ICCVAM Recommendations on the Usefulness and Limitations of the Cytosensor® Microphysiometer (CM) Test Method for Ocular Safety Testing

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Abstract

ICCVAM recently evaluated several *in vitro* test methods as potential replacements for the rabbit eye test for identifying potential ocular hazards. None of the methods were considered adequate as complete replacements. However, ICCVAM concluded that test substances within a defined limited applicability domain (water-soluble surfactants, surfactant-containing formulations, and nonsurfactants) that are positive for severe effects in CM can be classified as ocular corrosives/severe irritants (EPA Category I, EU R41, GHS Category 1). False positive rates ranged from 0% (0/17, 0/18) to 10% (3/29) and false negative rates from 9% (2/23) to 50% (6/12) depending on the hazard classification system used. ICCVAM also concluded that test substances within an even more restricted applicability domain (water-soluble surfactant chemicals and certain types of surfactant-containing formulations, but **not** nonsurfactants) can be considered as not classified for ocular hazards (EPA Category IV, EU Not Labeled, FHSA Not Labeled) without any further testing if they are negative in CM. Although false positive rates were high (50% [3/6] to 69% [18/26]), false negative rates ranged from 0% (0/27, 0/28, or 0/40) to 2% (1/46 or 1/47) depending on the hazard classification system used. A chemical that produces a response in CM between these two extremes would require additional testing (*in vitro* and/or *in vivo*) to establish a definitive classification. CM is not considered valid for identification of mild or moderate ocular irritants (EPA Categories II/III; EU R36; GHS Categories 2A/2B). ICCVAM also recommended a standardized CM protocol and future studies to expand the applicability domain of CM. These recommendations have been forwarded to Federal agencies. If accepted, CM will be the first *in vitro* test method available in the U.S. for identifying substances that do not require ocular hazard labeling.

Introduction

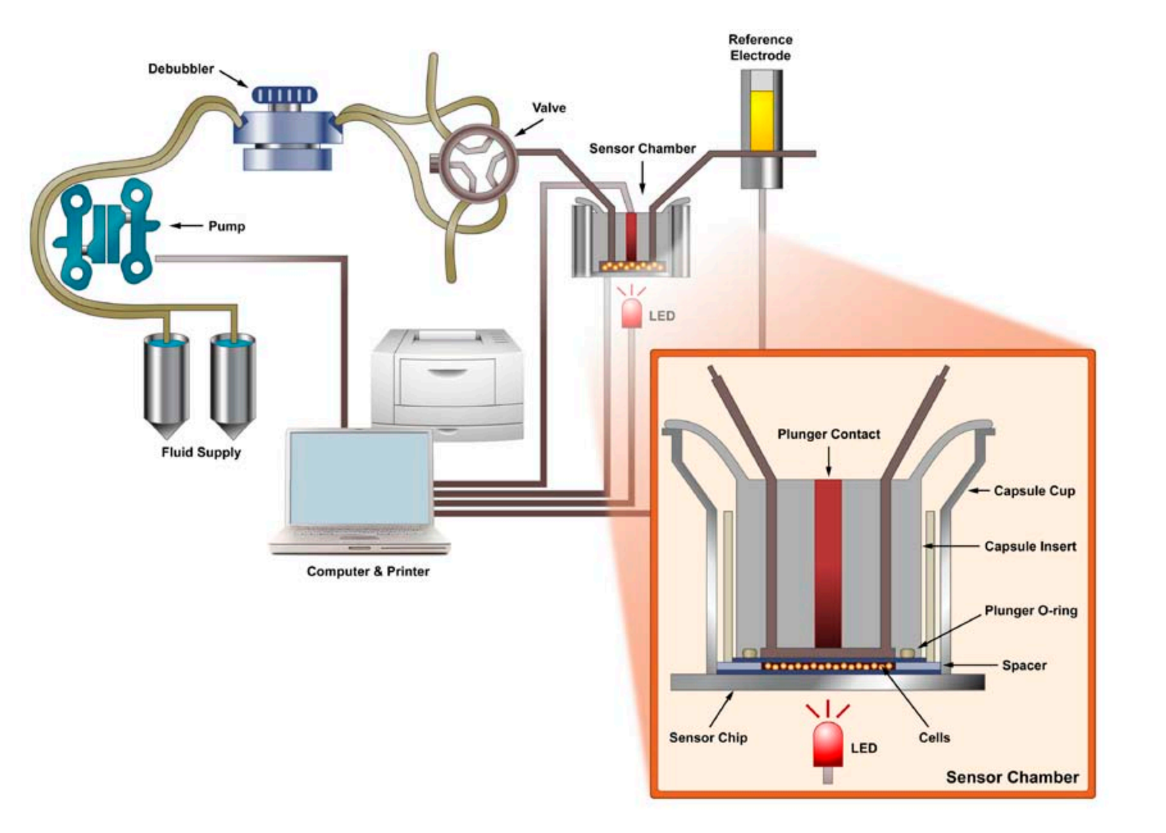
- The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is charged with evaluating the scientific validity of new, revised, and alternative toxicological test methods applicable to U.S. Federal agency safety testing requirements (ICCVAM 2000).
  - ICCVAM forwards recommendations to Federal agencies.
  - Agencies must respond to ICCVAM within 180 days.

