新規試験法提案書

2013年改訂OECD TG 438
ニワトリの摘出眼球を用いた眼刺激性試験
（ICE法：Isolated Chicken Eye Test）

平成27年１月

国立医薬品食品衛生研究所

新規試験法提案書

平成 27 年 1 月 6 日

No.　2014-01

2013 年改訂 OECD TG 438 ニワトリの摘出眼球を用いた眼刺激性試験
(ICE 法:　Isolated Chicken Eye Test)に関する提案

平成 26 年 10 月 28 日に東京、国立医薬品食品衛生研究所にて開催された新規試験法評価会議（通称：JaCVAM 評価会議）において以下の提案がなされた。

提案内容：　TG 438（2013）は、化学物質による眼刺激性を評価でき、トップダウン方式においてUN GHS区分 1 物質（重篤な眼の傷害を引き起こす物質）ならびにボトムアップ方式におけるUN GHS区分外物質（眼刺激性物質として分類されない）の範囲において行政的利用は可能であると考える。

この提案書は、OECD TG 438およびStreamed Summary Document Supporting OECD TG 438 on the Isolated Chicken Eye for Eye Irritation/Corrosion. Series on Testing and Assessment No. 188 (Part 1 and Part 2)をもとに、眼刺激性試験資料編纂委員会によりまとめられた文書を用いてJaCVAM評価会議が評価および検討した結果、その有用性が確認されたことから作成された。

以上の理由により、行政当局の安全性評価方法として「2013 年改訂 OECD TG 438 ニワトリの摘出眼球を用いた眼刺激性試験」の使用を提案するものである。

大野泰雄

JaCVAM 評価会議　議長

西川秋佳

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

大野泰雄	(運営委員会推薦)：座長
五十嵐良明	(国立医薬品食品衛生研究所　生活衛生化学部)
一鬼　勉	(日本化学工業協会)
篠田和俊	(独立行政法人　医薬品医療機器総合機構)
杉山真理子	(日本化粧品工業連合会)
谷川浩子	(日本動物実験代替法学会)
西川秋佳	(国立医薬品食品衛生研究所　安全性生物試験研究センター)
平賀秀明	(独立行政法人　医薬品医療機器総合機構)
牧　栄二	(日本免疫毒性学会)
増田光輝	(座長推薦)
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横関博雄	(日本皮膚アレルギー・接触皮膚炎学会)
吉田武美	(日本毒性学会)
吉田　緑	(国立医薬品食品衛生研究所　安全性生物試験研究センター　病理部)
吉村　功	(座長推薦)
渡部一人	(日本製薬工業協会)

任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日

JaCVAM 運営委員会

西川秋佳　（国立医薬品食品衛生研究所　安全性生物試験研究センター）：委員長
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束野正明　（厚生労働省　医薬食品局　化学物質安全対策室）
中村高敏　（独立行政法人　医薬品医療機器総合機構）
日田　充　（厚生労働省　医薬食品局　化学物質安全対策室）
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本間正充　（国立医薬品食品衛生研究所　安全性生物試験研究センター　変異遺伝部）
小島　肇　（国立医薬品食品衛生研究所　安全性生物試験研究センター　薬理部　新規試験表評価室）：事務局

JaCVAM statement on
Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

At the meeting concerning the above method, held on 28 October 2014 at the National Institute of Health Sciences (NIHS), Tokyo, Japan, the members of the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage is considered to have sufficient accuracy and reliability for prediction of eye irritating test substances for regulatory use.

Following the review of the results of OECD TG No. 438 and Streamed Summary Document Supporting OECD TG 438 on the Isolated Chicken Eye for Eye Irritation/Corrosion. Series on Testing and Assessment No. 188 (Part 1 and Part 2), it is concluded that Isolated Chicken Eye Test Method such as rritation testing are clearly beneficial.

The JaCVAM Regulatory Acceptance Board has been regularly kept informed of the progress of the study, and this endorsement is based on an assessment of various documents, including, in particular, the evaluation report prepared by the JaCVAM ad hoc peer review panel for eye irritation testing.

Yasuo Ohno
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

6 January, 2015

The JaCVAM Regulatory Acceptance Board was established by the JaCVAM Steering Committee, and is composed of nominees from the industry and academia.

This statement was endorsed by the following members of the JaCVAM Regulatory Acceptance Board:

Mr. Yasuo Ohno (nominee by JaCVAM Steering Committee) : Chairperson
Mr. Hideaki Hiraga (Pharmaceuticals and Medical Devices Agency)
Mr. Tsutomu Ichiki (Japan Chemical Industry Association)
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Eiji Maki (Japanese Society of Immunotoxicology)
Mr. Mitsuteru Masuda (nominee by Chairperson)
Mr. Takeshi Morita (Japanese Environmental Mutagen Society)
Mr. Akiyoshi Nishikawa (NIHS)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Ms. Koko Tanigawa (Japanese Society for Animal Experimentation)
Mr. Takashi Yamada (National Institute of Technology and Evaluation)
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)
Ms. Midori Yoshida (NIHS)
Mr. Takemi Yoshida (Japanese Society of Toxicology)
Mr. Isao Yoshimura (nominee by Chairperson)
Mr. Kazuto Watanabe (Japan Pharmaceutical Manufacturers Association)

Term: From 1st April 2014 to 31st March 2016

This statement was endorsed by the following members of the JaCVAM steering Committee after receiving the report from JaCVAM Regulatory Acceptance Board:

Mr. Akiyoshi Nishikawa (BSRC, NIHS): Chairperson
Mr. Toru Kawanishi (NIHS)
Mr. Mitsuru Hida (Ministry of Health, Labour and Welfare)
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)
Mr. Jun Kanno (Division of Cellular and Molecular Toxicology, BSRC, NIHS)
Mr. Kenji Kuramochi (Ministry of Health, Labour and Welfare)
Mr. Takatoshi Nakamura (Pharmaceutical & Medical Devices Agency)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Ms. Yuko Sekino (Division of Pharmacology, BSRC, NIHS)
Mr. Atsuya Takagi (Animal Management Section of the Division of Cellular and Molecular Toxicology, BSRC, NIHS)
Mr. Masaaki Tsukano (Ministry of Health, Labour and Welfare)
Mr. Nobuo Uemura (Ministry of Health, Labour and Welfare)
Mr. Hajime Kojima (Section for the Evaluation of Novel Methods, Division of Pharmacology, BSRC, NIHS): Secretary

2013 年改訂 OECD TG 438 ニワトリの摘出眼球を用いた眼刺激性試験
(ICE 法 ：Isolated Chicken Eye Test)

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2013 年改訂 OECD TG 438 ニワトリの摘出眼球を用いた眼刺激性試験
(ICE 法:　Isolated Chicken Eye Test)

JaCVAM 評価会議

平成 26 年（2014 年）10 月 28 日

1

JaCVAM 評価会議

大野泰雄　（運営委員会推薦）：座長

五十嵐良明（国立医薬品食品衛生研究所　生活衛生化学部）

一鬼　勉　（日本化学工業協会）

篠田和俊　（独立行政法人　医薬品医療機器総合機構）

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吉田武美　（日本毒性学会）

吉田　緑　（国立医薬品食品衛生研究所　安全性生物試験研究センター　病理部）

吉村　功　（座長推薦）

渡部一人　（日本製薬工業協会）

任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日

ニワトリの摘出眼球を用いた眼刺激性試験(Isolated Chicken Eye Test、以下 ICE 法)は、ウサギを用いた Draize 眼刺激性試験(以下、Draize 法)の代替試験法である。2003 年から 2006 年にかけて、トップダウン方式における重篤な眼の傷害を引き起こす化学物質(UN GHS 区分 1 物質)を同定するための試験法として、回顧的バリデーションが NICEATM/ICCVAM によりなされ、2009 年に OECD TG 438 (以下、TG438(2009)[1])として採択された。

これとは別に NICEATM/ICCVAM は、ECVAM および JaCVAM の協力の下、2006 年から 2010 年にかけて、ボトムアップ方式における区分外物質の同定法としての適用性に関する回顧的バリデーションを新たに行った。バリデーションには、初回に使用された ICCVAM データ(2006)[2]がそのまま用いられて解析がなされたが、ICE 法拡大適用の提言には至らなかった。

その後、2012 年、OECD 専門委員会により UN GHS 区分下において再評価が実施された。初回に使用されたデータベースにおける *in vivo* および *in vitro* 両方のデータが見直されたほか、*in vitro* の区分外基準が正式に設定された。そこで改めて評価が行われた結果、ICE 法はトップダウン方式における UN GHS 区分 1 物質の同定のみならず、ボトムアップ方式における区分外物質の同定法として使用することが可能であると判断され、2013 年 7 月 26 日付けで 2013 年改訂 TG 438(以下、TG438(2013)[3])として採択された。

TG 438(2013)における主な改訂点は、1) ボトムアップ方式による UN GHS 区分外物質同定への適用および 2) 適用限界、である。JaCVAM 評価会議は、この改訂の妥当性について検討した。

１．試験法の定義

名称：　2013 年改訂 OECD TG 438（TG438（2013）[3]）

ニワトリの摘出眼球を用いた眼刺激性試験 (ICE 法: Isolated Chicken Eye Test)

代替する対象毒性試験：　Draize 法

試験法の概略：　ICE 法は、ニワトリから摘出した眼球に被験物質を曝露し、その結果、眼球に生じる角膜の変性を、角膜の腫脹、混濁度およびフルオレセイン染色性の変化としてとらえる。これら 3 評価項目の変化をそれぞれ個別のスコアに変換して得られる総合評価をもとに *in vivo* での眼刺激性を予測する。

各評価項目の変化は、A)角膜の腫脹：光学的厚度計を装着した細隙灯顕微鏡にて角膜の厚さを測定し、曝露 240 分後までの経時的な変化率を定量的に求める。B)角膜の混濁度：細隙灯顕微鏡にて角膜混濁度の経時的な変化を曝露 240 分後まで観察する。C)フルオレセイン染色性：細隙灯顕微鏡にて曝露 30 分後の角膜表面のフルオレセイン染色性を調べる。各項目の結果を傷害の程

度により、眼刺激性の最も弱いクラスIから最も強いクラスIVの4段階に分類し、それらの分類結果を総合して、被験物質の眼刺激性を判定する。

２．評価に用いた資料および評価内容の科学的妥当性

NICEATM/ICCVAMによる回顧的バリデーションを経てOECD にてTG 438(2009)として採択された後、2012年にOECD専門委員会によりUN GHS区分下において再評価が実施され、2013年にはTG 438(2013)として採択されており、その評価に用いた資料[1-5]と評価内容に科学的妥当性がある。また、今回の改訂に先立ちOECD専門委員会により、前回評価を行ったICCVAM(2006)バリデーションデータベースの175物質について、*in vivo*および*in vitro*の個々のデータについて再評価されたが、最終的にボトムアップ方式の評価には175物質中152物質（単一物質：72、混合物質：80）が、トップダウン方式の評価には140物質（単一物質：65、混合物質：75）が用いられ、十分な数を用いて評価されている。

３．本試験法の有用性と適用限界

第三者評価報告書(ICCVAM 2006[4]、第三者評価報告書2012[6])で報告されているとおり、トップダウン方式およびボトムアップ方式における施設内・施設間再現性は高い。また、適用限界に関しては、防汚有機溶媒含有塗料、固体物質、界面活性剤およびアルコール類については、高い偽陰性率あるいは偽陽性率が示されたが、以下の理由からこれらの化学物質にもICE法を適用することができると報告された。

ボトムアップ方式：

・防汚有機溶媒含有塗料で陽性2物質のうち、1物質（TNO-94）が偽陰性であったが、用途の類似した化合物（TNO-93）は陽性であった。従って、ICE法への適用領域から除外するには根拠が不十分であり、防汚有機溶媒含有塗料を適用領域から除外する必要はない。

・単一物質、混合物質、液体、固体等における偽陰性率は5%以下と低い。偽陽性率については、ICE法では33%と他の試験法に比べ低いことが示された（BCOP（Bovine Corneal Opacity and Permeability）法; 69%、CM (Cytosensor Microphysiometer)法：68%[5]）。単一物質および界面活性剤についての偽陽性率は高いが、ボトムアップ方式において陽性と判断された場合には、他の適切な試験法による確認が必要とされていることから、これらの化学物質を適用領域から除外する必要はない。

トップダウン方式：

・偽陰性率は固体物質および界面活性剤において高く、偽陽性率はアルコール類において高かった。しかし、これらの分類においても眼刺激性が正確に予測された物質があること、さらにトップダウン方式において陰性と判断された場合には他の適切な試験法による確認が必要とされていることから、固体物質および界面活性剤を適用領域から除外する必要はない。ただし、アルコール類で陽性結果が得られた場合は、結果の解釈は慎重にすべきである。

以上から、ICE 法はボトムアップ方式での区分外物質およびトップダウン方式での UN GHS 区分 1 物質の同定法として、すべての種類の化学物質に適用できるとされた。

４．目的とする物質又は製品の毒性を評価する試験法としての、行政上利用及び社会的受け入れの可能性

社会的受け入れ性：

ICE 法では、食用として屠殺されたニワトリの眼球を用いるため Draize 法よりも社会的受け入れ性は高い。今回の改訂においては、動物福祉の観点からの変更がないことから、本改訂法の社会的受け入れ性は、改訂前と変わらない。

行政上の利用性：

TG 438（2013）は、化学物質による眼刺激性を評価でき、トップダウン方式においてUN GHS区分 1 物質（重篤な眼の傷害を引き起こす物質）ならびにボトムアップ方式におけるUN GHS区分外物質（眼刺激性物質として分類されない）の範囲において行政的利用は可能であると考える。

参考文献

1） OECD Guidelines for The Testing of Chemicals, Isolated Chicken Eye Test for Identifying Ocular Corrosives and Severe Irritants, TG438 (Adopted 7 September 2009)

2） ICCVAM, ICCVAM Test Method Evaluation Report: Current Validation Status of *In Vitro* Test Methods Proposed for Identifying Eye Injury Hazard Potential of Chemicals and Products. NIH Publication No. 10-7553. Volume 1 and Volume 2 (2010).

