新規試験法提案書

改訂 OECD TG No. 437 牛摘出角膜の混濁および透過性試験法

(BCOP 法：Bovine Corneal Opacity and Permeability Test)

平成26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 26 年 1 月 20 日

No.　2013-05

改訂 OECD TG　No.437 牛摘出角膜の混濁および透過性試験法
（BCOP 法：Bovine Corneal Opacity and Permeability Test）に関する提案

平成 25 年 10 月 21 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：BCOP 法は、化学物質による直接的な眼刺激性を評価でき、その範囲は、①UN GHS 区分 1（重篤な眼の傷害を起こす）及び ②UN GHS 区分外（眼の傷害を引き起こさない）である。これら ①及び②の範囲において、行政的な利用は可能である。

この提案書は、OECD (Organisation for Economic Co-operation and Development) Test Guideline 437 およびStreamed Summary Document Supporting OECD Guideline 437 on the Bovine Corneal Opacity and Permeability for Eye Irritation/Corrosionをもとに、眼刺激性試験評価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「改訂 OECD TG　No.437 牛摘出角膜の混濁および透過性試験法」の使用を提案するものである。

吉田武美

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

吉田武美　（日本毒性学会）：座長
浅野哲秀　（日本環境変異原学会）
五十嵐良明　（国立医薬品食品衛生研究所　生活衛生化学部）
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小野寺博志　（独立行政法人　医薬品医療機器総合機構）
黒澤　努　（日本動物実験代替法学会）
杉山真理子　（日本化粧品工業連合会）
谷田智子　（独立行政法人　医薬品医療機器総合機構）*
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）
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増田光輝　（座長推薦）
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任期：平成 24 年 4 月 1 日～平成 26 年 3 月 31 日
*: 平成 25 年 4 月 1 日～平成 26 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
川西　徹　（国立医薬品食品衛生研究所）
小川久美子　（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）
菅野　純　（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　毒性部）
倉持憲路　（厚生労働省　医薬食品局　化学物質安全対策室）*
斎藤和幸　（独立行政法人　医薬品医療機器総合機構）
佐々木正広　（厚生労働省　医薬食品局　化学物質安全対策室）
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高木篤也　（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　毒性部　動物管理室）
広瀬明彦　（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　総合評価研究室）
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）
光岡俊成　（厚生労働省　医薬食品局　審査管理課）
山本順二　（厚生労働省　医薬食品局　化学物質安全対策室）*
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

*：平成 25 年 8 月 1 日より

JaCVAM statement
on Bovine Corneal Opacity and Permeability 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 21 October 2013 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:

Bovine Corneal Opacity and Permeability 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 (Organisation for Economic Co-operation and Development) Test Guideline revised No. 437 and STREAMLINED SUMMARY DOCUMENT SUPPORTING OECD GUIDELINE 437 ON THE BOVINE CORNEAL OPACITY AND PERMEABILITY FOR EYE IRRITATION/CORROSION, it is concluded that Bovine Corneal Opacity and Permeability 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.

Takemi Yoshida
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

20 January, 2014

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. Takemi Yoshida (Japanese Society of Toxicology): Chairperson
Mr. Norihide Asano (Japanese Environmental Mutagen Society)
Mr. Tsutomu Ichiki (Japan Chemical Industry Association)*
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Tsutomu Miki Kurosawa (Japanese Society for Animal Experimentation)
Mr. Eiji Maki (Japanese Society of Immunotoxicology)
Mr. Mitsuteru Masuda (nominee by Chairperson)
Mr. Akiyoshi Nishikawa (NIHS)
Mr. Yasuo Ohno (nominee by Chairperson)*
Mr. Hiroshi Onodera (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Ms. Tomoko Tanita (Pharmaceuticals and Medical Devices Agency)*
Mr. Takashi Yamada (National Institute of Technology and Evaluation)*
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)
Ms. Midori Yoshida (NIHS)
Mr. Isao Yoshimura (nominee by Chairperson)
Mr. Kazuto Watanabe (Japan Pharmaceutical Manufacturers Association)

Term: From 1st April 2012 to 31st March 2014
*: From 1st April 2013 to 31st March 2014