- As part of a series of activities relevant to ocular safety testing nominated by EPA in 2003, ICCVAM recently completed a review of the current validation status of CM for the identification of substances that cause reversible and/or irreversible eye injuries.
- ICCVAM recommendations were published in September 2010.
- *Test Method Evaluation Report: Current Validation Status of In Vitro Test Methods Proposed for Identifying Eye Injury Hazard Potential of Chemicals and Products* (ICCVAM 2010):
  - CM usefulness and limitations
  - CM protocol
  - Recommended future studies

Test Method Description

- Estimates changes in cellular metabolism (i.e., glucose utilization rate) of mouse L929 fibroblasts by monitoring the rate of excretion of acid byproducts as measured by the resulting decrease in pH of the surrounding medium in an enclosed chamber (**Figures 1 and 2**).
  - Rate of pH change per unit time approximates the metabolic rate of the cell population
- Test substance concentration that results in a 50% reduction in acidification rate (i.e., MRD_{50} [metabolic rate decrement of 50%]) is the endpoint used as a correlate to potential eye irritation (**Figure 3**).
- Testing is restricted to water-soluble substances.

Figure 1. Diagram of the Operating Components of CM[1]



[1] Figure modified from the CM manual

Figure 2. ICCVAM-Recommended Protocol for CM[1]

Prepare Instrument and Cells

1. Prepare instrument and check background rates
2. Seed each capsule cup with L929 mouse fibroblast cells in seeding medium
3. Incubate L929 cells at 37±1°C in 5±1% CO_2 for 16-32 hrs in seeding medium
4. Prior to the start of the test, medium in the capsule cups is changed to low-buffered treatment medium

Prepare Test Substance

1. Evaluate test substance suitability:
   - Must be water-soluble
   - May not be viscous or a suspension
   - Other considerations depending on test objective
2. Prepare seven concentrations of test substance
   - Serial three-fold dilutions starting at 10 mg/mL in low-buffered treatment medium