3) OECD Guidelines for The Testing of Chemicals, Isolated Chicken Eye Test for Identifying Ocular Corrosives and Severe Irritants, TG438 (Adopted 26 July 2013)

4） Streamlined Summary Document Supporting OECD Test Guideline 438 on the Isolated Chicken Eye for Eye Irritation/Corrosion. Series on Testing and Assessment No. 188 (Part 1 and Part 2), OECD, Paris. (21 June 2013)

5） ICCVAM, Background Review Document: Current Status of *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants: Isolated Chicken Eye Test Methods. NIH Publication No. 06-4513 (2006).

6） JaCVAM, 眼刺激性試験代替法の第三者評価報告書 - 評価対象試験：眼に対する腐食性および強刺激性評価のためのニワトリ摘出眼球を用いた眼刺激性試験法, AATEX-JaCVAM J1, 16-29 (2012)

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眼刺激性試験代替法評価報告書

2013 年改訂 OECD TG 438 ニワトリの摘出眼球を用いた眼刺激性試験

（ICE 法：Isolated Chicken Eye Test）

平成 26 年 9 月 12 日

JaCVAM 眼刺激性試験資料編纂委員会

JaCVAM 眼刺激性試験資料編纂委員会

委員長	小坂　忠司	（残留農薬研究所）
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	加藤　雅一	（株式会社ジャパン･ティッシュ・エンジニアリング）
	細井　一弘	（参天製薬株式会社）
	山本　直樹	（藤田保健衛生大学）
	吉村　功	（東京理科大学名誉教授）

略語

BCOP:	Bovine Corneal Opacity and Permeability
BRD:	Background Review Document
CM:	Cytosensor Microphysiometer
CV:	Coefficient of Variation
EURL ECVAM:	European Union Reference Laboratory for Alternatives to Animal Testing
GHS:	Globally Harmonized System of Classification and Labeling of Chemicals
ICCVAM:	Interagency Coordinating Committee on the Validation of Alternative Methods
ICE:	Isolated Chicken Eye Test
JaCVAM:	Japanese Centre for the Validation of Alternative Methods
NICEATM:	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD:	Organization for Economic Co-operation and Development
SSD:	Streamlined Summary Document
TG:	Test Guideline
UN:	United Nations

用　語

トップダウン方式：　重度の刺激性を正確に識別できる試験法から始める評価方式。本ガイドラインでは、重篤な眼の損傷を引き起こすと疑われる化学物質に適用される段階的な評価方式を指し、まず、重篤な眼の損傷を起こす物質を、それ以外の物質から正確に識別できる試験法で判別することから始める（TG438 ANNEX 1, DEFINITIONS）。

ボトムアップ方式：　非刺激性を正確に識別できる試験法から始める評価方式。本ガイドラインでは、眼刺激性あるいは重篤な眼の傷害性ではないと予測される化学物質に適用される段階的な評価方式を指し、まず、眼刺激性あるいは重篤な眼の傷害性ではない物質を、それ以外の物質から正確に識別できる試験法で判別することから始める（TG438 ANNEX 1, DEFINITIONS）。

区分1物質:　UN (United Nations) GHS (Globally Harmonized Systems of Classification and Labeling of　Chemicals) 区分体系下、重篤な眼の傷害を引き起こす物質として区分される化学物質

区分外物質:　UN GHS区分体系下、眼刺激性物質として区分されない化学物質

１．改訂の背景

ニワトリの摘出眼球を用いた眼刺激性試験（Isolated Chicken Eye Test、以下 ICE 法）は、ウサギを用いた Draize 眼刺激性試験法（以下、Draize 法）の代替試験法である。2003 年から 2006 年にかけて、トップダウン方式における重篤な眼の傷害を引き起こす化学物質（区分 1 物質）を同定するための試験法として、回顧的バリデーションが NICEATM (NTP Interagency Center for the Evaluation of Alternative Toxicological Methods) / ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) によりなされ、2009 年に OECD (Organization for Economic Co-operation and Development) テストガイドライン（TG: Test Guideline）438（TG 438, 2009）として採択された。

これとは別に NICEATM/ICCVAM は EURL ECVAM (European Union Reference Laboratory for Alternatives to Animal Testing) および JaCVAM (Japanese Centre for the Validation of Alternative Methods) の協力の下、2006 年から 2010 年にかけて、ボトムアップ方式における区分外物質の同定法としての適用性に関する回顧的バリデーションが新たに行われた。バリデーションには、初回に使用された ICCVAM データ（2006）がそのまま用いられて解析がなされたが、ICE 法の拡大適用の提言には至らなかった。

その後、2012 年、OECD 専門委員会により UN (United Nations) GHS (Globally Harmonized System of Classification and Labeling of Chemicals) 区分下において再評価が実施された。初回に使用されたデータベースにおける *in vivo* および *in vitro* 両方のデータが見直されたほか、*in vitro* の区分外基準が正式に設定された。そこで改めて評価が行われた結果、ICE 法はトップダウン方式における区分 1 物質の同定のみならず、ボトムアップ方式における区分外物質の同定法として使用することが可能であると判断され、2013 年 7 月 26 日付けで改訂 TG　438 (TG 438, 2013) として採択された。

TG 438（2013）の主な改訂点は、1) ボトムアップ方式による UN GHS 区分外物質同定への適用および 2) 適用限界である。本資料編纂委員会は、この TG 438（2013）の改訂点について検討した。以下にその結果を報告する。

２．試験の概要

ICE 法では、ニワトリから摘出した眼球に被験物質を暴露し、その結果、眼球に生じる角膜の変性を、角膜の腫脹、混濁度およびフルオレセイン染色性の変化としてとらえる。これら 3 評価項目の変化をそれぞれ個別のスコアに変換して得られる総合評価をもとに *in vivo* での眼刺激性を予測する。

各評価項目の変化としては、A) 角膜の腫脹（光学的厚度計を装着した細隙灯顕微鏡にて角膜の厚さを測定して暴露 240 分後までの経時的な変化率を定量的に求める）、B) 角膜の混濁度（細隙灯顕微鏡にて角膜混濁度の経時的な変化を暴露 240 分後まで観察する）、C) フルオレセイン染色性（細隙灯顕微鏡にて暴露 30 分後の角膜表面のフルオレセイン染色性）を測定することである。各項目の結果を傷害の程度により、眼刺激性の最も弱いクラス I から最も強いクラス IV の 4 段階に分類し、それらの分類結果を総合して、被験物質の眼刺激性を判定する。

３．改訂点

３−１．*In vivo – in vitro* データベースの見直し

TG 438 （2013）の改訂に先立ち OECD 専門委員会により、前回評価を行った ICCVAM (2006)バリデーションデータベースをもとにまとめられた SSD (Streamlined Summary Document), Appendix 1 (OECD,

2013) から重複する 3 物質を除外した 175 物質について、*in vivo* および *in vitro* の個々のデータについて再評価された。*In vivo* データについては、生データが欠如している 15 物質を対象から除外し、さらに個々のデータを見直すことにより UN GHS 区分での再判定がなされた。また、*in vitro* データではデータの見直しと新たに設定した UN GHS 区分（３－２－１項）に基づく再判定がなされた。以上の再評価において、最終的にボトムアップ方式の評価には 175 物質中 152 物質（単一物質：72、混合物質：80）が、トップダウン方式の評価には 140 物質（単一物質：65、混合物質：75）が用いられた。

３－２．ボトムアップ方式での UN GHS 区分外物質同定への適用

３－２－１． UN GHS 区分外物質評価の新たな判定基準の設定

TG 438 (2013)では、新たなボトムアップ方式による UN GHS 区分外物質の同定に対応するため、区分外物質評価のための判定基準が新たに設定された。追加された判定基準を表 1 に示す。

表 1.　UN GHS 区分外物質評価のための判定基準

UN GHS 区分	3 評価項目の組み合わせ*
区分外	3 項目ともクラス I に分類される 2 項目がクラス I に分類され、1 項目がクラス II に分類される

＊）3 評価項目：角膜の腫脹、角膜混濁、角膜のフルオレセイン染色性

クラス I：最も弱い眼刺激性

３－２－２．ボトムアップ方式での正確性

上記のように評価した新たなデータベースを用い、ボトムアップ方式における区分外物質を同定する場合の正確性について評価された。結果を表 2 に示す。

区分外物質の同定において、Draize 法の結果と比較すると、ICE 法の正確度は 82%（125/152）、感度 99% （72/73）、特異度は 67%（53/79）、偽陰性率は 1%（1/73）および偽陽性率は 33%（26/79）を示した。偽陰性率は低く、偽陰性を示した物質は防汚有機溶媒含有塗料 3 物質中 1 物質（TNO-94）のみであった。一方、偽陽性率は高いが、ボトムアップ方式では陽性結果が得られた場合、他の適切な試験法による確認が必要とされている。

表 2. ボトムアップ方式における ICE 法の正確性－UN GHS 区分法での区分外物質の同定 [1]

ボトムアップ方式	No.	一致度		感度		偽陰性率		特異度		偽陽性率	
		%	No.	%	No.	%	No.	%	No.	%	No.
被験物質	152	82	125/152	99	72/73	1	1/73	67	53/79	33	26/79
防汚有機溶媒含有塗料を除外した場合	149	83	123/149	100	71/71	0	0/71	67	52/78	33	26/78

No. = 被験物質数

[1]UN GHS 区分: (区分外物質) 対 (区分外物質以外)

3－2－3． ボトムアップ方式における施設内・施設間再現性

第三者評価報告書（第三者評価報告書 2012、ICCVAM BRD : Background Review Document 2006）で報告されているとおり、ICE 法の角膜腫脹を指標とした場合の施設内 CV 値は 1.8%～6.3%であった。

被験物質のうち、ボトムアップ方式およびトップダウン方式とも、全ての施設において UN GHS 区分が一致した被験物質は 75%を占めた。また、UN GHS 区分との一致を指標とした場合、主導施設と他の参加施設との間の相関係数は 0.829～0.849 であった。

3－3．トップダウン方式での UN GHS 区分 1 物質同定への適用

3－3－1．トップダウン方式での正確性

トップダウン方式における区分 1 物質を同定する場合の正確性について、新たなデータベースを用いて評価された。結果を表 3 に示す。

区分 1 物質の同定において Draize 法の結果と比較すると一致度は 86%（120/140）、偽陰性率は 48%（13/27）、偽陽性率は 6%（7/113）であった。アルコール、固体および界面活性剤を除外した場合、一致度は上がり偽陰性率と偽陽性率は低下した。

表 3. トップダウン方式における ICE 法の正確性－UN GHS 区分 1 物質の同定 [1]

トップダウン方式	No.	一致度		感度		偽陰性率		特異度		偽陽性率	
		%	No.	%	No.	%	No.	%	No.	%	No.
被験物質	140	86	120/140	52	14/27	48	13/27	94	106/113	6	7/113
アルコール、固体および界面活性剤を除外した場合	82	94	77/82	71	5/7	29	2/7	96	72/75	4	3/75

No. = 被験物質数

[1]UN GHS 区分: (区分 1 物質) 対 (区分 1 物質以外)

3－3－2． トップダウン方式における施設内・施設間再現性

第三者評価報告書（第三者評価報告書 2012、ICCVAM BRD 2006）で報告されているとおり、トップダウン方式における施設内・施設間再現性はボトムアップ方式と同様な結果が得られている。

3－4．適用限界

防汚有機溶媒含有塗料、固体物質、界面活性剤およびアルコール類については、高い偽陰性率あるいは偽陽性率が示されたが、以下の理由からこれらの化学物質にも ICE 法を適用することができると報告された。

ボトムアップ方式：

- 防汚有機溶媒含有塗料で陽性 2 物質のうち、1 物質（TNO-94）が偽陰性であり、用途の類似した化合物（TNO-93）には認められなかった。従って、ICE 法への適用領域から除外するには根拠が不

十分であり、防汚有機溶媒含有塗料を適用領域から除外する必要はないとされた。

- 単一物質、混合物質、液体、固体等における偽陰性率は 5%以下と低く、偽陽性率については 33%と他の試験法（BCOP: Bovine Corneal Opacity and Permeability； 69%、CM : Cytosensor Microphysiometer；68%）に比べ低いことが示された。単一物質および界面活性剤についての偽陽性率は高いが、ボトムアップ方式においては陽性と判断された場合、他の適切な試験法による確認が必要とされていることから、これらの化学物質を適用領域から除外する必要はないとされた。