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. 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. Toru Kawanishi (NIHS)
Mr. Kenji Kuramochi (Ministry of Health, Labour and Welfare)*
Mr. Toshinari Mitsuoka (Ministry of Health, Labour and Welfare)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Mr. Kazuyuki Saito (Pharmaceutical & Medical Devices Agency)
Mr. Masahiro Sasaki (Ministry of Health, Labour and Welfare)
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. Junji Yamamoto (Ministry of Health, Labour and Welfare)*
Mr. Hajime Kojima (Section for the Evaluation of Novel Methods, Division of Pharmacology, BSRC, NIHS): Secretary

* Arrival at post day: 1st August 2013

改訂 OECD TG No.437 牛摘出角膜の混濁および透過性試験法
(BCOP 法：Bovine Corneal Opacity and Permeability Test)

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改訂 OECD TG　No.437 牛摘出角膜の混濁および透過性試験法（BCOP 法：Bovine Corneal Opacity and Permeability Test）の評価会議報告書

JaCVAM 評価会議

平成 25 年 10 月 21 日

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JaCVAM 評価会議

吉田武美　（日本毒性学会）：座長
浅野哲秀　（日本環境変異原学会）
五十嵐良明（国立医薬品食品衛生研究所　生活衛生化学部）
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大野泰雄　（座長推薦）*
小野寺博志（独立行政法人　医薬品医療機器総合機構）
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吉村　功　（座長推薦）
渡部一人　（日本製薬工業協会）

任期：平成 24 年 4 月 1 日～平成 26 年 3 月 31 日
*: 平成 25 年 4 月 1 日～平成 26 年 3 月 31 日

以上

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牛摘出角膜の混濁および透過性試験法（BCOP 法：Bovine Corneal Opacity and Permeability Test）は、ドレイズ眼刺激性試験の代替法として、トップダウンアプローチにおける重篤な眼の傷害を起こす化学物質（UN GHS 区分 1、US EPA 区分 1、EU DSD R43）を同定するための試験法として 2009 年に OECD（Organization for Economic Co-operation and Development）にテストガイドライン（TG）No.437 として採択された。さらに、2013 年には、BCOP 法がボトムアップアプローチでの眼の傷害を引き起こさない化学物質（GHS 等の区分外物質）の同定法として正式に採択された。JaCVAM 眼刺激性評価委員会は、今回の改訂の妥当性について検討した。

１．試験法の定義

名称：　改訂牛摘出角膜の混濁および透過性試験法（BCOP 法：Bovine Corneal Opacity and Permeability Test）

代替する対象毒性試験：ドレイズ眼刺激性試験

試験法の概略：BCOP 法は、牛眼球から摘出した角膜に被験物質を一定時間曝露させ、角膜の変性の指標となる角膜混濁度と角膜上皮のバリア機能の指標となる角膜透過度を測定し、それら測定値から計算式によって求められる IVIS（*in vitro* 刺激性スコア）を基にウサギを用いた Draize 法での眼刺激性を予測する代替法である。

２．評価に用いた資料および評価内容の科学的妥当性

NICEATM（NTP Interagency Center for the Evaluation of Alternative Toxicological Methods ）/ICCVAM（Interagency Coordinating Committee on the Validation of Alternative Methods）による回顧的なバリデーションを経て、2009 年に NICEATM/ICCVAM の第三者評価委員会で評価されており、その評価内容に科学的妥当性がある。バリデーションでは、評価物質を 213 物質（単一物質／混合物質 : 113 物質／100 物質）に増やしており十分な数が評価されている。

３．本試験法の有用性と適用限界

2009 年の TG では、施設間・施設内の再現性は高く、トップダウンアプローチにおける眼の傷害を起こす化学物質を同定できる試験法であるとされた。アルコール・ケトン類および固体物質の適用については前回のトップダウンアプローチの評価では本試験法の限界として挙げられていたが、今回の TG 改訂では、アルコール・ケトン類で陽性結果が得られた場合は、高い偽陽性率を考慮し、その結果は慎重に検討する必要があるとの注釈が付された上で、適用限界はなくなった。固

