likelihood that an animal will show a positive response for a given substance. Importantly, it is a ‘population’ response rate, not the response rate observed in a given sample of 3 to 6 animals. However, for a specified value of p , it is possible to compute the likelihood of observing various responses in a given sample using binomial probabilities. This is illustrated in **Table 3-4** for a general p , and for $p = 20\%$ and $p = 60\%$ to provide specific examples. For example, for a substance with an underlying positive response rate of $p = 60\%$, the likelihood is 0.311 (31.1%) that there will be exactly 4 positive animals in a sample of 6 animals.

Table 3-4 Probability of Observing 0 to 6 Positive Animals in a Sample of $n = 3$ or $n = 6$ for Various Population Positive Response Rates (p) Assuming a Binomial Model

No. Positive Animals in a Sample	Probability of Response in Sample		Probability of Response in Sample			
	$n = 3$	$n = 6$	$n = 3$	$n = 3$	$n = 6$	$n = 6$
			$p = 20\%$	$p = 60\%$	$p = 20\%$	$p = 60\%$
0	$(1-p)^3$	$(1-p)^6$	0.512	0.064	0.262	0.004
1	$3p(1-p)^2$	$6p(1-p)^5$	0.384	0.288	0.393	0.037
2	$3p^2(1-p)$	$15p^2(1-p)^4$	0.096	0.432	0.246	0.138
3	p^3	$20p^3(1-p)^3$	0.008	0.216	0.082	0.276
4	-	$15p^4(1-p)^2$	-	-	0.015	0.311
5	-	$6p^5(1-p)$	-	-	0.002	0.187
6	-	p^6	-	-	<0.001	0.047

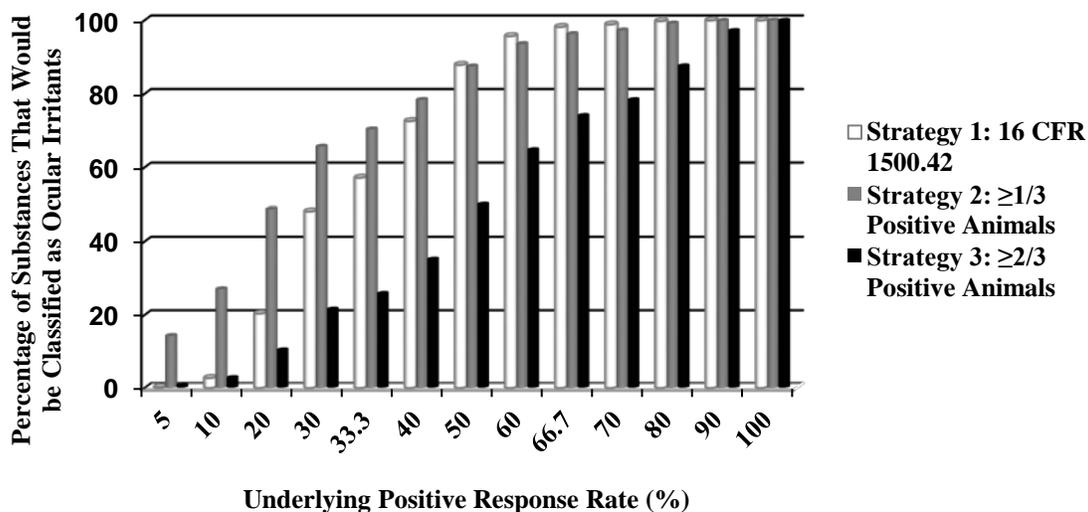
Table 3-5 presents the likelihood of classifying a substance as an eye irritant for various underlying values of p . However, it does not show whether or not this classification is ‘correct’ because this would require knowledge of the underlying positive response rate that differentiates irritants from nonirritants. However, because the underlying positive response rates in a population that are characteristic of an irritant or a nonirritant are not definitively known (see **Table 3-3**), a range of different underlying positive response rates were compared (**Table 3-5**) and presented graphically in **Figure 3-1**.