トップダウン方式：

- 偽陰性率は固体物質（n=34, 55%=6/11）および界面活性剤（n=21, 67%=6/9）において高く、偽陽性率はアルコール類（n=12, 0%=4/10）において高かった。しかし、これらの分類においても眼刺激性が正確に予測された物質があること、さらにトップダウン方式において陰性と判断された場合は他の適切な試験法による確認が必要とされていることから、固体物質および界面活性剤を適用領域から除外する必要はないとされた。ただし、アルコール類で陽性結果が得られた場合は、結果の解釈は慎重にすべきとされた。

以上から、ICE 法はボトムアップ方式での区分外物質およびトップダウン方式での区分 1 物質の同定法として、すべての種類の化学物質に適用できるとされた。

3－5．その他

- 習熟度確認物質の追加：
  TG 438（2009）では、ICE 法の習熟度チェック用物質として 10 物質が推奨された。しかし、10 推奨物質の中に、区分外物質が含まれていなかったことから、本改訂版では、3 種類の区分外物質が追加され、以下のような習熟度確認物質としてまとめられた（表 4）。
- 眼球の運搬温度（外界温度）として“18℃から 25℃”の温度範囲が示された。
- 被験物質暴露後の眼球洗浄において、“追加の洗浄”が認められた。
- 報告書への記載項目が追加された。
- 語句の定義について追加・訂正がなされた。
- ICE 法判定に関する用語として、カテゴリーがクラスという用語に置き換わった。

表 4. ICE 法の習熟度確認物質

化学物質	CAS番号	分類	物理的状態	*In vivo*での区分	ICEでの区分
塩化ベンザルコニウ（5%） Benzalkonium chloride (5%)	8001-54-5	オニウム化合物類 Onium compound	液体 Liquid	区分1 Category 1	区分1 Category 1
クロルヘキシジン Chlorhexidine	55-56-1	アミン類 アミジン類 Amine, Amidine	固体 Solid	区分1 Category 1	区分1 Category 1
ジベンゾイル-L-酒石酸 Dibenzoyl-L- tartaric acid	2743-38-6	カルボン酸類 エステル類 Carboxylic acid, Ester	固体 Solid	区分1 Category 1	区分1 Category 1
イミダゾール Imidazole	288-32-4	ヘテロサイクリック化合物類 Heterocyclic	固体 Solid	区分1 Category 1	区分1 Category 1
トリクロロ酢酸（30%） Trichloroacetic acid (30%)	76-03-9	カルボン酸類 Carboxylic acid	液体 Liquid	区分1 Category 1	区分1 Category 1
2,6-ジクロロベンゾイルクロリド Dichlorobenzoyl chloride	4659-45-4	アシルハライド類 Acyl halide	液体 Liquid	区分2A Category 2A	区分不可
硝酸アンモニウム Ammonium nitrate	6484-52-2	無機塩 Inorganic salt	固体 Solid	区分2B Category 2B	区分不可
エチル-2-メチルアセト酢酸 Ethyl-2-methylacetoacetate	609-14-3	ケトン類 エステル類 Ketone, Ester	液体 Liquid	区分2B Category 2B	区分不可
ジメチルスルホキシド Dimethyl sulfoxide	67-68-5	有機硫黄化合物 Organic sulphur compound	液体 Liquid	区分外 Not Classified	区分外 Not Classified
グリセロール Glycerol	56-81-5	アルコール類 Alcohol	液体 Liquid	区分外 Not Classified	区分外 Not Classified
メチルシクロペンタン Methylcyclopentane	96-37-7	炭化水素（環状） Hydrocarbon (cyclo)	液体 Liquid	区分外 Not Classified	区分外 Not Classified
n-ヘキサン n-Hexane	110-54-3	炭化水素（鎖状） Hydrocarbon (acylic)	液体 Liquid	区分外 Not Classified	区分外 Not Classified
トリアセチン Triacetin	102-76-1	脂質 Lipid	液体 Liquid	区分外 Not Classified	区分外 Not Classified

４．結論

TG 438（2013）では、その正確性と再現性の結果から、UN GHS の眼刺激性区分においてトップダウン方式における区分 1 物質の同定のみならず、ボトムアップ方式における区分外物質の同定に ICE 法を適用することは可能であり、どちらの方式においても適用物質に制限を設ける必要はないとされている。

眼刺激性資料編纂委員会としても、トップダウン方式における UN GHS 区分 1 物質ならびにボトムアップ方式における UN GHS 区分外物質の同定に、Draize 法の代替試験法として TG 438（2013）を適用することは可能であると考えた。

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Adopted:
26 July 2013

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

INTRODUCTION

1. The Isolated Chicken Eye (ICE) test method was evaluated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Centre for the Validation of Alternative Methods (JaCVAM), in 2006 and 2010 (1) (2) (3). In the first evaluation, the ICE was endorsed as a scientifically valid test method for use as a screening test to identify chemicals (substances and mixtures) inducing serious eye damage (Category 1) as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (1) (2) (4). In the second evaluation, the ICE test method was evaluated for use as a screening test to identify chemicals not classified for eye irritation or serious eye damage as defined by UN GHS (3) (4). The results from the validation study and the peer review panel recommendations maintained the original recommendation for using the ICE for classification of chemicals inducing serious eye damage (UN GHS Category 1), as the available database remained unchanged since the original ICCVAM validation. At that stage, no further recommendations for an expansion of the ICE applicability domain to also include other categories were suggested. A re-evaluation of the *in vitro* and *in vivo* dataset used in the validation study was made with the focus of evaluating the usefulness of the ICE to identify chemicals not requiring classification for eye irritation or serious eye damage (5). This re-evaluation concluded that the ICE test method can also be used to identify chemicals not requiring classification for eye irritation and serious eye damage as defined by the UN GHS (4) (5). This Test Guideline (adopted in 2009 and updated in 2013) includes the recommended uses and limitations of the ICE test method based on these evaluations. The main differences between the original 2009 version and the 2013 updated version include, but are not limited to, the use of the ICE test method to identify chemicals not requiring classification according to the UN GHS Classification System, an update to the test report elements, an update of Annex 1 on definitions, and an update to Annex 2 on the proficiency chemicals.

2. It is currently generally accepted that, in the foreseeable future, no single *in vitro* eye irritation test will be able to replace the *in vivo* Draize eye test to predict across the full range of irritation for different chemical classes. However, strategic combinations of several alternative test methods within a (tiered) testing strategy may be able to replace the Draize eye test (6). The Top-Down approach (7) is designed to be used when, based on existing information, a chemical is expected to have high irritancy potential, while the Bottom-Up approach (7) is designed to be used when, based on existing information, a chemical is expected not to cause sufficient eye irritation to require a classification. The ICE test method is an *in vitro* test method that can be used, under certain circumstances and with specific limitations as described in paragraphs 8 to 10 for eye hazard classification and labelling of chemicals. While it is not considered valid as a stand-alone replacement

for the *in vivo* rabbit eye test, the ICE test method is recommended as an initial step within a testing strategy such as the Top-Down approach suggested by Scott *et al*. (7) to identify chemicals inducing serious eye damage, i.e., chemicals to be classified as UN GHS Category 1 without further testing (4). The ICE test method is also recommended to identify chemicals that do not require classification for eye irritation or serious eye damage as defined by the UN GHS (No Category, NC) (4), and may therefore be used as an initial step within a Bottom-Up testing strategy approach (7). However, a chemical that is not predicted as causing serious eye damage or as not classified for eye irritation/serious eye damage with the ICE test method would require additional testing (*in vitro* and/or *in vivo*) to establish a definitive classification. Furthermore, the appropriate regulatory authorities should be consulted before using the ICE in a bottom up approach under other classification schemes than the UN GHS.

3. The purpose of this Test Guideline is to describe the procedures used to evaluate the eye hazard potential of a test chemical as measured by its ability to induce or not toxicity in an enucleated chicken eye. Toxic effects to the cornea are measured by (i) a qualitative assessment of opacity, (ii) a qualitative assessment of damage to epithelium based on application of fluorescein to the eye (fluorescein retention), (iii) a quantitative measurement of increased thickness (swelling), and (iv) a qualitative evaluation of macroscopic morphological damage to the surface. The corneal opacity, swelling, and damage assessments following exposure to a test chemical are assessed individually and then combined to derive an Eye Irritancy Classification.

4. Definitions are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. This Test Guideline is based on the protocol suggested in the OECD Guidance Document 160 (8), which was developed following the ICCVAM international validation study (1) (3) (9), with contributions from the European Centre for the Validation of Alternative Methods, the Japanese Center for the Validation of Alternative Methods, and TNO Quality of Life Department of Toxicology and Applied Pharmacology (Netherlands). The protocol is based on information obtained from published protocols, as well as the current protocol used by TNO (10) (11) (12) (13) (14).

6. A wide range of chemicals has been tested in the validation underlying this Test Guideline and the empirical database of the validation study amounted to 152 chemicals including 72 substances and 80 mixtures (5). The Test Guideline is applicable to solids, liquids, emulsions and gels. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Gases and aerosols have not been assessed yet in a validation study.

7. The ICE test method can be used to identify chemicals inducing serious eye damage, i.e., chemicals to be classified as UN GHS Category 1 (4). When used for this purpose, the identified limitations for the ICE test method are based on the high false positive rates for alcohols and the high false negative rates for solids and surfactants (1) (3) (9). However, false negative rates in this context (UN GHS Category 1 identified as not being UN GHS Category 1) are not critical since all test chemicals that come out negative would be subsequently tested with other adequately validated *in vitro* test(s), or as a last option in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach. It should be noted that solids may lead to variable and extreme exposure conditions in the *in vivo* Draize eye irritation test, which may result in irrelevant predictions of their true irritation potential (15). Investigators could consider using this test method for all types of chemicals, whereby a positive result should be accepted as indicative of serious eye

damage, i.e., UN GHS Category 1 classification without further testing. However, positive results obtained with alcohols should be interpreted cautiously due to risk of over-prediction.

8. When used to identify chemicals inducing serious eye damage (UN GHS Category 1), the ICE test method has an overall accuracy of 86% (120/140), a false positive rate of 6% (7/113) and a false negative rate of 48% (13/27) when compared to *in vivo* rabbit eye test method data classified according to the UN GHS classification system (4) (5).

9. The ICE test method can also be used to identify chemicals that do not require classification for eye irritation or serious eye damage under the UN GHS classification system (4). The appropriate regulatory authorities should be consulted before using the ICE in a bottom up approach under other classification schemes. This test method can be used for all types of chemicals, whereby a negative result could be accepted for not classifying a chemical for eye irritation and serious eye damage. However, on the basis of one result from the validation database, anti-fouling organic solvent-containing paints may be under-predicted (5).

10. When used to identify chemicals that do not require classification for eye irritation and serious eye damage, the ICE test method has an overall accuracy of 82% (125/152), a false positive rate of 33% (26/79), and a false negative rate of 1% (1/73), when compared to *in vivo* rabbit eye test method data classified according to the UN GHS (4) (5). When test chemicals within certain classes (*i.e.*, anti-fouling organic solvent containing paints) are excluded from the database, the accuracy of the ICE test method is 83% (123/149), the false positive rate 33% (26/78), and the false negative rate of 0% (0/71) for the UN GHS classification system (4) (5).

11. The ICE test method is not recommended for the identification of test chemicals that should be classified as irritating to eyes (*i.e.*, UN GHS Category 2 or Category 2A) or test chemicals that should be classified as mildly irritating to eyes (UN GHS Category 2B) due to the considerable number of UN GHS Category 1 chemicals underclassified as UN GHS Category 2, 2A or 2B and UN GHS No Category chemicals overclassifed as UN GHS Category 2, 2A or 2B. For this purpose, further testing with another suitable method may be required.

12. All procedures with chicken eyes should follow the test facility's applicable regulations and procedures for handling of human or animal-derived materials, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions are recommended (16).

13. Whilst the ICE test method does not consider conjunctival and iridal injuries as evaluated in the rabbit ocular irritancy test method, it addresses corneal effects which are the major driver of classification *in vivo* when considering the UN GHS Classification. Also, although the reversibility of corneal lesions cannot be evaluated *per se* in the ICE test method, it has been proposed, based on rabbit eye studies, that an assessment of the initial depth of corneal injury may be used to identify some types of irreversible effects (17). In particular, further scientific knowledge is required to understand how irreversible effects not linked with initial high level injury occur. Finally, the ICE test method does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

14. This Test Guideline will be updated periodically as new information and data are considered. For example, histopathology may be potentially useful when a more complete characterization of corneal damage is needed. To evaluate this possibility, users are encouraged to preserve eyes and prepare histopathology specimens that can be used to develop a database and decision criteria that may further improve the accuracy of this test method. The OECD has developed a Guidance Document on

the use of *in vitro* ocular toxicity test methods, which includes detailed procedures on the collection of histopathology specimens and information on where to submit specimens and/or histopathology data (8).

15. For any laboratory initially establishing this assay, the proficiency chemicals provided in Annex 2 should be used. A laboratory can use these chemicals to demonstrate their technical competence in performing the ICE test method prior to submitting ICE data for regulatory hazard classification purposes.