体物質の偽陰性も他の試験法で補完できることから、トップダウンアプローチでは考慮する必要がないとされた。

今回の TG 改訂で、ボトムアップアプローチでの眼の傷害を引き起こさない化学物質の同定を行うための試験法であるとされている。その結果から、高い偽陽性率を示したが、追加試験による分類判断が必要となることから、必ずしも問題はならないと判断された。トップダウンアプローチの場合と異なり、ボトムアップアプローチでも、適用限界はないとされた。

４．当該試験法は、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、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：

元来 BCOP 法は、食用などの用途で屠殺されたウシの角膜を用いた動物実験代替法であるため、ドレイズ眼刺激性試験法よりも倫理的に優れており社会的受け入れ性は高い。今回の改訂において、動物福祉の観点からの変更はないことから、本改訂法の社会的受け入れ性も、原法と同様高いものと考えられる。ただし、日本では牛角膜の入手が容易ではなく、この点が普及の妨げになる可能性が示唆された。

行政上の利用性：

BCOP 法は、化学物質による直接的な眼刺激性を評価でき、評価できる眼刺激性の範囲は、①UN GHS 区分 1（重篤な眼の傷害を起こす）及び②UN GHS 区分外（眼の傷害を引き起こさない）である。これら①及び②の範囲において、行政的な利用は可能である。

参考文献

1) JaCVAM 眼刺激性評価委員会：眼刺激性代替法の第三者評価報告書、改訂 OECD TG　No.437 牛摘出角膜の混濁および透過性試験法（BCOP 法：Bovine Corneal Opacity and Permeability Test）平成 23 年 10 月 11 日
2) 牛摘出角膜を用いた眼刺激性試験代替法（BCOP法 : Bovine Corneal Opacity and Permeability Test）の評価会議報告書（平成21年12月17日、平成23年4月20日改定）
3) JaCVAM眼刺激性評価委員会：眼刺激性代替法の第三者評価報告書、牛摘出角膜の混濁および透過性試験法（BCOP法：Bovine Corneal Opacity and Permeability Test）平成21年10月14日
4) OECD Test Guideline, Revised TG437（2013）

眼刺激性代替法　改訂 OECD TG　No.437 牛摘出角膜の混濁および透過性試験法（BCOP 法：Bovine Corneal Opacity and Permeability Test）の評価報告書

平成 25 年 7 月 19 日

眼刺激性代替法評価委員会

委員名：

竹内　小苗　（P&G イノベーション合同会社）
小坂　忠司　（残留農薬研究所）
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）
簾内　桃子　（国立医薬品食品衛生研究所）
細井　一弘　（参天製薬株式会社）
増田　光輝
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 Test
BRD:	Background Review Document
DSD:	Dangerous Substances Directive
EPA:	Environmental Protection Agency
EU:	European Union
GHS:	Globally Harmonized Systems of Classification and Labeling
ICCVAM:	Interagency Coordinating Committee on the Validation of Alternative Methods
IVIS:	In Vitro Irritancy Score
NICEATM:	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD:	Organization for Economic Co-operation and Development
UN:	United Nations
US:	United States

１．改訂の背景

牛摘出角膜の混濁および透過性試験法（Bovine Corneal Opacity and Permeability Test; 以下、BCOP 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 年の NICEATM（NTP Interagency Center for the Evaluation of Alternative Toxicological Methods）/ICCVAM （Interagency Coordinating Committee on the Validation of Alternative Methods）による回顧的なバリデーションを経て、トップダウンアプローチにおける重篤な眼の傷害を起こす化学物質［UN（United Nations） GHS（Globally Harmonized Systems of Classification and Labeling）区分 1、US（United States） EPA（Environmental Protection Agency）区分 1、EU（European Union） DSD（Dangerous Substances Directive） R43］を同定するための試験法として 2009 年に OECD（Organization for Economic Co-operation and Development）テストガイドライン（TG）No.437 として採択された。