   Prepare positive control to be run concurrently

Perform Dose Range-finding Test

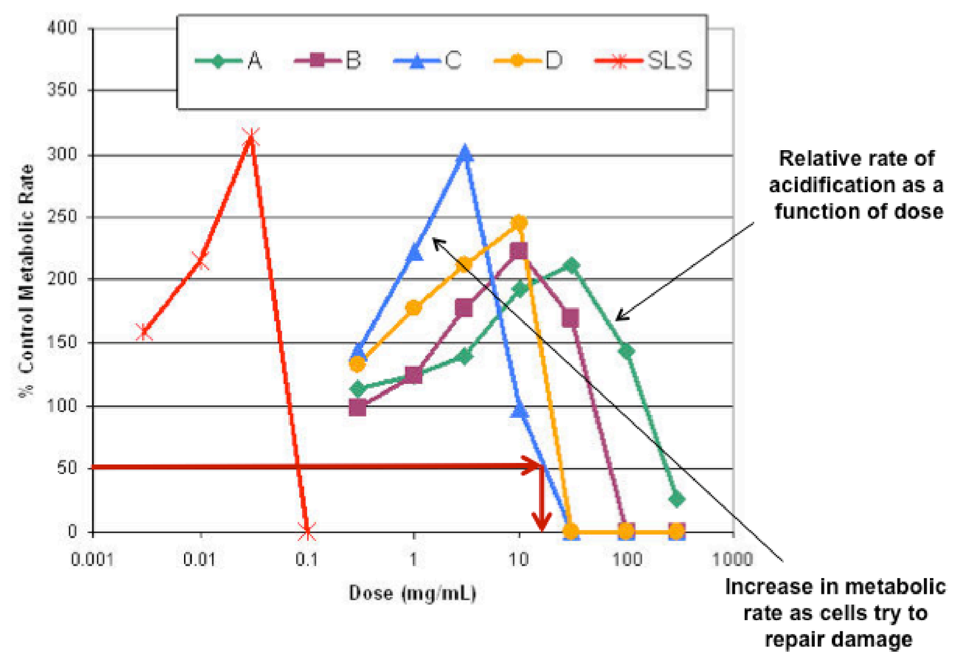
1. Expose cells to test substance starting with lowest concentration:
   - Exposure time 13 min 30 sec (flow rate 100 µL/min for first minute, then 20 µL/min)
   - Washout time 6 minutes (100 µL/min)
   - Measure rate of pH change for 25 sec (0 µL/min)
2. Repeat cycles until highest concentration is reached or the concentration that leads to a 50% decline in the basal metabolic rate of the population (the MRD_{50}) has been surpassed
3. If possible, calculate the MRD_{50} from the dose range-finding test

Perform Definitive Test

1. Repeat test as above at least two more times with seven concentrations of test substance:
   - Three concentrations above expected MRD_{50}
   - One concentration of expected MRD_{50}
   - Three concentrations below expected MRD_{50}
   - If the MRD_{50} could not be calculated from the dose range-finding test, minimum concentration will be 270 mg/mL
2. Calculate the MRD_{50}
3. Assign hazard classification using decision criteria in Table 1

[1] For use of CM, ICCVAM recommends using the updated ICCVAM CM INVITTOX Protocol 102. The protocol, summarized above, is available in the *ICCVAM Test Method Evaluation Report* (ICCVAM 2010).

Figure 3. Example of CM Data and MRD_{50} Calculation[1,2]



Abbreviations: MRD_{50} = metabolic rate decrement of 50%; the concentration of test substance (w/v) required to cause 50% inhibition of the basal acidification (metabolic) rate. SLS = 10% (w/v) sodium lauryl sulfate or positive control.

[1] Figure courtesy of Dr. Rodger Curren (Institute for In Vitro Sciences, Inc.).

[2] Letters A, B, C, and D represent different test substances.

Table 1. Decision Criteria for the EPA, GHS, and EU Classification Systems Used for CM Evaluation

MRD_{50} (mg/mL)	EPA	GHS	EU
>80	Category IV (No hazard label required)	NA	NA
>2; ≤80	No prediction can be made	NA	NA
>10	NA	Not Classified	Not Labeled
>2; ≤10	NA	No prediction can be made	No prediction can be made
≤2	Category I (Severe/corrosive)	Category 1 (Severe/corrosive)	R41 (Severe/corrosive)

Abbreviations: EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = U.N. Globally Harmonized System; MRD_{50} = metabolic rate decrement of 50%; NA = not applicable for this particular classification and labeling system

[1]EPA classification system (EPA 2007); GHS classification system (UN 2009); EU classification system (EU 2001).

Validation Database

- Accuracy assessments were conducted for each of two distinct databases.
- The database of 53 surfactant substances (tested in seven different laboratories) included:
  - 21 surfactant chemicals
  - 32 surfactant-containing formulations
- The database of 29 nonsurfactant substances (tested in seven different laboratories) included:
  - 27 nonsurfactant chemicals, which included a range of chemical classes (e.g., acids, alcohols, alkalis, and ketones)
  - 2 nonsurfactant formulations

Test Method Accuracy

Distinguishing Substances Not Labeled as Irritants From All Other Hazard Categories

- For surfactant-containing substances, accuracy ranged from 66% (35/53) to 93% (43/46) (**Table 2**).
- For nonsurfactant substances, accuracy ranged from 63% (15/24) to 76% (22/29) (**Table 3).**

Distinguishing Ocular Corrosives and Severe Irritants From All Other Hazard Categories

- For surfactant-containing substances, accuracy ranged from 85% (44/52) to 94% (50/53) (**Table 4).**
- For nonsurfactant substances, accuracy ranged from 79% (23/29) to 92% (23/25) (**Table 5**).