PRINCIPLE OF THE TEST

16. The ICE test method is an organotypic model that provides short-term maintenance of the chicken eye *in vitro*. In this test method, damage by the test chemical is assessed by determination of corneal swelling, opacity, and fluorescein retention. While the latter two parameters involve a qualitative assessment, analysis of corneal swelling provides for a quantitative assessment. Each measurement is either converted into a quantitative score used to calculate an overall Irritation Index, or assigned a qualitative categorization that is used to assign an *in vitro* ocular hazard classification, either as UN GHS Category 1 or as UN GHS non-classified. Either of these outcomes can then be used to predict the potential *in vivo* serious eye damage or no requirement for eye hazard classification of a test chemical (see Decision Criteria). However, no classification can be given for chemicals not predicted as causing serious eye damage or as not classified with the ICE test method (see paragraph 11).

Source and Age of Chicken Eyes

17. Historically, eyes collected from chickens obtained from a slaughterhouse where they are killed for human consumption have been used for this assay, eliminating the need for laboratory animals. Only the eyes of healthy animals considered suitable for entry into the human food chain are used.

18. Although a controlled study to evaluate the optimum chicken age has not been conducted, the age and weight of the chickens used historically in this test method are that of spring chickens traditionally processed by a poultry slaughterhouse (*i.e.,* approximately 7 weeks old, 1.5 - 2.5 kg).

Collection and Transport of Eyes to the Laboratory

19. Heads should be removed immediately after sedation of the chickens, usually by electric shock, and incision of the neck for bleeding. A local source of chickens close to the laboratory should be located so that their heads can be transferred from the slaughterhouse to the laboratory quickly enough to minimize deterioration and/or bacterial contamination. The time interval between collection of the chicken heads and placing the eyes in the superfusion chamber following enucleation should be minimized (typically within two hours) to assure meeting assay acceptance criteria. All eyes used in the assay should be from the same group of eyes collected on a specific day.

20. Because eyes are dissected in the laboratory, the intact heads are transported from the slaughterhouse at ambient temperature (typically between 18^{o}C and 25^{o}C) in plastic boxes humidified with tissues moistened with isotonic saline.

Selection Criteria and Number of Eyes Used in the ICE

21. Eyes that have high baseline fluorescein staining (*i.e.,* > 0.5) or corneal opacity score (*i.e.,* > 0.5) after they are enucleated are rejected.

22. Each treatment group and concurrent positive control consists of at least three eyes. The negative control group or the solvent control (if using a solvent other than saline) consists of at least one eye.

23. In the case of solid materials leading to a GHS NC outcome, a second run of three eyes is recommended to confirm or discard the negative outcome.

PROCEDURE

Preparation of the Eyes

24. The eyelids are carefully excised, taking care not to damage the cornea. Corneal integrity is quickly assessed with a drop of 2% (w/v) sodium fluorescein applied to the corneal surface for a few seconds, and then rinsed with isotonic saline. Fluorescein-treated eyes are then examined with a slit-lamp microscope to ensure that the cornea is undamaged (*i.e.,* fluorescein retention and corneal opacity scores ≤ 0.5).

25. If undamaged, the eye is further dissected from the skull, taking care not to damage the cornea. The eyeball is pulled from the orbit by holding the nictitating membrane firmly with surgical forceps, and the eye muscles are cut with a bent, blunt-tipped scissor. It is important to avoid causing corneal damage due to excessive pressure (*i.e.,* compression artifacts).

26. When the eye is removed from the orbit, a visible portion of the optic nerve should be left attached. Once removed from the orbit, the eye is placed on an absorbent pad and the nictitating membrane and other connective tissue are cut away.

27. The enucleated eye is mounted in a stainless steel clamp with the cornea positioned vertically. The clamp is then transferred to a chamber of the superfusion apparatus (18). The clamps should be positioned in the superfusion apparatus such that the entire cornea is supplied with the isotonic saline drip (3-4 drops per minute or 0.1 to 0.15 mL/min). The chambers of the superfusion apparatus should be temperature controlled at 32 ± 1.5°C. Annex 3 provides a diagram of a typical superfusion apparatus and the eye clamps, which can be obtained commercially or constructed. The apparatus can be modified to meet the needs of an individual laboratory (*e.g.,* to accommodate a different number of eyes).

28. After being placed in the superfusion apparatus, the eyes are again examined with a slit-lamp microscope to ensure that they have not been damaged during the dissection procedure. Corneal thickness should also be measured at this time at the corneal apex using the depth measuring device on the slit-lamp microscope. Eyes with; (i), a fluorescein retention score of > 0.5; (ii) corneal opacity > 0.5; or, (iii), any additional signs of damage should be replaced. For eyes that are not rejected based on any of these criteria, individual eyes with a corneal thickness deviating more than 10% from the mean value for all eyes are to be rejected. Users should be aware that slit-lamp microscopes could yield different corneal thickness measurements if the slit-width setting is different. The slit-width should be set at 0.095 mm.

29. Once all eyes have been examined and approved, the eyes are incubated for approximately 45 to 60 minutes to equilibrate them to the test system prior to dosing. Following the equilibration period, a zero reference measurement is recorded for corneal thickness and opacity to serve as a baseline (*i.e.,* time = 0). The fluorescein score determined at dissection is used as the baseline measurement for that endpoint.

Application of the Test Chemical

30. Immediately following the zero reference measurements, the eye (in its holder) is removed from the superfusion apparatus, placed in a horizontal position, and the test chemical is applied to the cornea.

31. Liquid test chemicals are typically tested undiluted, but may be diluted if deemed necessary (*e.g.,* as part of the study design). The preferred solvent for diluted test chemicals is physiological saline. However, alternative solvents may also be used under controlled conditions, but the appropriateness of solvents other than physiological saline should be demonstrated.

32. Liquid test chemicals are applied to the cornea such that the entire surface of the cornea is evenly covered with the test chemical; the standard volume is 0.03 mL.

33. If possible, solid test chemicals should be ground as finely as possible in a mortar and pestle, or comparable grinding tool. The powder is applied to the cornea such that the surface is uniformly covered with the test chemical; the standard amount is 0.03 g.

34. The test chemical (liquid or solid) is applied for 10 seconds and then rinsed from the eye with isotonic saline (approximately 20 mL) at ambient temperature. The eye (in its holder) is subsequently returned to the superfusion apparatus in the original upright position. In case of need, additional rinsing may be used after the 10-sec application and at subsequent time points (*e.g.,* upon discovery of residues of test chemical on the cornea). In general the amount of saline additionally used for rinsing is not critical, but the observation of adherence of chemical to the cornea is important.

Control Substances

35. Concurrent negative or solvent/vehicle controls and positive controls should be included in each experiment.

36. When testing liquids at 100% or solids, physiological saline is used as the concurrent negative control in the ICE test method to detect non-specific changes in the test system, and to ensure that the assay conditions do not inappropriately result in an irritant response.

37. When testing diluted liquids, a concurrent solvent/vehicle control group is included in the test method to detect non-specific changes in the test system, and to ensure that the assay conditions do not inappropriately result in an irritant response. As stated in paragraph 31, only a solvent/vehicle that has been demonstrated to have no adverse effects on the test system can be used.

38. A known ocular irritant is included as a concurrent positive control in each experiment to verify that an appropriate response is induced. As the ICE assay is being used in this Test Guideline to identify corrosive or severe irritants, the positive control should be a reference substance that induces a severe response in this test method. However, to ensure that variability in the positive control response across time can be assessed, the magnitude of the severe response should not be excessive. Sufficient

in vitro data for the positive control should be generated such that a statistically defined acceptable range for the positive control can be calculated. If adequate historical ICE test method data are not available for a particular positive control, studies may need to be conducted to provide this information.

39. Examples of positive controls for liquid test chemicals are 10% acetic acid or 5% benzalkonium chloride, while examples of positive controls for solid test chemicals are sodium hydroxide or imidazole.

40. Benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative irritancy potential of an ocular irritant within a specific range of irritant responses.

Endpoints Measured

41. Treated corneas are evaluated prior to treatment and at 30, 75, 120, 180, and 240 minutes (± 5 minutes) after the post-treatment rinse. These time points provide an adequate number of measurements over the four-hour treatment period, while leaving sufficient time between measurements for the requisite observations to be made for all eyes.

42. The endpoints evaluated are corneal opacity, swelling, fluorescein retention, and morphological effects (*e.g.,* pitting or loosening of the epithelium). All of the endpoints, with the exception of fluorescein retention (which is determined only prior to treatment and 30 minutes after test chemical exposure) are determined at each of the above time points.

43. Photographs are advisable to document corneal opacity, fluorescein retention, morphological effects and, if conducted, histopathology.

44. After the final examination at four hours, users are encouraged to preserve eyes in an appropriate fixative (*e.g.,* neutral buffered formalin) for possible histopathological examination (see paragraph 14 and reference (8) for details).

45. Corneal swelling is determined from corneal thickness measurements made with an optical pachymeter on a slit-lamp microscope. It is expressed as a percentage and is calculated from corneal thickness measurements according to the following formula:

$$\left(\frac{corneal\ thickness\ at\ time\ t \ - \ corneal\ thickness\ at\ time = 0}{corneal\ thickness\ at\ time = 0} \right) \times 100$$

46. The mean percentage of corneal swelling for all test eyes is calculated for all observation time points. Based on the highest mean score for corneal swelling, as observed at any time point, an overall category score is then given for each test chemical (see paragraph 51).

47. Corneal opacity is evaluated by using the area of the cornea that is most densely opacified for scoring as shown in Table 1. The mean corneal opacity value for all test eyes is calculated for all observation time points. Based on the highest mean score for corneal opacity, as observed at any time point, an overall category score is then given for each test chemical (see paragraph 51).

Table 1. Corneal opacity scores.

Score	Observation
0	No opacity
0.5	Very faint opacity
1	Scattered or diffuse areas; details of the iris are clearly visible
2	Easily discernible translucent area; details of the iris are slightly obscured
3	Severe corneal opacity; no specific details of the iris are visible; size of the pupil is barely discernible
4	Complete corneal opacity; iris invisible

48. Fluorescein retention is evaluated at the 30 minute observation time point only as shown in Table 2. The mean fluorescein retention value of all test eyes is then calculated for the 30-minute observation time point, and used for the overall category score given for each test chemical (see paragraph 51).

Table 2. Fluorescein retention scores.

Score	Observation
0	No fluorescein retention
0.5	Very minor single cell staining
1	Single cell staining scattered throughout the treated area of the cornea
2	Focal or confluent dense single cell staining
3	Confluent large areas of the cornea retaining fluorescein

49. Morphological effects include “pitting” of corneal epithelial cells, “loosening” of epithelium, “roughening” of the corneal surface and “sticking” of the test chemical to the cornea. These findings can vary in severity and may occur simultaneously. The classification of these findings is subjective according to the interpretation of the investigator.

DATA AND REPORTING

Data Evaluation

50. Results from corneal opacity, swelling, and fluorescein retention should be evaluated separately to generate an ICE class for each endpoint. The ICE classes for each endpoint are then combined to generate an Irritancy Classification for each test chemical.

Decision Criteria

51. Once each endpoint has been evaluated, ICE classes can be assigned based on a predetermined range. Interpretation of corneal swelling (Table 3), opacity (Table 4), and fluorescein

retention (Table 5) using four ICE classes is done according to the scales shown below. It is important to note that the corneal swelling scores shown in Table 3 are only applicable if thickness is measured with a Haag-Streit BP900 slit-lamp microscope with depth-measuring device no. 1 and slit-width setting at 9½, equalling 0.095 mm. Users should be aware that slit-lamp microscopes could yield different corneal thickness measurements if the slit-width setting is different.

Table 3. ICE classification criteria for corneal swelling.

Mean Corneal Swelling (%)	ICE Class
0 to 5	I
>5 to 12	II
>12 to 18 (>75 min after treatment)	II
>12 to 18 (≤75 min after treatment)	III
>18 to 26	III
>26 to 32 (>75 min after treatment)	III
>26 to 32 (≤75 min after treatment)	IV
>32	IV

*Highest mean score observed at any time point

Table 4. ICE classification criteria for opacity.

Maximum Mean Opacity Score*	ICE Class
0.0-0.5	I
0.6-1.5	II
1.6-2.5	III
2.6-4.0	IV

*Maximum mean score observed at any time point (based on opacity scores as defined in Table 1).

Table 5. ICE classification criteria for mean fluorescein retention.

Mean Fluorescein Retention Score at 30 minutes post-treatment*	ICE Class
0.0-0.5	I
0.6-1.5	II
1.6-2.5	III
2.6-3.0	IV

*Based on scores as defined in Table 2.

52. The *in vitro* classification for a test chemical is assessed by reading the GHS classification that corresponds to the combination of categories obtained for corneal swelling, corneal opacity, and fluorescein retention as described in Table 6.

Table 6. Overall *in vitro* classifications.

UN GHS Classification	**Combinations of the 3 Endpoints**
No Category	3 x I
	2 x I, 1 x II
No prediction can be made	Other combinations
Category 1	3 x IV
	2 x IV, 1 x III
	2 x IV, 1 x II*
	2 x IV, 1 x I*
	Corneal opacity ≥ 3 at 30 min (in at least 2 eyes)
	Corneal opacity = 4 at any time point (in at least 2 eyes)
	Severe loosening of the epithelium (in at least 1 eye)

*Combinations less likely to occur.

Study Acceptance Criteria

53. A test is considered acceptable if the concurrent negative or vehicle/solvent controls and the concurrent positive controls are identified as GHS Non-Classified and GHS Category 1, respectively.