さらに、2006 年から 2009 年にかけ、ボトムアップアプローチにおける重篤な眼の傷害を起こす物質および眼刺激性物質とは分類されない物質（以下、区分外物質）の同定法としての適用性について、回顧的なバリデーションが行われた。その結果、2009 年、NICEATM/ICCVAM の第三者評価委員会で、UN GHS および EU DSD の分類における区分外物質を同定する方法として有用であるという結論を得た。この結果を受け、OECD では BCOP 法を重篤な眼の傷害を起こす化学物質だけでなく、区分外物質の同定方法として用いることを検討するため、TG No.437 の改訂検討プロジェクトがたちあげられた。このプロジェクトで組織された眼刺激性専門家会議において、トップダウンアプローチのための BRD（Background Review Document）に新たなデータを追加した Issue Paper に基づいて、BCOP 法のボトムアップアプローチでの性能が検討された。その結果、2013 年、BCOP 法がボトムアップアプローチでの区分外物質の同定法として正式に改定 TG No.437 に採択された。このとき、適用限界と陽性対照物質等についての改訂も同時に行われた。このようにしてできたのが改定 TG No.437 である。本評価委員会は、この改定 TG No.437 の妥当性を検討した。以下にその検討結果を報告する。

２．試験の概要

BCOP 法は、牛眼球から摘出した角膜に被験物質を一定時間曝露させ、角膜の変性の指標となる角膜混濁度と角膜上皮のバリア機能の指標となる角膜透過度を測定し、それら測定値から計算式によって求められる IVIS（In Vitro Irritancy Score ：*in vitro* 刺激性スコア）を基にウサギを用いた Draize 法での眼刺激性を予測する代替眼刺激性試験法である。

試験群あたり 3 個の角膜を使用し、被験物質が液体の場合は原液（100%、ただし界面活性剤の場合は 10%希釈液）を、固体の場合は 20%の溶液または懸濁液を、それぞれ 10 分間、または 4 時間曝露させる。その後、混濁度としてオパシトメータで角膜の光透過度を、また、透過度としてフルオレセインナトリウムの透過度（OD_{490}）を測定する。これら 2 つ

の測定値を以下の計算式に当てはめ IVIS 値を算出する。

（計算式）IVIS = mean opacity + (15 x mean OD_{490})

IVIS の値が 3 以下の場合、その被験物質は GHS 区分外物質と判断され（追加された基準)、IVIS の値が 55 を超える場合は区分 1 と判断される。IVIS の値が $3 < IVIS \leq 55$ の場合、本試験法の結果のみでは眼刺激性の予測はできないため、追加の試験を必要とする。

３．改訂点

３－１．ボトムアップアプローチでの GHS 区分外物質の同定

３－１－１． ボトムアップアプローチでの正確性

前回評価を行った ICCVAM のバリデーションデータベースに 66 の抗菌洗浄製品の結果を新たに追加したデータベースを用いた正確性評価の結果を表 1 に示す。

表 1. BCOP 法の正確性－GHS 分類法での区分外物質の同定

Chemicals	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
All	196	69	135/196	100	107/107	0	0/107	31	28/89	69	61/89
Substances	104	72	75/104	100	55/55	0	0/55	41	20/49	59	29/49
Mixtures	92	65	60/92	100	52/52	0	0/52	20	8/40	80	32/40
All without alcohols, ketones and solids	135	64	87/135	100	75/75	0	0/75	20	12/60	80	48/60
Substances without alcohols, ketones and solids	45	64	29/45	100	24/24	0	0/24	24	5/21	76	16/21
Mixtures without alcohols, ketones and solids	90	64	58/90	100	51/51	0	0/51	18	7/39	82	32/39

Abbreviations: No. = data used to calculate the percentage.
[1]UN GHS classification system (UN 2011) (4): No Category vs. Category 1/2A/2B.

区分外物質の同定において、Draize 法の結果を比較対照にすると、BCOP 法の正確度は 69%（135/196）、偽陽性率 69%（61/89）、偽陰性率 0%（0/107）となった。偽陽性率は高いが、ボトムアップアプローチでは陽性結果が得られた場合、すなわち区分外物質と判定されなかった場合は、他の方法による追加試験が必要となることから、偽陽性率が高いことは必ずしも問題にはならないと判断した。

３－１－２．ボトムアップアプローチでの施設内・施設間再現性

BCOP 法の IVIS 値の施設内再現性は十分であるという判断は前回の評価でされている。

ボトムアップアプローチにおける区分外物質を同定する場合の施設間再現性について、3 つのバリデーション試験の結果を表 2 に示す。その結果、80%（103/128）の物質において、それぞれの試験参加施設すべての判定が 100%一致した。ボトムアップアプローチでの施設間再現性は十分であると判断した。