Table 3-5 Percentage of Substances Classified as Eye Irritants Based on Various Population Positive Response Rates (*p*) for the Three Strategies

Population Positive Response Rate (<i>p</i>)	Percentage of Substances That Would be Classified as Eye Irritants		
	Strategy 1 16 CFR 1500.42	Strategy 2 ≥1/3 Positive Animals	Strategy 3 ≥2/3 Positive Animals
1.7% ^a	0.0%	5.0%	0.1%
5%	0.2%	14.3%	0.8%
10%	2.7%	27.1%	2.8%
20%	20.4%	48.8%	10.4%
30%	48.2%	65.7%	21.6%
33.3%	57.2%	70.4%	25.9%
40%	72.6%	78.4%	35.2%
50% ^a	87.9%	87.5%	50.0%
60%	95.7%	93.6%	64.8%
66.7%	98.2%	96.3%	74.1%
70%	98.9%	97.3%	78.4%
80%	99.8%	99.2%	87.6%
90%	100%	99.9%	97.2%
97.8% ^a	100%	100%	99.9%
100%	100%	100%	100%

^a Estimated underlying positive response rates for the NICEATM database (see **Table 3-7**)

Figure 3-1 Strategy 2 Provides Eye Hazard Classification the Same as or Greater Than Current Testing Procedures (16 CFR 1500.42)



For purposes of illustration, consider $p = 20\%$. **Table 3-6** summarizes all the possible ways in which Strategy 1 could lead to a negative classification for a substance with a 20% population positive

response rate. The probabilities in **Table 3-6** are derived from **Table 3-4**. Thus, by subtraction from 1.0, the likelihood of a positive classification for Strategy 1 for $p = 20\%$ is $1 - 0.796$, or 0.204 or 20.4% (see **Table 3-5**).

Table 3-6 Probability That Strategy 1 Will Result in a Negative Classification for $p = 20\%$

Strategy 1 Test Result			Probability
Test 1	Test 2	Test 3	
0/6	-	-	0.262
1/6	-	-	0.393
2/6	0/6	-	$(0.246)(0.262) = .00645$
3/6	0/6	-	$(0.082)(0.262) = 0.0215$
2/6	1/6	0/6	$(0.246)(0.393)(0.262) = 0.0253$
3/6	1/6	0/6	$(0.082)(0.393)(0.262) = 0.0084$
2/6	2/6	0/6	$(0.246)(0.246)(0.262) = 0.0159$
3/6	2/6	0/6	$(0.082)(0.246)(0.262) = 0.0053$
Total	-	-	0.796

These calculations are much simpler for Strategies 2 and 3. The likelihood of a positive classification using Strategy 2, assuming $p = 20\%$, is just the likelihood of observing 1/3, 2/3, or 3/3 positive responses. Using the probabilities in **Table 3-4**, the likelihood is $0.384 + 0.096 + 0.008 = 0.488$, or 48.8% (see **Table 3-5**). For Strategy 3 and $p = 20\%$, the likelihood of a positive classification is the sum of the likelihood of observing 2/3 or 3/3 positive responses, which is $0.096 + 0.008 = 0.104$, or 10.4% (see **Table 3-5**).

Even though it uses fewer animals, Strategy 2 is more powerful than current testing procedures (16 CFR 1500.42) for detecting positive response rates of up to 40% and has approximately the same power for response rates of 50% and greater (see **Figure 3-1**). Strategy 3 identifies far fewer irritants than Strategy 2 for underlying positive response rates of 80% and less. Strategy 3 considers a single positive response (1/3) to not indicate an irritant response. Strategy 3 also has lower power than current testing procedures for underlying positive response rates of 20% to 80%.

These calculations were based on a variety of underlying positive response rates without consideration of whether or not they reflect the positive response rates seen in practice. Rather than assuming that each irritant and nonirritant has its own unique (and unknown) underlying positive response rate, a potentially useful approach is to derive a mathematical model that accurately describes the observed distribution of positive responses seen for a large database of test substances. If a definitive structure can be imposed upon the data (and if the model fits the data), then the model parameters can be used to estimate over- and underprediction rates. With this in mind, NICEATM-ICCVAM analyzed a database of 481 rabbit eye test studies that each used 6 animals per test. This database includes a wide range of chemical and product categories (Haseman et al. 2011).

To calculate the estimated over- and underprediction rates for the three strategies using the NICEATM database, the first step was to find a model that fit the observed outcomes (**Table 3-7**), some of which are irritants and some of which are nonirritants. NICEATM-ICCVAM used a model that assumed a mixture of three binomial distributions because it is unlikely that every irritant has exactly the same likelihood of producing a positive response in an animal. Irritants were categorized into two groups. Irritants with a high underlying positive response rate in an animal were designated

as Type I irritants. Irritants with a smaller underlying positive response rate in an animal were designated as Type II irritants.