Test Report

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

Test Chemical and Control Substances

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

- Treatment of the test chemical/control substances prior to testing, if applicable (*e.g.,* warming, grinding);

- Stability, if known;

Information Concerning the Sponsor and the Test Facility

- Name and address of the sponsor, test facility and study director;

- Identification on the source of the eyes (*e.g.,* the facility from which they were collected);

Test Method Conditions

- Description of test system used;

- Slit-lamp microscope used (*e.g.,* model) and instrument settings for the slit-lamp microscope used;

- Reference to historical negative and positive control results and, if applicable, historical data demonstrating acceptable concurrent benchmark control ranges;

- The procedure used to ensure the integrity (*i.e.,* accuracy and reliability) of the test method over time (*e.g.,* periodic testing of proficiency chemicals)).

Eyes Collection and Preparation

- Age and weight of the donor animal and if available, other specific characteristics of the animals from which the eyes were collected (*e.g.,* sex, strain);

- Storage and transport conditions of eyes (*e.g.,* date and time of eye collection, time interval between collection of chicken heads and placing the enucleated eyes in superfusion chamber);

- Preparation & mounting of the eyes including statements regarding their quality, temperature of eye chambers, and criteria for selection of eyes used for testing.

Test Procedure

- Number of replicates used;

- Identity of the negative and positive controls used (if applicable, also the solvent and benchmark controls);

- Test chemical dose, application and exposure time used;

- Observation time points (pre- and post- treatment);

- Description of evaluation and decision criteria used;

- Description of study acceptance criteria used;
- Description of any modifications of the test procedure.

Results

- Tabulation of corneal swelling, opacity and fluorescein retention scores obtained for each individual eye and at each observation time point, including the mean scores at each observation time of all tested eyes;
- The highest mean corneal swelling, opacity and fluorescein retention scores observed (from any time point), and its relating ICE class.
- Description of any other effects observed;
- The derived *in vitro* GHS classification;
- If appropriate, photographs of the eye;

Discussion of the Results

Conclusion

LITERATURE

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ANNEX 1

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of "relevance." The term is often used interchangeably with "concordance", to mean the proportion of correct outcomes of a test method.

Benchmark substance: A substance used as a standard for comparison to a test chemical. A benchmark substance should have the following properties; (i), a consistent and reliable source(s); (ii), structural and functional similarity to the class of substances being tested; (iii), known physical/chemical characteristics; (iv) supporting data on known effects; and (v), known potency in the range of the desired response

Bottom-Up Approach: step-wise approach used for a chemical suspected of not requiring classification for eye irritation or serious eye damage, which starts with the determination of chemicals not requiring classification (negative outcome) from other chemicals (positive outcome).

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

Corneal opacity: Measurement of the extent of opaqueness of the cornea following exposure to a test chemical. Increased corneal opacity is indicative of damage to the cornea.

Corneal swelling: An objective measurement in the ICE test of the extent of distension of the cornea following exposure to a test chemical. It is expressed as a percentage and is calculated from baseline (pre-dose) corneal thickness measurements and the thickness recorded at regular intervals after exposure to the test material in the ICE test. The degree of corneal swelling is indicative of damage to the cornea.

Eye Irritation: Production of changes in the eye following the application of test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Interchangeable with "Reversible effects on the Eye" and with "UN GHS Category 2" (4).

False negative rate: The proportion of all positive substances falsely identified by a test method as negative. It is one indicator of test method performance.

False positive rate: The proportion of all negative substances that are falsely identified by a test method as positive. It is one indicator of test method performance.

Fluorescein retention: A subjective measurement in the ICE test of the extent of fluorescein sodium that is retained by epithelial cells in the cornea following exposure to a test substance. The degree of fluorescein retention is indicative of damage to the corneal epithelium.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

Irreversible effects on the eye: see "Serious eye damage" and "UN GHS Category 1".

Mixture: A mixture or a solution composed of two or more substances in which they do not react (4)

Negative control: An untreated replicate containing all components of a test system. This sample is processed with test chemical-treated samples and other control samples to determine whether the solvent interacts with the test system.

Not Classified: Substances that are not classified for eye irritation (UN GHS Category 2) or serious damage to eye (UN GHS Category 1). Interchangeable with "UN GHS No Category".

Positive control: A replicate containing all components of a test system and treated with a chemical known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the severe response should not be excessive.

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability.

Reversible effects on the Eye: see "Eye Irritation" and "UN GHS Category 2".

Serious eye damage: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test chemical to the anterior surface of the eye, which is not fully reversible within 21 days of application. Interchangeable with "Irreversible effects on the eye" and with "UN GHS Category 1" (4).

Slit-lamp microscope: An instrument used to directly examine the eye under the magnification of a binocular microscope by creating a stereoscopic, erect image. In the ICE test method, this instrument is used to view the anterior structures of the chicken eye as well as to objectively measure corneal thickness with a depth-measuring device attachment.

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

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition (4).

Surfactant: Also called surface-active agent, this is a substance, such as a detergent, that can reduce the surface tension of a liquid and thus allow it to foam or penetrate solids; it is also known as a wetting agent.

Top-Down Approach: step-wise approach used for a chemical suspected of causing serious eye damage, which starts with the determination of chemicals inducing serious eye damage (positive outcome) from other chemicals (negative outcome).

Test chemical: Chemical (substance or mixture) assessed in the test method.

Tiered testing strategy: A stepwise testing strategy where all existing information on a test chemical is reviewed, in a specified order, using a weight-of-evidence process at each tier to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier. If the irritancy potential of a test chemical can be assigned based on the existing information, no additional testing is required. If the irritancy potential of a test chemical cannot be assigned based on the existing information, a step-wise sequential animal testing procedure is performed until an unequivocal classification can be made.

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (4).

UN GHS Category 1: see "Serious damage to eyes" and/or "Irreversible effects on the eye".

UN GHS Category 2: see "Eye Irritation" and/or "Reversible effects to the eye".

UN No Category: Substances that do not meet the requirements for classification as UN GHS Category 1 or 2 (2A or 2B). Interchangeable with “Not classified”.

Validated test method: A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose.

Weight-of-evidence: The process of considering the strengths and weaknesses of various pieces of information in reaching and supporting a conclusion concerning the hazard potential of a chemical.

ANNEX 2

PROFICIENCY CHEMICALS FOR THE ICE TEST METHOD

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly identifying the eye hazard classification of the 13 substances recommended in Table 1. These substances were selected to represent the range of responses for eye hazards based on results from the *in vivo* rabbit eye test (TG 405) and the UN GHS classification system (*i.e.,* UN GHS Categories 1, 2A, 2B, or No Category) (4)(6). Other selection criteria were that substances are commercially available, there are high quality *in vivo* reference data available, and there are high quality data from the ICE *in vitro* method. Reference data are available in the SSD (5) and in the ICCVAM Background Review Documents for the ICE test method (9).

Table 1: Recommended substances for demonstrating technical proficiency with ICE

Chemical	CASRN	Chemical Class[1]	Physical Form	*In Vivo* Classification[2]	*In Vitro* Classification[3]
Benzalkonium chloride (5%)	8001-54-5	Onium compound	Liquid	Category 1	Category 1
Chlorhexidine	55-56-1	Amine, Amidine	Solid	Category 1	Category 1
Dibenzoyl-L-tartaric acid	2743-38-6	Carboxylic acid, Ester	Solid	Category 1	Category 1
Imidazole	288-32-4	Heterocyclic	Solid	Category 1	Category 1
Trichloroacetic acid (30%)	76-03-9	Carboxylic Acid	Liquid	Category 1	Category 1
2,6-Dichlorobenz-oyl chloride	4659-45-4	Acyl halide	Liquid	Category 2A	No predictions can be made [4]
Ammonium nitrate	6484-52-2	Inorganic salt	Solid	Category 2A[5]	No predictions can be made [4]
Ethyl-2-methylaceto-acetate	609-14-3	Ketone, Ester	Liquid	Category 2B	No predictions can be made [4]
Dimethyl sulfoxide	67-68-5	Organic sulphur compound	Liquid	No Category	No Category
Glycerol	56-81-5	Alcohol	Liquid	No Category	No Category (borderline)
Methylcyclopentane	96-37-7	Hydrocarbon (cyclic)	Liquid	No Category	No Category
n-Hexane	110-54-3	Hydrocarbon (acyclic)	Liquid	No Category	No Category
Triacetin	102-76-1	Lipid	Liquid	Not classified	No Category

Abbreviations: CASRN = Chemical Abstracts Service Registry Number

[1]Chemical classes were assigned to each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system (available at http//www.nlm.nih.gov/mesh)

[2]Based on results from the *in vivo* rabbit eye test (OECD TG 405) and using the UN GHS (4)(6).

[3]Based on results in ICE as described in table 6.

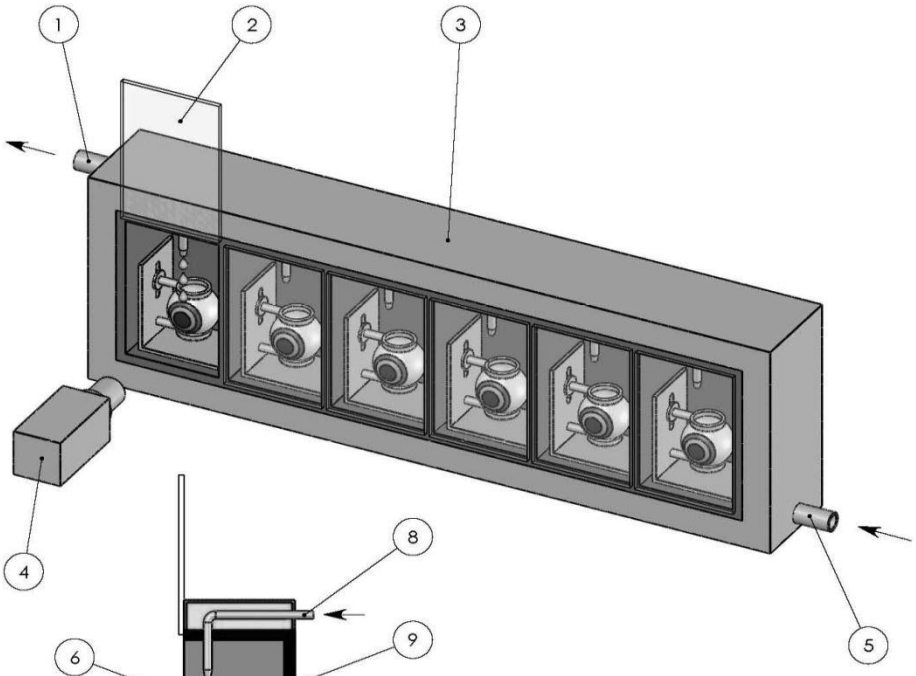
[4] Combination of ICE scores other than the ones described in table 6 for the identification of GHS no-category and GHS Category 1 (see table 6)

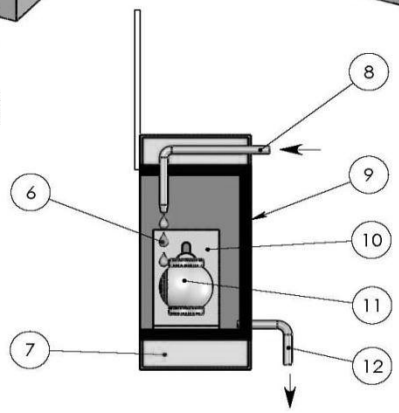
[5] Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, *i.e.* 1 out of 3 vs 2 out of 3 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* study included 3 animals. All endpoints apart from conjunctiva redness in one animal recovered to a score of zero by day 7 or earlier. The one animal that did not fully recover by day 7 had a conjunctiva redness score of 1 (at day 7) that fully recovered at day 10.

ANNEX 3

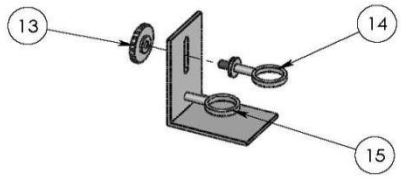
DIAGRAMS OF THE ICE SUPERFUSION APPARATUS AND EYE CLAMPS

(See Burton et al. (18) for additional generic descriptions of the superfusion apparatus and eye clamp)





CROSS SECTION COMPARTMENT



EYE HOLDER

ITEM NO.	DESCRIPTION
1	OUTLET WARM WATER
2	SLIDING DOOR
3	SUPERFUSION APPARATUS
4	OPTICAL MEASURING INSTRUMENT
5	INLET WARM WATER
6	SALINE SOLUTION
7	WARM WATER
8	INLET SALINE SOLUTION
9	COMPARTMENT
10	EYE HOLDER
11	CHICKEN EYE
12	OUTLET SALINE SOLUTION
13	SETSCREW
14	ADJUSTABLE UPPER ARM
15	FIXED LOWER ARM

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

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ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2013

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Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France**

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

FOREWORD

This streamlined summary document (SSD) was developed to provide summary information in support of OECD Test Guideline 438 on the Isolated Chicken Eye Test Method addressing the endpoint eye irritation/corrosion. This SSD was developed by a Secretariat consultant and submitted to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in March 2013, together with the updated version of TG 438 (originally adopted in 2009). The SSD provides useful and more detailed information than is otherwise available from the Test Guideline itself on: 1) the scientific basis of the test method, 2) the identified limitations, weaknesses and strengths, 3) the applicability domain, 4) the sensitivity, specificity and accuracy, and 5) the within-laboratory and between-laboratory reproducibility of the method.