表 2. BCOP 法の施設間再現性－GHS 分類法での区分外物質の同定 - ICCVAM(2010)より抜粋

Data Source	No. of Testing Labs	No. of Substances	Substances with 100% Agreement among Labs
Balls et al. (1995)	5	60	55 (92%)
Gautheron et al. (1994)	11 12	52	34 (65%)
Southee (1998)	3	16	14 (88%)
Total	-	128	103 (80%)

３－２．トップダウンアプローチでの GHS 区分１（重篤な眼の傷害を起こす化学物質）の同定

トップダウンアプローチにおける重篤な眼の傷害を起こす化学物質（区分 1）を同定する場合の正確性についても、新たなバリデーションデータベースでの結果を表 3 に示す。これによると、正確度、偽陽性率、偽陰性率とも、前回評価の結果が変わるほどの大きな違いはない。

なお、トップダウンアプローチの施設内・施設間再現性は十分であると前報にて判断した。

表 3. BCOP 法の正確性－GHS 分類法での区分 1 物質の同定

Chemicals	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
All	191	79	150/191	86	56/65	14	9/65	75	94/126	25	32/126
Substances	100	73	73/100	77	20/26	23	6/26	72	53/74	28	21/74
Mixtures	91	85	77/91	92	36/39	8	3/39	79	41/52	21	11/52
All without alcohols, ketones and solids	131	85	111/131	92	46/50	8	4/50	80	65/81	20	16/81
Substances without alcohols, ketones and solids	42	83	35/42	83	10/12	17	2/12	83	25/30	17	5/30
Mixtures without alcohols, ketones and solids	89	85	76/89	95	36/38	5	2/38	78	40/51	22	11/51

Abbreviations: No. = data used to calculate the percentage.
[1]UN GHS classification system (UN 2011) (4): Category 1 vs. No Category/Category 2A/2B.

３－３．適用限界

アルコール・ケトン類および固体物質の適用については前回のトップダウンアプローチの評価では本試験法の限界として挙げられていたが、以下の理由からアルコール・ケトン類および固体物質も適用することができると判断した。

- 重篤な眼の傷害を起こす化学物質を同定するのに用いる場合（トップダウンアプローチ)、バリデーションに用いられた化学物質のデータベースの中で、アルコール・ケトン類に対し高い偽陽性率、固体物質に対し高い偽陰性率を示した。ただ、これら化学物質類の中でも正確に予測されたものはある。また、偽陰性の結果を示した固体物質はすべて 3 < IVIS ≤ 55（眼刺激性予測不可）となり、結果として別の試験法での検討が必要とされる。アルコール・ケトン類で陽性結果が得られた場合は、高い偽陽性率を考慮し、その結果は慎重に検討する必要がある。結論としては、これら化学物質類を、適用領域から除外する必要はないと判断された。区分外物質を同定するのに用いる場合（ボトムアップアプローチ)、高い偽陽性率を示した。しかしながら、3 < IVIS ≤ 55（眼刺激性予測不可）となった物質は追加試験による分類判断が必要となることから、必ずしも問題はならないと判断した。トップダウンアプローチの場合と異なり、ボトムアップアプローチでは、アルコール・ケトン類、固体物質の結果が正

確性に影響を与えてはいない。

また、バリデーション試験では単一物質・混合物質がそれぞれ 113 物質、100 物質と十分な数が評価されており、どちらも適用可能であると判断されている。

３－４．　陽性対照

2009 年に本評価委員会が評価した BCOP 法は液体被験物質の陽性対照として 100%エタノールを用いていたが、OECD ガイドライン化の時点で 100%エタノールに替わり 1%水酸化ナトリウムが採用されていた。