Table 2. Accuracy of CM for Distinguishing Substances Not Labeled as Irritants[1] From all Other Irritant Classes for Surfactant-Containing Substances

Classification System[2]	N	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate[3]	
		%	No.	%	No.	%	No.	%	No.	%	No.
EPA	52	92	48/52	98	45/46	50	3/6	50	3/6	2	1/46
GHS	53	68	36/53	100	28/28	32	8/25	68	17/25	0	0/28
EU	53	66	35/53	100	27/27	31	8/26	69	18/26	0	0/27
FHSA-20%[4]	53	92	49/53	98	46/47	50	3/6	50	3/6	2	1/47
FHSA-67%[4]	46	93	43/46	100	40/40	50	3/6	50	3/6	0	0/40

Abbreviations: CM = Cytosensor Microphysiometer; EPA = U.S. Environmental Protection Agency; EU = European Union; FHSA = U.S. Federal Hazardous Substances Act; GHS = U.N. Globally Harmonized System; MRD_{50} = metabolic rate decrement of 50%; N = number of substances included in this analysis; No. = data used to calculate the percentage

[1] EPA classification system (EPA 2007): Category IV vs. Category I/II/III
GHS classification system (UN 2009): Not Classified vs. Category 1/2A/2B
EU classification system (EU 2001): Not Labeled vs. R41/R36
FHSA classification system (FHSA 2005): Not Labeled vs. Irritant

[2] EPA decision criteria: MRD_{50} >80 mg/mL = Category IV; MRD_{50} ≤2 mg/mL = Category I
GHS decision criteria: MRD_{50} >10 mg/mL = Not Classified; MRD_{50} ≤2 mg/mL = Category 1
EU decision criteria: MRD_{50} >10 mg/mL = Not Labeled; MRD_{50} ≤2 mg/mL = R41
FHSA decision criteria: Applied EPA decision criteria

[3] The one false negative substance based on *in vivo* data was EPA Category III or FHSA Irritant. For this substance, six test animals were included. One test animal had no observable effects, three test animals had conjunctival redness (score = 1), and two test animals had corneal opacity (score = 1) that cleared after one day.

[4] In order to maximize the number of substances included in the FHSA analyses, "proportionality" criteria (i.e., FHSA-20% and FHSA-67%) were applied for the purpose of assigning a FHSA classification for test results that would require additional testing according to the FHSA sequential testing strategy (FHSA 2005).

Table 3. Accuracy of CM for Distinguishing Substances Not Labeled as Irritants[1] From All Other Irritant Classes for Nonsurfactant Substances

Classification System[2]	N	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.	%	No.	%	No.	%	No.	%	No.
EPA	29	66	19/29	67	16/24	60	3/5	40	2/5	33	8/24
GHS	25	64	16/25	62	13/21	75	3/4	25	1/4	38	8/21
EU	29	76	22/29	76	16/21	75	6/8	25	2/8	24	5/21
FHSA-20%[3]	25	64	16/25	62	13/21	75	3/4	25	1/4	38	8/21
FHSA-67%[3]	24	63	15/24	60	12/20	75	3/4	25	1/4	40	8/20

Abbreviations: CM = Cytosensor Microphysiometer; EPA = U.S. Environmental Protection Agency; EU = European Union; FHSA = U.S. Federal Hazardous Substances Act; GHS = U.N. Globally Harmonized System; MRD_{50} = metabolic rate decrement of 50%; N = number of substances included in this analysis; No. = data used to calculate the percentage

[1] EPA classification system (EPA 2007): Category IV vs. Category I/II/III
GHS classification system (UN 2009): Not Classified vs. Category 1/2A/2B
EU classification system (EU 2001): Not Labeled vs. R41/R36
FHSA classification system (FHSA 2005): Not Labeled vs. Irritant