The SSD was approved by the WNT with a few changes to paragraph 11, including additional references 22, 23, 24 and 25, on 30 April 2013.

The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 14 June, 2013.

This document is published under the responsibility of the Joint Meeting of the Chemicals committee and the Working Party on Chemicals, Pesticides and Biotechnology.

STREAMLINED SUMMARY DOCUMENT

Description of applicability domain and performance, based on the retrospective validation studies and their revisions, of the Isolated Chicken Eye (ICE) Test Method (Test Guideline 438) for identifying i) chemicals inducing serious eye damage and ii) chemicals not requiring classification for eye irritation or serious eye damage

INTRODUCTION AND BACKGROUND

The 2003-2006 Validation Studies

1. Between 2003 and 2006, a retrospective evaluation was carried out concerning the validation status of the Isolated Chicken Eye (ICE) test method for identifying chemicals (substances and mixtures) inducing serious eye damage ("ocular corrosives and severe irritants"), *i.e.*, its usefulness and limitations for initiating a Top-Down approach (1). This evaluation, counting with a total of 175 chemicals, was performed by the US-Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the US-National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), in collaboration with the EU-European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM). For a full description, see the Background Review Document (BRD) (2) and the ICCVAM Test Method Evaluation Report (TMER) (3).

2. The study aimed at characterising the reproducibility and predictive capacity of the ICE for the following classification systems: UN GHS (Category 1) (4), US EPA (Category I) (5) and EU DSD (R41) (6) (the EU CLP classification system (7) based on UN GHS had not yet been adopted at that time). On the basis of all collected data and information the ICE was considered as scientifically valid (reliable and relevant) for identifying chemicals inducing serious eye damage (*i.e.*, to initiate a Top-Down approach (1)) and was recommended for regulatory hazard classification and labelling purposes. Chemicals inducing serious eye damage are defined as those that produce tissue damage in the eye, or serious physical decay of vision, following application to the anterior surface of the eye in the *in vivo* Draize rabbit eye test (8), which is not (or not expected to be) fully reversible within 21 days of application. Following these recommendations, the OECD officially adopted the ICE test method as OECD Test Guideline (TG) 438 for identifying chemicals inducing serious eye damage in September 2009 (9).

The 2006-2009 Validation Studies

3. NICEATM/ICCVAM, in collaboration with ECVAM and JaCVAM, further evaluated between 2006 and 2009 the usefulness and limitations of the ICE test method for the additional identification of chemicals not causing sufficient effects on the eye to require hazard classification and labelling according to the UN GHS (4), US EPA (5), US FHSA and EU DSD (6) classification systems (the EU CLP classification system (7) based on UN GHS had not been adopted at that time), *i.e.*, its usefulness and limitations for initiating a Bottom-Up approach (1). The ICE validation database remained unchanged and comprised 175 chemicals (90 substances and 85 mixtures) collected from five individual studies (Prinsen and Koëter 1993 (10), Balls et al. 1995 (11), Prinsen 1996 (12), Prinsen 2000 (13) and Prinsen 2005 (14)), which were used to determine the predictive capacity of the ICE test method (ICCVAM BRD from April 2009 (15)).

4. In May 2009, NICEATM/ICCVAM convened an independent international scientific peer review panel (PRP) on alternative ocular safety testing methods, composed of members from EU, USA, Japan and

Canada. The PRP maintained the original recommendation for using the ICE for classification of serious eye damage (16). At that stage however, no further recommendations were made for an expansion of the ICE applicability domain to also include other classification categories, and in particular the identification of non-classified chemicals (16). This was due to the false negatives rates (6% or 4/62 for the UN GHS classification system) and the fact that amongst the false negatives there was one substance was classified as GHS Cat 1 based on Draize rabbit eye test data (16).

Revisions of the Validation Dataset

5. On the basis of revisions of the validation dataset carried out in 2012 it was found that the individual *in vitro* and *in vivo* classifications of a number of chemicals deserved further considerations. In particular discrepancies were found in the final *in vivo* and *in vitro* classifications for a number of chemicals in the ICCVAM BRD from April 2009 (15) which had an impact on the number of false negative chemicals (1 out of 73 false negative instead of 4 out of 62; see Appendixes 1, 2 and 3). In addition, it was felt important to recognize the limitations of the *in vivo* Draize rabbit eye irritation test and their implications for validation purposes (17). Some of the drawbacks of the Draize rabbit eye test referred in literature include (see extract from Eskes (18) in Appendix 3 for details):

- The fact that the *in vivo* rabbit eye irritation/corrosion test has no standardized exposure regimen, so that the duration of exposure of the test substance with the rabbit eyes remains unknown and can vary from a few minutes to several hours. In addition, for solids and sticky chemicals it is unclear how much of the compound (solid, paste or liquid) stays in contact with the eye (19);
- The limited reproducibility of the Draize rabbit eye test method;
- The subjectivity in the allocation of the rabbit ocular tissue scores;
- The type of exposure which does not reflect a potential human accidental exposure;
- The differences in physiology and sensitivity to tested chemicals between rabbit and human eyes;
- Ethical issues and the fact that the Draize test can be very painful to the rabbits.

Adoption of TG 438 for the Identification of Chemicals Not Classified for Eye Irritation or Serious Eye Damage

6. In April 2012, following a proposal from the Netherlands and the European Commission, a project for updating TG 438 (9) was included in the work plan of the OECD Test Guidelines Programme. The aim of the project was, taking into account the review of the individual *in vivo* and *in vitro* data, to reassess the performances of ICE and address a possible update of TG 438 to allow its use also for the identification of chemicals not requiring classification for eye irritation or serious eye damage under the UN GHS classification system. An initial re-evaluation of the *in vitro* and *in vivo* ICE dataset was carried out by the Netherlands, followed by the preparation of an Issue Paper and its addendum by a consultant to the OECD Secretariat with the aim to review existing ICE data and make a recommendation on the use of TG 438 for the identification of chemicals not requiring classification for eye irritation or serious eye damage (Appendixes 3 and 4). The ICE Issue Paper and its addendum reviewed both the ICE data presented in the ICCVAM-NICEATM ICE BRDs (2) (15) as well as the evaluation carried out by The Netherlands in the first draft version of this SSD (17). The Issue Paper and its addendum were discussed by the eye irritation/corrosion expert group at an OECD expert meeting held on 6-7 December 2012. The present SSD represents a compilation of all relevant data on the ICE test method evaluation and takes into account all comments received at and after the eye irritation expert meeting, as well as the comments received from the two commenting rounds on the proposed revised TG 438.

7. In 2013, the OECD approved the updated version of TG 438 allowing the use of ICE for identifying chemicals inducing serious eye damage as well as for identifying chemicals not requiring classification for eye irritation or serious eye damage.

SCIENTIFIC BASIS FOR THE ICE TEST METHOD

8. The ICE test method (TG 438) is an organotypic model that provides short-term maintenance of the chicken eye *in vitro*. Damage to the cornea is assessed by determination of corneal swelling, corneal opacity, and fluorescein retention in a test that typically takes less than 8 hours to perform. Analysis of corneal swelling provides for a quantitative assessment, whereas corneal opacity and fluorescein retention involve a qualitative assessment based nevertheless on slit-lamp observations. Each endpoint is then either converted into a quantitative score or assigned a qualitative categorization (ICE Classes) which are then combined together and used to assign an *in vitro* ocular hazard classification (9) (20).

Data Interpretation Procedures

9. If the criteria used to attribute the ICE classes (I to IV) for the three endpoints (corneal swelling, corneal opacity and fluorescein retention) remained unchanged, the Data Interpretation Procedure (DIP) proposed by ICCVAM and in the OECD Guidance Document 160 (15) (20) differed slightly from the one proposed by The Netherlands (see Appendix 3). Following consultation of the OECD Expert Group on Eye Irritation, it was recommended to make use of the DIP proposed by ICCVAM and by Guidance Document 160 (15) (20) complemented with some effects proposed by The Netherlands as described in Table 1.

Table 1. Data Interpretation Procedures applied for the ICE test method for identifying *i)* chemicals inducing serious eye damage and *ii)* chemicals not requiring classification for eye irritation or serious eye damage.

UN GHS Classification	Combinations of the 3 Endpoints*
No Category	3 x I 2 x I, 1 x II
No prediction can be made	Other combinations
Category 1	3 x IV 2 x IV, 1 x III 2 x IV, 1 x II** 2 x IV, 1 x I** Corneal opacity ≥ 3 at 30 min (in at least 2 eyes) Corneal opacity = 4 at any time point (in at least 2 eyes) Severe loosening of the epithelium (in at least 1 eye)

* Based on the criteria proposed in the original TG 438 (9) and in the Guidance Document 160 (20)

**Combinations less likely to occur.

Comparison of the ICE Test Method with the In Vivo Rabbit Eye Test Method

10. In contrast to ICE, the *in vivo* rabbit eye test involves only qualitative evaluations based mainly on visual observations of the severity of adverse effects on the cornea, the iris, and the conjunctiva, as well as the reversibility of any ocular effects detected at selected intervals up to 21 days after exposure. In ICE, liquids and solids are typically tested undiluted and are applied to evenly cover the entire surface of the cornea. In the *in vivo* rabbit eye test, liquid and solid test substances are also tested usually undiluted, however they are applied to the conjunctival sac of the rabbit eyes. Because rabbits blink and/or tear, exposure of the cornea to the test substance will be affected by these factors in terms of coverage or

duration. The neurogenic components that drive tear film production are not present in the ICE. When compared with an *in vivo* rabbit eye study, application of a test substance in the absence of this protective barrier might be expected to cause an increase in false positive outcomes. One of the conclusions from a workshop on mechanisms of eye irritation highlighted the need for additional research on the impact of chemicals on tear film and the consequences of tear film disruption. However, for some test substances (*e.g.,* solids), blinking can also induce mechanical damage *in vivo*, contributing to a higher degree of irritation. Thus, the ICE test method differs from the *in vivo* rabbit eye test method in the following significant ways:

- The ICE evaluates only corneal effects and does not assess effects on the iris and the conjunctiva as performed in the *in vivo* rabbit eye test. Measurements are performed quantitatively and qualitatively with the help of a slit-lamp in the ICE assay, while they are assessed only qualitatively based mainly on visual observations in the *in vivo* rabbit eye test.
- Corneal exposure conditions, including test substance concentration and exposure duration, are well defined in the ICE assay, whereas subject to potentially large variations *in vivo* due to the ill-defined exposure conditions, blink response and natural tearing of the eye in a live animal. Moreover, based on the unrealistic accidental *in vivo* exposure conditions, solids may lead to variable and extreme responses in the *in vivo* Draize eye irritation test, which may not reflect their true irritation potential in humans (19).
- The observation period of the ICE assay is typically of 4 hours, whereas ocular effects are typically evaluated in the *in vivo* rabbit eye test for a minimum of 72 hours and can extended up to 21 days.
- Reversibility/irreversibility of corneal effects induced by a test substance cannot be observed in the ICE assay *per se*, but histological evaluation of the exposed eyes may provide additional information about the depth and type of injury that could aid predictions, as to whether damage is irreversible. It has been proposed, based on rabbit eye studies, that an assessment of the initial depth of corneal injury may be used to identify some types of irreversible effects (21) although further scientific knowledge is required to understand how irreversible effects not linked with initial high level injury occur.
- Protective mechanisms of the eye, such as tear production and blinking (e.g., against drying and infection), are built into *in vivo* testing, but are absent in *in vitro* / *ex vivo* testing. However, if regeneration of the tear film might be important for the *in vivo* healing process, it may play a minor role in the ICE test method where the initial damage is measured rather than recovery.
- The ICE assay does not account for systemic effects following ocular instillation that may be noted with the *in vivo* rabbit eye test (*e.g.,* toxicity or lethality as in the case of certain pesticides). However, these effects are typically predicted from other acute toxicity test methods, and may not be relevant for the many consumer products that are formulated with well characterized raw materials of known systemic toxicity.

IDENTIFIED LIMITATIONS, WEAKNESSES AND STRENGTHS

11. The potential shortcomings of the ICE test method when used to identify chemicals inducing serious eye damage (UN GHS Category 1) in e.g., a Top-Down approach, are based on the high false positive rate for alcohols and the high false negative rates for solids and surfactants, as observed in the 2003-2006 retrospective validation study (2) (3). When substances within these chemical and physical classes are excluded from the database, the accuracy of the ICE test method is substantially improved (2) (3) (see Table 6 below). However, since not all alcohols are over-predicted (4 out of 10) and some are correctly predicted as UN GHS Category 1, this organic functional group is not considered to be out of the applicability domain of the test method. Positive results obtained with alcohols should nevertheless be

interpreted cautiously due to potential over-prediction. Similarly, given the fact that *i)* some solids and surfactants are correctly predicted by the ICE test method as UN GHS Category 1, *ii)* that not all solids and surfactants are underpredicted (6 underpredictions out of 11 solids, and 6 out of 9 surfactants), and that *iii)* the underpredicted solids and surfactants would need to be subsequently tested with other suitable test method(s) in a sequential testing strategy (as none of the GHS Cat 1 solid and surfactant materials in the validation database are underpredicted as GHS non-classified), solids and surfactants are also not considered to be out of the applicability domain of the ICE test method. Evidence suggests that there is a certain probability that Cat 1 are predicted as Cat 2 due to the variability of individual animal responses within the same test (22). Although not based on the same dataset, the resulting probability seems to be in the same range as the BCOP/ICE under-prediction rate for identifying UN GHS Category 1. However, variability between laboratories can further contribute to the variability of *in vivo* responses (23)(24)(25). Quantitative estimates for such uncertainties, both for the in vivo tests and for their in vitro alternatives, should be considered in the future development of testing strategies for serious eye damage/eye irritation.