From the observed distribution of positive animals in a 6-animal test, five key parameters were estimated: the underlying positive response rates for nonirritants and Type I and Type II irritants, and the percentage of Type I and Type II irritants in the database (the percentage of nonirritants in the database can then be calculated by subtraction from 100%). The following parameter estimates provided the best fit to the NICEATM database (**Tables 3-7** and **3-8**):

- Type I irritants: underlying positive response rate = 97.8%
- Type II irritants: underlying positive response rate = 50.0%
- Nonirritants: underlying positive response rate = 1.7%
- Percentage of Type I irritants in the sample: 54% or 260 substances
- Percentage of Type II irritants in the sample: 12.9% or 62 substances
- Percentage of nonirritants in the sample: 33.1% or 159 substances

Given this excellent fit to the data as indicated in **Table 3-7**, NICEATM–ICCVAM calculated the percentage of substances that would be classified as eye irritants using each of the three strategies (**Table 3-8**). The likelihood that a Type I irritant would be classified as an eye irritant is close to 100% for all three strategies. The likelihood that a Type II irritant would be classified as an eye irritant is approximately 88% for Strategies 1 and 2 but 50% for Strategy 3. The likelihood of classifying a nonirritant as an eye irritant is 0% for Strategy 1, 5.0% for Strategy 2, and 0.1% for Strategy 3 (**Table 3-8**).

Table 3-7 Goodness of Fit for a Database of 481 Test Results Using a Mixture of Three Binomial Distributions

Number of Positive Animals in a 6-Animal Test	Predicted Type I Irritants	Predicted Type II Irritants	Predicted Nonirritants	Total Predicted by NICEATM Model	Total Observed in NICEATM Database
0	0	1.0	143.4	144.4	142
1	0	5.8	15.0	20.8	21
2	0	14.5	0.6	15.1	19
3	0.1	19.4	0	19.5	15
4	1.7	14.5	0	16.2	20
5	30.7	5.8	0	36.5	35
6	227.5	1.0	0	228.5	229
Total	260 (54.0%)	62 (12.9%)	159 (33.1%)	481	481

Table 3-8 Percentage of Substances Classified as Eye Irritants Based on Estimated Underlying Positive Response Rates for Three Strategies: Three Binomial Distributions

Binomial Distribution	Estimated Underlying Positive Response Rate	Percentage of Substances That Would be Classified as Eye Irritants		
		Strategy 1 16 CFR 1500.42	Strategy 2 ≥1/3 Positive Animals	Strategy 3 ≥2/3 Positive Animals
Nonirritants	1.7%	0%	5.0%	0.1%
Type II Irritants	50%	87.9%	87.5%	50.0%
Type I Irritants	97.8%	100%	100%	99.9%

Based on these outcomes, the underlying over- and underprediction rates associated with this model were then calculated. All three strategies have a very low underprediction rate for Type I irritants. However, for Type II irritants, Strategies 1 and 2 have underprediction rates of approximately 12%, while Strategy 3 has a 50% underprediction rate. For nonirritants, Strategies 1 and 3 have very low overprediction rates, while the overprediction rate for Strategy 2 is 5% (Table 3-9).

It is important to note that this approach is similar to the approach used by Springer et al. (1993) except for the fact that NICEATM–ICCVAM assumed two different underlying positive response rates for irritants, whereas Springer et al. used only one (i.e., they assumed that every irritant has exactly the same likelihood of producing a positive response in an animal). Based on the distribution of positive animals in a 6-animal test in the NICEATM database, the use of two different underlying positive response rates for irritants provided a much better fit to the data.

Table 3-9 Percentage of Substances That Would be Over- and Underpredicted for the Three Strategies

Three Binomial Distribution	Strategy 1 16 CFR 1500.42	Strategy 2 ≥1/3 Positive Animals	Strategy 3 ≥2/3 Positive Animals
Percentage of Substances That Would be Overpredicted			
Nonirritant	0%	5.0%	0.1%
Percentage of Substances That Would be Underpredicted			
Type II Irritants	12.1%	12.5%	50.0%
Type I Irritants	0%	0%	0.1%

3.4 Previous Proposals to Reduce the Number of Animals Used for Eye Safety Testing

Results from DeSousa et al. (1984) and Talsma et al. (1988) showed that using 3 rabbits per test provided accuracy of up to 94% in predicting a 6-animal test (using subsets of 3 animals). Springer et al. (1993) also conducted analyses to determine if the standard group size of 6 rabbits for eye safety testing could be reduced in order to use fewer animals and concluded that a 3-animal test and a decision rule requiring at least 2 positive animals to classify a substance as an irritant yielded accuracy of 98%. As indicated above, the model used by Springer et al. assumed two mutually exclusive populations, irritants and nonirritants, each population having a single underlying positive response rate estimated from the data. Springer et al. fit a mixture of two binomial models to each of four different databases, but the only database with a distribution of outcomes that closely matched

the NICEATM database of 481 rabbit eye test studies was an EPA database of 48 substances. Springer et al. reported the following parameter estimates for the EPA database:

- Irritants: underlying positive response rate = 95.0%
- Nonirritants: underlying positive response rate = 8.6%
- Percentage of nonirritants in the sample: 35%
- Percentage of irritants in the sample: 65%

Note that the estimated percentage of nonirritants in the EPA database (35%) is very similar to NICEATM–ICCVAM’s estimate (33.1%) for the much larger NICEATM database, but the Springer et al. model does not differentiate between Type I and Type II irritants. As a result, their parameter estimates provided a poor fit to the NICEATM database of 481 studies (**Table 3-10**). In fact, NICEATM–ICCVAM found that the Springer et al. model did not provide a good fit to the EPA data upon which their parameter estimates were based (e.g., predicting only 0.2 outcomes when three outcomes were actually observed for 3/6 positive responses, a 15-fold underprediction). This lack of model fit was more apparent using the NICEATM database of 481 substances, which was approximately 10-fold larger than the Springer et al. (1993) EPA database.

The largest database used by Springer et al. (1993) was the 139-substance Marzulli and Ruggles database, but the pattern of response seen in this database was quite different from that seen in the NICEATM database of 481 studies. Even so, the best-fitting Springer et al. model showed the same lack-of-fit problem. For example, ten 3/6 positive responses were observed compared with only 3.1 predicted by the best-fitting Springer et al. model.

It is important to understand the factors that led to different conclusions in the NICEATM–ICCVAM evaluation, which favored Strategy 2, and that of Springer et al. (1993), which favored Strategy 3. For example, Table 1 in Springer et al. suggests that Strategy 2 may have an unacceptably high overprediction rate.

Table 3-10 Lack of Fit Using the Springer et al. (1993) Model on the NICEATM Database

Number of Positive Animals in a 6-Animal Test	Springer Model Predicted Irritants	Springer Model Predicted Nonirritants	Total Predicted by Springer Model	Total Observed in NICEATM Database
0	0	98.2	98.2	142
1	0	55.5	55.5	21
2	0	13.0	13.0	19
3	0.7	1.6	2.3	15
4	9.5	0.1	9.6	20
5	72.6	0	72.6	35
6	229.8	0	229.8	229
Total	312.6 (65%)	168.4 (35%)	481	481

The primary reason for the different conclusions is that the EPA 48-substance database was of insufficient size to detect the Type II irritants that were producing positive response rates of approximately 50%. By not taking these irritants into account, the Springer et al. (1993) model underestimated the underprediction rate for Strategy 3 because this strategy does not perform well for detecting positive response rates of approximately 50% (see **Table 3-5**).

Another consequence of Springer et al. (1993) ignoring the Type II irritants was a 5-fold overestimation of the positive response rate of nonirritants. This difference is important because the overprediction rate of Strategy 2 increases substantially as the assumed positive response rate for nonirritants increases (see **Table 3-5**). It is the Springer et al. overestimation of the positive response rate for nonirritants that produced the artificially high overprediction rate for Strategy 2 shown in their Table 1.

3.5 Animal Welfare Considerations

This analysis (Haseman et al. 2011) reduces animal use because it should facilitate regulatory decisions on classification criteria that will support the adoption of test methods using fewer animals. It also harmonizes the number of animals used for eye safety testing with current EPA (1998) and OECD (2002) testing guidelines, thereby reducing the number of tests that should need to be performed.

4.0 ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process provides numerous opportunities for public stakeholder involvement, including submission of written comments and oral comments at the public SACATM meetings. **Table 4-1** lists the four opportunities for public comments that were provided during the ICCVAM evaluation process (**Appendix A**). The number of public comments received in response to each of the opportunities is indicated. Three public comments were received. Comments received in response to or related to the *Federal Register* notices are included in **Appendix B** and are accessible on the NICEATM–ICCVAM website (<http://iccvam.niehs.nih.gov/>). The following sections briefly discuss the public comments received.

Table 4-1 Opportunities for Public Comment

Opportunity for Public Comment	Date	Number of Public Comments Received
75 FR 26757: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	May 12, 2010	0
SACATM Meeting, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina	June 17–18, 2010	1
SACATM Meeting, Hilton Arlington, Arlington, Virginia	June 16–17, 2011	0
76 FR 50220: Availability of Draft ICCVAM Recommendations; Request for Comments	August 12, 2011	2

4.1 Public Comments in Response to 75 FR 26757 (May 12, 2010)

Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

The SACATM meeting was announced, and written and public oral comments on the agenda topics were requested.