[2] EPA decision criteria: MRD_{50} >80 mg/mL = Category IV; MRD_{50} ≤2 mg/mL = Category I
GHS decision criteria: MRD_{50} >10 mg/mL = Not Classified; MRD_{50} ≤2 mg/mL = Category 1
EU decision criteria: MRD_{50} >10 mg/mL = Not Labeled; MRD_{50} ≤2 mg/mL = R41
FHSA decision criteria: Applied EPA decision criteria

[3] In order to maximize the number of substances included in the FHSA analyses, "proportionality" criteria (i.e., FHSA-20% and FHSA-67%) were applied for the purpose of assigning a FHSA classification for test results that would require additional testing according to the FHSA sequential testing strategy (FHSA 2005).

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health

NIEHS National Institute of Environmental Health Sciences

NTP National Toxicology Program
U.S. Department of Health and Human Services

Table 4. Accuracy of CM for Distinguishing Corrosives/Severe Irritants[1,2] From All Other Irritant Classes for Surfactant-Containing Substances

Classification System[3]	N	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.	%	No.	%	No.	%	No.	%	No.
EPA	52	85	44/52	78	18/23	90	26/29	10	3/29	22	5/23
GHS	53	94	50/53	91	21/23	97	29/30	3	1/30	9	2/23
EU	53	89	47/53	81	21/26	96	26/27	4	1/27	19	5/26

Abbreviations: CM = Cytosensor Microphysiometer; EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = U.N. Globally Harmonized System; MRD_{50} = metabolic rate decrement of 50%; N = number of substances included in this analysis; No. = data used to calculate the percentage

[1] EPA classification system (EPA 2007): Category I vs. Category II/III/IV
GHS classification system (UN 2009): Category 1 vs. Category 2A/2B/NC
EU classification system (EU 2001): R41 vs. R36/NL

2 The FHSA ocular hazard category that is assigned based on results from the rabbit eye test does not distinguish between ocular corrosives/severe irritants and less severe irritants. For this reason, an evaluation of CM as a screening test to identify ocular corrosives/severe irritants using the FHSA classification system is not possible.

[3] EPA decision criteria: MRD_{50} >80 mg/mL = Category IV; MRD_{50} ≤2 mg/mL = Category I
GHS decision criteria: MRD_{50} >10 mg/mL = Not Classified; MRD_{50} ≤2 mg/mL = Category 1
EU decision criteria: MRD_{50} >10 mg/mL = Not Labeled; MRD_{50} ≤2 mg/mL = R41

Table 5. Accuracy of CM for Distinguishing Corrosives/Severe Irritants[1,2] From All Other Irritant Classes for Nonsurfactant Substances

Classification System[3]	N	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.	%	No.	%	No.	%	No.	%	No.
EPA	25	92	23/25	71	5/7	100	18/18	0	0/18	29	2/7
GHS	29	83	24/29	55	6/11	100	18/18	0	0/18	45	5/11
EU	29	79	23/29	50	6/12	100	17/17	0	0/17	50	6/12

Abbreviations: CM = Cytosensor Microphysiometer; EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = U.N. Globally Harmonized System; MRD_{50} = metabolic rate decrement of 50%; N = number of substances included in this analysis; No. = data used to calculate the percentage

[1] EPA classification system (EPA 2007): Category I vs. Category II/III/IV
GHS classification system (UN 2009): Category 1 vs. Category 2A/2B/NC
EU classification system (EU 2001): R41 vs. R36/NL

[2] The FHSA ocular hazard category that is assigned based on results from the rabbit eye test does not distinguish between ocular corrosives/severe irritants and less severe irritants. For this reason, an evaluation of CM as a screening test to identify ocular corrosives/severe irritants using the FHSA classification system is not possible.

[3] EPA decision criteria: MRD_{50} >80 mg/mL = Category IV; MRD_{50} ≤2 mg/mL = Category I
GHS decision criteria: MRD_{50} >10 mg/mL = Not Classified; MRD_{50} ≤2 mg/mL = Category 1
EU decision criteria: MRD_{50} >10 mg/mL = Not Labeled; MRD_{50} ≤2 mg/mL = R41

Test Method Reliability

Intralaboratory Reproducibility

- Assessed quantitatively based on calculated coefficients of variation (CVs) for MRD_{50} values for 16 test substances using the data from the European Commission/Home Office (EC/HO; Balls et al. 1995) and European Cosmetic, Toiletry and Perfumery Association (COLIPA; Brantom et al. 1997) validation studies (i.e., two experiments with ≥3 replicates per experiment)
- Mean CVs ranged from 10% to 24%.