12. When used to identify chemicals not requiring classification for eye irritation or serious eye damage under the UN GHS classification system, in e.g., a Bottom-Up approach, anti-fouling organic solvent containing paint were found to risk under-prediction (1 out of 2 classified anti-fouling solvent containing paint was found to be under-predicted as non-classified). When chemicals within this product classes are excluded from the database, the accuracy of the ICE test method is only slightly improved (see Table 7 below). In addition, the only underpredicted material (TNO-94) was classified *in vivo* as GHS Cat 1 due to unusual effects, i.e., a residue of paint got attached to the cornea most probably caused by grooming/scratching of the eye by the rabbit and the type of exposure of the rabbit eyes (i.e., adding the paint in the conjunctival sac of the eye and holding the eye lids together). This was observed in only one animal, whereas the two other animals only showed GHS Cat 2-type effects (see Appendix 5). The OECD Expert Group decided to add a warning sentence for this category of materials, but not to exclude them from the applicability domain of the ICE test method for the following reasons: i) there was insufficient evidence to exclude those types of formulations (only two classified chemicals from this category), ii) the type of exposure to this material is unlikely to occur in humans, and iii) sticky materials present similar difficulties to test either *in vitro* and *in vivo*. Regarding the false positive rates, surfactants (5 out of 8) may risk overprediction. However due to *i)* the fact that not all surfactants were overpredicted, *ii)* that some surfactants were correctly predicted, and that *iii)* surfactants not predicted as GHS non-classified would need to be subsequently tested with other suitable test method(s) in a sequential testing strategy (2) (as none of the *in vivo* GHS non-classified chemicals (n=73) was overpredicted as GHS Cat. 1), surfactants are not considered to be out of the applicability domain of the ICE test method.

13. Although the ICE test takes into account some of the ocular effects evaluated in the *in vivo* test method and to some degree their severity, it does not consider conjunctival and iridal injuries. Nevertheless, the ICE directly addresses corneal effects, which are the major driver of classification *in vivo* when considering the UN GHS classification system (see Annex 6 of SSD for TG 437). In addition, Burton (26) found a direct relation between corneal swelling and the conjunctival reactions in a study with 600 rabbits and approximately 100 test substances. Prinsen (12) also reported a high correlation between conjunctival reactions and the endpoints assessed in the ICE test method after parallel testing of test substances *in vivo* and *in vitro*. Furthermore, although the reversibility of corneal lesions cannot be evaluated *per se* in the ICE test method it has been proposed, based on rabbit eye studies, that an assessment of the initial depth of corneal injury (*e.g.*, through histological evaluation) may be used to identify some types of irreversible effects (21). Finally, the ICE does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

14. The ICE test method is not recommended for the identification of test substances classified as irritating to eyes (UN GHS Category 2 or Category 2A) or test substances classified as mildly irritating to eyes (UN GHS Category 2B) due to the considerable number of UN GHS Category 1 chemicals underclassified as UN GHS Category 2, 2A or 2B and UN GHS No Category chemicals overclassifed as UN GHS Category 2, 2A or 2B. For this purpose, further testing with another suitable method may be required.

Table 2: Physicochemical properties and compatibility with the ICE

Physicochemical property	Is a material with this property compatible with the ICE assay?
Fixative	Yes
Solvent	Yes
Extreme pH	Yes
Gases	No
Liquids	Yes
Solid materials	Yes
Emulsions	Yes
Granular materials	Yes
Suspensions	Yes
Coloured materials	Yes
Diluted concentrations of chemicals	Yes
Highly viscous materials	Yes
Volatile materials	Yes
Reactive chemistries	Yes
Hydrophobic/lipophilic chemicals	Yes
Neat concentrations of chemicals	Yes

APPLICABILITY DOMAIN

15. The Test Guideline can be used for testing all types of substances and mixtures, provided there is no evidence that the method is not valid for the chemical tested**.**

Categories of Irritancy

16. Based on the conclusions of the 2003-2006 and 2006-2009 retrospective validation studies (3) (16), TG 438 was adopted in 2009 for classification of chemicals inducing serious eye damage (UN GHS Category 1). In addition, following the re-evaluations carried out in 2012 and their review by the OECD Expert Group on Eye Irritation, TG 438 was also approved in 2013 for the identification of chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category), under the UN GHS classification system.

Potential Role in an ITS

17. The ICE can be used as a validated *ex vivo* test method in a tiered testing approach as described in the addendum to TG 405 (8) with the purpose of identifying chemicals inducing serious eye damage (UN GHS Category 1), as well as chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category). No published studies have been found at the current date, on the combination of ICE with other *in vitro* test methods in testing strategies for the prediction of ocular hazards.

Mode of Action (MoA)

18. An expert meeting held at EC-ECVAM in 2005 (1) recommended to expand the concept of defining the applicability domain as not only chemical classes, but also as a function of the mechanism of eye

irritation. The four identified MoA that were discussed included: (i) cell membrane lysis (breakdown of membrane integrity as might occur from exposure to membrane active materials, *e.g.,* surfactants), (ii) saponification (breakdown of lipids by alkaline action), (iii) coagulation (precipitation/denaturation of macromolecules, particularly protein, characteristic of acid, alkali, or organic solvent exposure), and (iv) actions on macromolecules (chemicals that react with cellular constituents/organelles that may or may not lead to overt lysis or coagulation, *e.g.*, peroxides, mustards and bleaches). The ICE test method addresses the three first MoAs. In addition, it may also address the fourth MoA (actions on macromolecules) when histopathological information is available.

Table 3: Summary of events involved in chemical-induced eye irritation *in vivo*

Events involved in chemical-induced eye irritation	Modelled by the ICE assay?
Chemical interaction with tear film	No
Chemical binding to the conjunctival epithelium	No
Adhesion molecules compromised	Yes
Corneal epithelial damage	Yes
Inhibition of receptor-mediated transport	Yes
Compromise of cell membrane integrity of upper corneal epithelium	Yes
Cell **membrane lysis** of all corneal epithelial layers	Yes
Hydration of corneal stroma	Yes
Cross-linking *of proteins in corneal stroma*	Yes
Erosion of corneal stroma	Yes
Cell damage to corneal epithelium and limbus	Yes
Dilation and increased lymphatic leakage from scleral vasculature	No
Stimulation of nerve endings, i.e*., enhanced blinking, tearing*	No
Erosion of nerve endings in corneal and sclera	No
Duration of the response, *i.e.*, length of time cell responses deteriorate. Duration of response covers the effects of **reactive chemicals**, which can cause **coagulation**, **saponification**, that are effects, which develop and increase over time.	Yes
Recovery from response, *i.e.*, length of time for cell responses to return to control levels	No

Chemical Classes

19. The ICE validation dataset comprised a total of 175 individual chemicals (90 substances and 85 mixtures) collected from Prinsen and Koëter 1993 (10), Balls et al. 1995 (11), Prinsen 1996 (12), Prinsen 2000 (13) and Prinsen 2005 (14) as described in the retrospective validation studies (2) (15). Tables 4 and 5 show the chemical and product classes representation within the ICE validation database. Although the single components from the mixtures used in the validation dataset could not be disclosed due to proprietary reasons they represented relevant to current commerce mixtures and formulations. In addition, details on the product categories and chemical classes of most of the tested mixtures and substances were available as described in the ICCVAM BRD from April 2009 (15). Out of the 175 chemicals, 85 (including mixtures) could not be assigned a specific chemical class and 23 (including substances) could not be assigned a specific product category. Detailed information, including chemical name, Chemical Abstracts Service Registry Number (CASRN), chemical class, product category, physical state, purity, concentration(s) tested, *in vivo* GHS classification, *in vitro* GHS classification, *in vitro* raw data, *in vitro* categories and literature reference using the chemical are provided in Appendix 1.

Table 4: Chemical classes tested in the ICE test method*.

Chemical Class	# of Chemicals	Chemical Class	# of Chemicals
Acetate	1	Inorganic Chloride Compound	1
Acid	5	Inorganic Salt	3
Acyl halide	1	Inorganic Silver / Nitrogen Compound	1
Alcohol	15	Ketone	4
Aldehyde	2	Lactone	1
Alkali	3	Lipid	1
Amide /Amidine	7	Nitrile	1
Amino acid	1	Nitro Compound	1
Boron compound	1	Not Classified	85
Carbohydrate	2	Onium Compound	8
Carboxylic acid	12	Organic Silicon Compound	2
Ester	10	Organic Sulfur Compound	3
Ether	1	Organometallic	2
Heterocyclic	9	Organophosphrous Compound	1
Hydrocarbon	5	Polycyclic	4
Imide	2	Polyether	5
Inorganic Chemical	1	Urea Compound	1

* Revised from (15) based on information presented in Appendix 1.

Table 5: Product classes tested in the ICE test method*.

Product Class	# of Chemicals	Product Class	# of Chemicals
Adhesive	2	Fertilizer	1
Antifungal	3	Food additive	1
Antihistamine	1	Fungicide / Germicide / Bactericide	8
Anti-infective	3	Industrial Chemical, Intermediate or Formulation	19
Antiseptic	2	Not Classified	23
Caustic Agent	4	Optical Resolution Agent	1

Chlorination by-product	1	Paint	4
Cleaner	8	Pesticide / Herbicide	17
Copolymer	8	Preservative	6
Cosmetic Ingredient	1	Pharmaceutical Compounds / Intermediates	6
Detergent	8	Raw Material	9
Developer	1	Reagent	4
Disinfectant	5	Resin	2
Dyes & Stains	10	Silicon Resin	1
Elastomer	2	Soap	9
Enzyme Inhibitor	1	Surfactant	25
Enzyme Solution	3	Solvent	37

* Revised from (15) based on information presented in Appendix 1.

SENSITIVITY, SPECIFICITY AND ACCURACY

20. Within the ICE validation database a total of 152 chemicals (72 substances and 80 mixtures) had sufficient *in vivo* and *in vitro* data to assess the ICE predictive capacity (Appendix 1). Their distribution according to the UN GHS classification categories are described below.

- Identification of GHS NC (Bottom-Up approach):
  152 chemicals (72 substances + 80 mixtures)
  79 NC + 73 classified chemicals (30 Cat 1 + 6 Cat 1/2 + 27 Cat 2A + 8 Cat 2B + 2 Cat 2A/2B)
- Identification of GHS Cat 1 (Top-Down approach):
  139 chemicals (65 substances + 75 mixtures)
  27 Cat 1 + 113 non-Cat 1 (26 Cat 2A + 8 Cat 2B + 79 Non-Classified (NC))

Out of the 175 chemicals from the validation dataset, a number of chemicals (n=15) had no raw *in vivo* data to allocate a UN GHS Classification. In addition, a number of chemicals (n=13) had Study Criteria Not Met (SCNM) to assign an *in vivo* classification (e.g., incomplete dataset to assess reversibility / irreversibility of effects at day 21). For a large number of them (n=11), the *in vivo* scores suggested the need for classification even if not possible to allocate a specific classification category (i.e., GHS Cat 2B versus 2A versus 1). These chemicals were used for the evaluation of the predictive capacity of the ICE test method in a bottom-up approach, but not for the top-down approach due to uncertainty as to which classification category to assign (i.e., GHS Cat 1 versus GHS Cat 2). Chemicals that had a SCNM and were estimated to be non-classified based on expert judgement (n=2), were not included in any of the analyses for precautionary reasons (although in the original evaluation they were considered as NC). A total of 5 chemicals that were classified as Eye GHS Cat 1 based on data from skin corrosion studies were not included for the purposes of the Test Guideline, in order to consider only chemicals for which high quality *in vivo* ocular data was available. Finally, two chemicals had a borderline GHS Cat 1 / Cat 2 classification so that they could only be used for the evaluation of the predictive capacity of the ICE test method in a bottom-up approach, and not in a top-down approach.

Overall Predictive Capacity

21. Due to discrepancies found in a number of *in vitro* and *in vivo* classifications from previous validation studies (for details see Appendixes 1, 2 and 3), the predictive capacities of the ICE test method were re-calculated for *i)* the identification of GHS Category 1 chemicals (Top-Down approach) and *ii)* the identification of non-classified chemicals (Bottom-up approach) as shown in Tables 6 and 7. The analyses were based on the outcome of individual test substances (and not on individual laboratory outcome), as recommended by the Expert Group on Eye Irritation, in order to be in alignment with previous ICCVAM evaluations and with the analyses carried out in the context of the revisions of the BCOP Test Guideline.

Table 6: Predictive capacity of the ICE test method for distinguishing chemicals (substances and mixtures) inducing serious eye damage (UN GHS[1] Category 1) from all other categories.