No written public comments were received in response to this *Federal Register* notice.

4.2 Public and SACATM Comments: SACATM Meeting on June 17–18, 2010

The SACATM meeting included a discussion of the current issues in the validation of alternative methods for assessing chemically induced eye injuries, which included an overview of the analysis (Haseman et al. 2011) conducted to determine classification criteria based on results from a 3-animal test that would maintain eye hazard classification equivalent to that provided by current testing procedures specified in 16 CFR 1500.42 (CPSC 2010).

SACATM Comment

SACATM members and two *ad hoc* experts praised the statistical analysis (Haseman et al. 2011) and agreed that the proposed 33% positive response rate provides appropriate criteria for eye safety testing compared to current testing procedures (16 CFR 1500.42).

Public Comment

One oral comment relevant to this discussion was provided.

An individual shared her story of having been in a serious automobile accident many years ago in which airbags deployed, and she suffered chemical burns to her eyes from the chemical powder in the airbag. The chemical eye injuries caused permanent damage, with complete loss of vision in one eye and severe visual impairment in the other eye. She urged the committee to bring more attention to the

danger of serious eye injuries associated with the chemicals in airbags in older cars and the importance of warnings for consumers about the presence of chemicals that can cause severe or permanent eye injuries.

ICCVAM Response

ICCVAM appreciates her unique perspective on the importance of eye hazard labeling and her willingness to share her story, which emphasizes the need for accurate testing and appropriate hazard classification and labeling.

4.3 Public and SACATM Comments: SACATM Meeting on June 16–17, 2011

The NICEATM–ICCVAM update presented at the SACATM meeting included a brief summary of the analysis (Haseman et al. 2011) conducted to determine classification criteria based on results from a 3-animal test that would maintain eye hazard classification equivalent to that provided by current testing procedures (16 CFR 1500.42).

SACATM Comment

No SACATM member comments specific to this agenda topic were provided.

Public Comment

No public comments specific to this agenda topic were provided.

4.4 Public Comments in Response to 76 FR 50220 (August 12, 2011)

Availability of Draft ICCVAM Recommendations on Using Fewer Animals to Identify Chemical Eye Hazards: Revised Criteria Necessary to Maintain Equivalent Hazard Classification; Request for Comments

NICEATM requested public comments on the draft ICCVAM recommendations that were based on the analysis (Haseman et al. 2011) conducted to determine classification criteria based on results from a 3-animal test that would maintain eye hazard classification equivalent to that provided by current testing procedures (16 CFR 1500.42).

NICEATM received two written comments in response to this *Federal Register* notice.

A comment from an individual supported using fewer animals but further encouraged the use of non-animal methods.

ICCVAM Response

Current U.S. animal welfare laws, regulations, and policies require that the fewest animals necessary for statistically significant results should be used. Investigators proposing the use of animals for eye testing must provide written documentation of their consideration of alternative methods that can reduce or avoid the use of animals and lessen or avoid unrelieved pain and distress. Alternative methods should be used when determined to be appropriate. Adequate consideration and appropriate use of available reduction, refinement, and replacement alternatives must be documented and approved by Institutional Animal Care and Use Committees before tests are conducted in animals (OLAW 2002). However, while currently approved *in vitro* test methods can identify some eye hazards (OECD 2009a, 2009b), they are not currently sufficiently validated to completely replace all animal testing. Until there are valid *in vitro* alternatives that can completely replace the use of animals for eye safety testing, reduction and refinement strategies will be critical to promoting animal welfare (ICCVAM 2010).

A second comment provided by the Association for Research in Vision and Ophthalmology supported the draft ICCVAM recommendation that eye safety testing should adhere to the 3-animal procedure, as described in Test Guideline 405 (OECD 2002) and by the EPA (1998). The association stated that the proposed regulatory change is in the spirit of “the 3Rs” and would be good for harmonizing

guidelines. The association also found the statistical approach used in the analysis (Haseman et al. 2011) to be reasonable.

ICCVAM Response

ICCVAM appreciates the time and effort that was dedicated to review of the analysis (Haseman et al. 2011) and thanks the association for their support of the draft ICCVAM recommendations and the statistical approach. ICCVAM agrees that harmonizing the number of animals used for eye safety testing with current testing guidelines for the EPA (1998) and the OECD (2002) is in the spirit of “the 3Rs” and will reduce the number of tests that should need to be performed.

5.0 References

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