Interlaboratory Reproducibility

- Assessed using the data from the EC/HO (Balls et al. 1995) and COLIPA (Brantom et al. 1997) validation studies (**Table 6**)
- Mean CVs ranged from 16% to 37% for surfactant substances and up to 51% for nonsurfactant substances.

Table 6. Interlaboratory Reproducibility of CM for All Ocular Hazard Categories for the EPA Classification System

Material Type	Number of Labs	Number of Substances	Agreement Among Laboratories	Maximum Mean CV	Study
Surfactants	4	11	100% agreement for 6 of 11 substances (55%)	37%	EC/HO: (Balls et al. 1995)
			75% agreement for 3 of 11 substances (27%)		
			50% agreement for 2 of 11 substances (18%)		
Nonsurfactants		23	100% agreement for 11 of 23 substances (48%)	51%	
			75% agreement for 5 of 23 substances (22%)		
			67% agreement for 1 of 23 substances (4%)		
			50% agreement for 3 of 23 substances (13%)		
			0% agreement for 3 of 23 substances (13%)		
Surfactants	2	10	100% agreement for 9 of 10 substances (90%)	23%	COLIPA: (Brantom et al. 1997)
			0% agreement for 1 of 10 substances (10%)		
Surfactant-based formulations and mixtures		7	100% agreement for 7 of 7 substances (100%)	16%	
Nonsurfactants		9	100% agreement for 7 of 9 substances (78%)	51%	
			0% agreement for 2 of 9 substances (22%)		

Ocular Peer Review Panel Meeting

- A public meeting of an international independent scientific peer review panel organized by NICEATM and ICCVAM was held at Consumer Product Safety Commission Headquarters in Bethesda, MD, on May 19–21, 2009.
- The peer panel report was published in July 2009 (ICCVAM 2009).

Charge to the Peer Review Panel

- Review the background review documents describing the validation status of each test method, including accuracy and reliability
- Provide conclusions and recommendations on the current validation status of each test method with respect to their usefulness and limitations, standardized protocols, performance standards, and future studies

Peer Review Panel Conclusions

- CM can be used as a screening test to identify water-soluble surfactant substances as ocular corrosives and severe irritants and substances not labeled as irritants in a tiered-testing strategy, as part of a weight-of-evidence approach.
  - Expressed concern regarding the availability of the instrumentation (it has been discontinued)
- CM applicability domain is restricted to water-soluble surfactants and surfactant-based formulations (e.g., cosmetics and personal care products).
- The complete ocular peer review panel report can be accessed at: http://iccvam.niehs.nih.gov/docs/ocutox_docs/OcularPRPRept2009.pdf

ICCVAM Recommendations: Usefulness and Limitations

Evaluation as a Screening Test to Identify Substances Not Labeled as Irritants

- Water-soluble surfactant chemicals and certain types of surfactant-containing formulations:
  - ICCVAM concludes that the accuracy and reliability of CM are sufficient to support its use as a screening test to identify these types of substances (e.g., cosmetics and personal care product formulations, but not pesticide formulations) as substances not labeled as irritants (i.e., EPA Category IV, EU Not Labeled, FHSA Not Labeled) and distinguish them from all other hazard categories (i.e., EPA Category I, II, III; EU R41, R36, FHSA Irritant) when results are to be used specifically for hazard classification and labeling purposes under the EPA, EU, and FHSA classification systems (EPA 2007; EU 2001; FHSA 2005). False negative rates ranged from 0% (0/27) to 2% (1/47).
- Water-soluble nonsurfactant substances and formulations:
  - Because of high false negative rates (24% [5/21] to 40% [8/20]), CM is **not** recommended for these types of substances.