Top-.Down Approach	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
Overall	140	86	120/140	52	14/27	48	13/27	94	106/113	6	7/113
Without alcohols, solids and surfactants	82	94	77/82	71	5/7	29	2/7	96	72/75	4	3/75

Abbreviations: No. = data used to calculate the percentage.
[1]UN GHS classification system (4): Category 1 vs. Non-Category 1 (No Category + Cat. 2B + Cat. 2A).

Table 7: Predictive capacity of the ICE test method for distinguishing chemicals (substances and mixtures) not requiring classification for eye irritation or serious eye damage (UN GHS[1] Non-Classified) from all other irritant categories.

Bottom-Up Approach	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
Overall	152	82	125/152	99	72/73	1	1/73	67	53/79	33	26/79
Without anti-fouling organic-solvent containing paints	149	83	123/149	100	71/71	0	0/71	67	52/78	33	26/78

Abbreviations: No. = data used to calculate the percentage.
[1]UN GHS classification system (4): No Category vs. Classified Chemicals (Cat. 1 + Cat. 2A + Cat. 2B).

22. For the Top-Down approach, alcohols were found to risk over-prediction (4 alcohols out of 10 non-Category 1 were over-predicted as Category 1) whereas solids and surfactants were found to risk under-prediction (6 out of 11 Category 1 solids were found to be under-predicted, and 6 out of 9 Category 1 surfactants were found to be under-predicted). Table 8 shows the false positive and false negative rates

obtained for specific chemical classes and properties of interest, including mixtures and substances based on the revised dataset (Appendix 1). Substances, mixtures, liquids and solids all showed false positive rates below or equal to 15% suggesting an appropriate identification of GHS Category 1. The rate of false negatives was found to be particularly high (i.e., higher than 50% for 5 chemicals or more), for solids and surfactants. However due to the fact that the underpredicted solids and surfactants would need to be subsequently tested with other suitable test method(s) in a sequential testing strategy (as none of the GHS Cat 1 solid and surfactant materials in the validation database are underpredicted as GHS non-classified), solids and surfactants are not considered to be out of the applicability domain of the ICE test method.

23. For the Bottom-up approach, anti-fouling organic solvent containing paint were found to risk under-prediction (1 out of 2 classified anti-fouling solvent containing paint was found to be under-predicted as non-classified). As explained in paragraph 12, a warning sentence was included in the Test Guideline for this category of materials but they were not to excluded from the applicability domain of the ICE test method for the following reasons: i) there was insufficient evidence (only two classified chemicals from this category), ii) the type of exposure is unlikely to occur in humans, and iii) sticky materials present similar difficulties to test either *in vitro* and *in vivo*. Table 9 shows the false positive and false negative rates obtained for specific chemical classes and properties of interest, including mixtures and substances based on the revised dataset (Appendix 1). Substances, mixtures, liquids and solids all showed false negative rates below or equal to 5% suggesting an appropriate identification of GHS Non-classified chemicals based on the criteria discussed by the OECD Expert Group on Eye Irritation. Regarding the false positive rates, the ICE test method was found to have a lower overall false positive rate as compared to other test methods accepted for this purpose (i.e., 33% for ICE versus 69% for BCOP and 68% for CM). The false positive rates was found nevertheless to be particularly high (i.e., higher than 50% for 5 chemicals or more) for surfactants. However due to the fact that the overpredicted surfactants would need to be subsequently tested with other suitable test method(s) in a sequential testing strategy (2) (as none of the *in vivo* GHS non-classified chemicals (n=73) was overpredicted as GHS Cat. 1), surfactants are not considered to be out of the applicability domain of the ICE test method.

Table 8: False positive and false negative rates of the ICE test method, by properties of interest, chemical class and product categories, for distinguishing chemicals (substances and mixtures) inducing serious eye damage (UN GHS[1] Category 1) from all other categories.

Top-Down Approach	N[2]	False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]
Overall	140	6	7/113	48	13/27
Properties of interest					
Substances	65	15	6/40	44	11/25
Mixtures	75	1	1/73	100*	2/2[4,*]
Liquids[5]	95	8	6/80	40	6/15
Solids[5]	34	0	0/23	55	6/11
Emulsions and gels[5]	7	14	1/7	n.a.	n.a.
Chemical Classes[6]					
Alcohol	12	40	4/10	50*	1/2*
Amine/Amidine	5	0*	0/1*	50*	2/4*
Carboxylic acid	10	0*	0/3*	43	3/7
Ester	9	13	1/8	0*	0/1*
Heterocyclic	9	0*	0/3*	50	3/6
Onium compound	8	0*	0/2*	50	3/6
Polyether	5	25*	1/4*	100*	1/1*

Product categories					
Cleaners	4	0*	0/3*	100*	1/1*
Copolymer	8	13	1/8	n.a.	n.a.
Detergent	7	0*	0/4*	100*	3/3*
Dyes & stains	8	0	0/7	0*	0/1*
Fungicide / Germicide / Bactericide	7	0*	0/1*	50	3/6
Industrial Chemical, Intermediate or Formulation	16	0	0/14	50*	1/2*
Pesticide / Herbicide	12	0	0/6	50	3/6
Preservative	5	0*	0/1*	25*	1/4*
Pharmaceutical compound or intermediate	4	50*	1/2*	50*	1/2*
Raw material	8	0	0/8	n.a.	n.a.
Soap	7	0	0/6	100*	1/1*
Solvent	34	19	6/31	0*	0/3*
Surfactant – Total[7] -cationic -nonionic -anionic	21 7 5 2	0 0* 0* 0*	0/12 0/1* 0/4* 0/1*	67 50 100* 100*	6/9 3/6 1/1* 1/1*

* Too small dataset to make definitive conclusions; n.a.: not applicable.
[1]GHS = Globally Harmonized System (UN 2011) (4).
[2]N = Number of chemicals.
[3]Data used to calculate the percentage.
[4] Only few formulations having severe effects are available.
[5]Physical form (*i.e.*, solid or liquid) not known for 4 chemicals.
[6]Chemical classes included in this Table are represented by at least five chemicals tested in the ICE test method and assignments are based on the MeSH categories (www.nlm.nih.gov/mesh) as described in (2) and (15).
[7]Combines single substances labelled as surfactants along with surfactant-containing mixtures.

Table 9: False positive and false negative rates of the ICE test method, by properties of interest, chemical class and product categories, for distinguishing chemicals (substances and mixtures) not requiring classification for eye irritation or serious eye damage (UN GHS[1] No Category) from all other irritant categories.

Bottom-Up Approach	**N[2]**	**False Positive Rate**		**False Negative Rate**	
		%	No.[3]	%	No.[3]
Overall	152	33	26/79	1	1/73
Properties of interest					
Substances	72	70	14/20	0	0/52
Mixtures	80	20	12/59	5	1/21
Liquids[4]	101	36	20/55	2	1/46
Solids[4]	38	18	3/17	0	0/21
Emulsions, gels and paste[4]	9	50*	2/4*	0	0/5
Chemical Classes[5]					
Alcohol	13	100*	4/4*	0	0/9
Amine/Amidine	6	100*	1/1*	0	0/5
Carboxylic acid	11	100*	2/2*	0	0/9

Ester	10	100*	4/4*	0	0/6
Heterocyclic	9	100*	2/2*	0	0/7
Onium compound	8	100*	1/1*	0	0/7
Polyether	5	100*	2/2*	0*	0/3*
Product categories					
Cleaners	5	100*	1/1*	0*	0/4*
Copolymer	8	33	2/6	0*	0/2*
Detergent	7	100*	2/2*	0	0/5
Dyes & stains	9	43	3/7	0*	0/2*
Fungicide / Germicide / Bactericide	8	100*	1/1*	0	0/7
Industrial Chemical, Intermediate or Formulation	18	50*	2/4*	0	0/14
Paints	4	0*	0/2*	50*	1/2*
Pesticide / Herbicide	14	50*	2/4*	0	0/10
Preservative	5	n.a.	n.a.	0	0/5
Pharmaceutical compound or intermediate	6	n.a.	n.a.	0	0/6
Raw material	9	20	1/5	0*	0/4*
Soap	8	25*	1/4*	0*	0/4*
Solvent	35	38	8/21	0	0/14
Surfactant – Total[6] -cationic -nonionic -anionic	22 7 5 2	63 100* 100* 100*	5/8 1/1* 2/2* 1/1*	0 0 0* 0*	0/14 0/6 0/3* 0/1*

* Too small dataset to make definitive conclusions; n.a.: not applicable.
[1]GHS = Globally Harmonized System (UN 2011) (4).
[2]N = Number of chemicals.
[3]Data used to calculate the percentage.
[4]Physical form (*i.e.*, solid or liquid) not known for 4 chemicals.
[5]Chemical classes included in this Table are represented by at least five chemicals tested in the ICE test method and assignments are based on the MeSH categories (www.nlm.nih.gov/mesh) as described in (2) and (15).
[6]Combines single substances labelled as surfactants along with surfactant-containing mixtures.

WITHIN- AND BETWEEN-LABORATORY REPRODUCIBILITY

24. A thorough evaluation of the ICE reproducibility was conducted in the 2003-2006 retrospective validation study (2). Based on a quantitative analysis of within-laboratory reproducibility of the ICE test method endpoints, the evaluation showed CV values for the corneal thickness measurement, when results were compared within experiments, varying from 1.8% to 6.3% (2) (3). The other endpoints evaluated produced ranges of CV values that were larger, with variability most prominent with the non-irritating substance. However, this can be explained by an exaggeration of variability given the relatively small values that were produced by chemicals not requiring classification relative to chemicals inducing eye irritation and serious eye damage (i.e., corneal swelling values of 2, 0, and 3 yield a higher CV than values of 11, 14, and 18). A similar discussion also can be applied to the variability among the qualitative endpoints (i.e., corneal opacity and fluorescein retention) given the small dynamic range of their scores (0-4 or 0-3, respectively).

25. Regarding the between-laboratory reproducibility, the retrospective studies showed median/mean % CV values to be 32%/35% for the Irritation Index, 36%/39% for fluorescein retention, 37/47% for corneal opacity, and 75%/77% for corneal swelling (2). All laboratories were in 100% agreement on the classification of 75% (44/59) of the substances according to the UN GHS classification system for both the top-down (2) and bottom-up approaches (15) according to the UN GHS classification. Finally, the EC/HO study showed the following inter-laboratory correlations between the ICE classification at TNO (lead laboratory) and the classifications obtained in three other laboratories: 0.829, 0.849 and 0.844 (11).

26. Specific issues were raised on the between-laboratory variability of the corneal swelling endpoint. This was due to the use of different slit-lamp measuring devices by the participating laboratories of the EC/HO study which, unless normalized, can contribute to the increased variability and/or the excessive values calculated for this endpoint (2). In particular, out of the four participating laboratories, two (that are no longer active in the area of toxicity testing) were reported to use different slit-lamps and different slit width settings resulting in different ranges of values for corneal swelling (see Appendix 4). In order to avoid potential variability issues linked to this endpoint, the use of a specific pachymeter and appropriate slit width, together with the use of proficiency chemicals are requested in both the adopted TG 438 (9) and the revised TG 438 (i.e., old paragraph 45, new paragraph 50: "*Corneal swelling scores are only applicable if thickness is measured with a Haag-Streit BP900 slit-lamp microscope with depth-measuring device no. I and slit-width setting at 9½, equalling 0.095 mm. Users should be aware that slit-lamp microscopes could yield different corneal thickness measurements if the slit-width setting is different.*").

Considerations on variability for the Bottom-Up approach

27. As shown in Table 7 only one chemical was identified as a false negative in the ICE test method for the identification of chemicals not requiring classification for eye irritation or serious eye damage in a Bottom-Up approach (i.e., TNO-94, a anti-fouling solvent containing paint). However, a total of eight chemicals that were correctly predicted as causing ocular effects that require a UN GHS classification, were found to be false negatives in some of the participating laboratories (Table 10).

Table 10. Chemicals showing one or more under-classification in the various participating laboratories.

N.	Chemical name	*In vivo* GHS Cat.	Physical state	Lab 22	Lab 25	Lab 24	Lab 27	Overall *in vitro* class
15	Captan 90	Cat 1	Solid	NC	2	2B	2B	2B
16	4- Carboxybenzaldehyde	Cat 2A	Solid	2B	1	NC	2	2A
36	Ethyl-2-methylacetoacetate	Cat 2B	Liquid	NC	2B	2B	NC	2B
46	Maneb	Cat 2A (EJ)	Solid	NC	2A	NC	2B	2B
50	Methyl cyanoacetate	Cat 2A	Liquid	NC	2A	NC	2B	2B
62	Quinacrine	Cat 1	Solid	2B	NC	2A	2B	2B
71	Sodium oxalate	Cat 1	Solid	2B	2B	NC	NC	2B
72	Sodium perborate	Cat 1	Solid	NC	2B	2B	2B	2B

EJ : classification based on expert judgment

Over the entire dataset, these chemicals represent 6 solids out of the 21 GHS classified solids present in the ICE validation dataset (i.e., 29%), and 2 liquids out of the 46 GHS classified liquids present in the ICE validation dataset (i.e., 4%). Due to higher probability of solids to have discordant classifications and in a

precautionary approach, the revised Test Guideline requires that "*In the case of solid materials leading to a GHS NC outcome, a second run of three eyes is recommended to confirm or discard the negative outcome*" (revised paragraph 22).

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