Evaluation as a Screening Test to Identify Ocular Corrosives and Severe Irritants

- Water-soluble surfactants, surfactant-containing formulations, and nonsurfactants:
  - ICCVAM recommends that CM can be used as a screening test to identify these types of substances as ocular corrosives and severe irritants (i.e., EPA Category I, EU R41, GHS Category 1[1]) in a tiered-testing strategy, as part of a weight-of-evidence approach.
    - False positive rates ranged from 0% (0/17 or 0/18) to 10% (3/29) and false negative rates ranged from 9% (2/23) to 50% (6/12).
    - A substance that tests negative with CM would need to be tested in the rabbit eye test to confirm whether the substance is or is not a corrosive/severe eye irritant, and if it is not, to distinguish between moderate and mild ocular irritants.
  - Users may want to consider using CM before using another *in vitro* ocular test method, since it can be used to identify ocular corrosives and severe irritants and substances not labeled as irritants for certain types of substances.
  - Because CM has a high false positive rate for substances not labeled as irritants (50% [3/6] to 69% [18/26] depending on the hazard classification system used), a high level of inconclusive results are likely, resulting in the need to retest in another validated system.

[1] The FHSA ocular hazard category that is assigned based on results from the rabbit eye test does not distinguish between ocular corrosives/severe irritants and less severe irritants. For this reason, an evaluation of CM as a screening test to identify ocular corrosives/severe irritants using the FHSA classification system is not possible.

ICCVAM Recommendations: Future Studies

- Expand the applicability domain of CM for the identification of ocular corrosives and severe irritants (i.e., EPA Category I, GHS Category 1) and substances not labeled as irritants (EPA Category IV, FHSA Not Labeled, GHS Not Classified)
  - For these studies, select from the ICCVAM-recommended reference substances for validation of *in vitro* ocular safety test methods for the evaluation of ocular corrosives and severe irritants (ICCVAM 2006).
  - Similarly, a set of reference substances could also be selected from this list for the evaluation of substances not labeled as irritants.
- Identify and test substances in the moderate and mild ocular irritant categories (i.e., EPA Category II, III; GHS Category 2A, 2B) to further evaluate the performance of CM for the identification of all ocular hazard categories
- Encourage users to provide ICCVAM with all data generated from future studies to assist with further characterization of the usefulness and limitations of CM for the evaluation of all ocular hazard categories

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Independent Scientific Peer Review Panel

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Middle Row: Maria Pilar Vinardell, Ph.D., Universitat de Barcelona, Barcelona, Spain; Donald Sawyer, D.V.M., Ph.D., DACVA, Retired, Michigan State University, East Lansing, MI; Denise Rodeheaver, Ph.D., DABT, Alcon Research Ltd., Ft. Worth, TX; Alison McLaughlin, M.Sc, DABT, Health Canada, Ottawa, Ontario, Canada; Sherry Ward, Ph.D., MBA, BioTred Solutions, and the International Foundation for Ethical Research, New Market, MD; J. Lynn Palmer, Ph.D., MD Anderson Cancer Center, Houston, TX; Richard Dubielzig, D.V.M., University of Wisconsin–Madison, Madison, WI

Front Row: Kirk Tarlo, Ph.D., DABT, Amgen, Inc., Thousand Oaks, CA; Paul Bailey, Ph.D., Bailey & Associates Consulting, Neshanic Station, NJ; William Stokes, D.V.M., DACLAM (NICEATM Director), NIEHS, Research Triangle Park, NC; A. Wallace Hayes, Ph.D., DABT, FATS, ERT (Panel Chair), Harvard School of Public Health, Andover, MA and Spherix Inc., Bethesda, MD; Marilyn Wind, Ph.D. (ICCVAM Chair), U.S. Consumer Product Safety Commission, Bethesda, MD; Robert Peiffer, Jr., D.V.M., Ph.D., DACVO, Merck Research Laboratories, Doylestown, PA

* Henry Edelhauser, Ph.D., Emory University School of Medicine, Atlanta, GA, Daryl Thake, D.V.M., DACVP, Midwest ToxPath Sciences Inc., Chesterfield, MO, and Scheffer Tseng, M.D., Ph.D., Tissue Tech, Inc. and Ocular Surface Center, Miami, FL were unable to attend the public meeting on May 19–21, 2009. However, they were involved in the peer review of the background review documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Evaluation of the Validation Status of Alternative Ocular Safety Testing Methods and Strategies* (ICCVAM 2009).

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