今回の改訂 TG　No.437 では陽性対照について以下の条件が必要とされた。すなわち、同じ試験系で陽性反応を誘発することが既知であることと、反応の変動を評価するために反応が過度でないことである。これにより、1%水酸化ナトリウムが陽性対照候補物質リストから除外され、100%エタノールがこのリストに加えられた。100%エタノールは、BCOP 法での眼刺激性分類が Draize 法と一致していないが、改訂 TG　No.437 の陽性対照の必要条件には合っており、BCOP 法での背景データが多くあることから、本評価委員会は 100%エタノールを液体被験物質の陽性対照物質とすることに問題はないと判断した。

３－５．その他

試験採用基準についても改訂がなされているが、本質的な部分ではなく、具体的なデータもないことからあえて評価は実施しなかった。

４．結論

本評価委員会はその正確性、再現性の結果より、BCOP 法を GHS の眼刺激性分類においてトップダウンアプローチでの重篤な眼の傷害を起こす化学物質を同定するための方法だけでなく、ボトムアップアプローチでの眼刺激性区分外物質を同定する方法としても用いることが可能であると判断した。また、どちらのアプローチにおいても、本試験法の適用物質に制限を設ける必要はなく、液体被験物質の陽性対照として 100%エタノールを用いることができると考えた。

結論として、トップダウンアプローチにおける GHS 区分 1 物質、およびボトムアップアプローチにおける GHS 区分外物質の同定に、Draize 法の代替法として改訂 TG No.437 を用いることは可能であると判断した。

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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Bovine Corneal Opacity and Permeability 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 Bovine Corneal Opacity and Permeability (BCOP) 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 Center for the Validation of Alternative Methods (JaCVAM), in 2006 and 2010 (1)(2). In the first evaluation, the BCOP test method was evaluated for its usefulness to identify chemicals (substances and mixtures) inducing serious eye damage (1). In the second evaluation, the BCOP test method was evaluated for its usefulness to identify chemicals (substances and mixtures) not classified for eye irritation or serious eye damage (2). The BCOP validation database contained 113 substances and 100 mixtures in total (2)(3). From these evaluations and their peer review it was concluded that the test method can correctly identify chemicals (both substances and mixtures) inducing serious eye damage as well as those not requiring classification for eye irritation or serious eye damage, as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (4), and it was therefore endorsed as scientifically valid for both purposes. Serious eye damage is the 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. Test chemicals inducing serious eye damage are classified as UN GHS Category 1. Chemicals not classified for eye irritation or serious eye damage are defined as those that do not meet the requirements for classification as UN GHS Category 1 or 2 (2A or 2B), i.e. they are referred to as UN GHS No Category. This Test Guideline (adopted in 2009 and updated in 2013) includes the recommended use and limitations of the BCOP test method based on its evaluations. The main differences between the original 2009 version and the 2013 updated version concern, but are not limited to: the use of the BCOP test method to identify chemicals not requiring classification according to UN GHS (paragraphs 2 and 7); clarifications on the applicability of the BCOP test method to the testing of alcohols, ketones and solids (paragraphs 6 and 7)

and of substances and mixtures (paragraph 8); clarifications on how surfactant substances and surfactant-containing mixtures should be tested (paragraph 28); updates and clarifications regarding the positive controls (paragraphs 39 and 40); an update of the BCOP test method decision criteria (paragraph 47); an update of the study acceptance criteria (paragraph 48); an update to the test report elements (paragraph 49); an update of Annex 1 on definitions; the addition of Annex 2 for the predictive capacity of the BCOP test method under various classification systems; an update of Annex 3 on the list of proficiency chemicals; and an update of Annex 4 on the BCOP corneal holder (paragraph 1) and on the opacitometer (paragraphs 2 and 3).

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 (5). The Top-Down approach (5) is designed to be used when, based on existing information, a chemical is expected to have high irritancy potential, while the Bottom-Up approach (5) 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 BCOP test method is an *in vitro* test method that can be used under certain circumstances and with specific limitations for eye hazard classification and labeling of chemicals. While it is not considered valid as a stand-alone replacement for the *in vivo* rabbit eye test, the BCOP test method is recommended as an initial step within a testing strategy such as the Top-Down approach suggested by Scott *et al.* (5) to identify chemicals inducing serious eye damage, i.e. chemicals to be classified as UN GHS Category 1, without further testing (4). The BCOP 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 (UN GHS No Category) (4) within a testing strategy such as the Bottom-up approach (5). However, a chemical that is not predicted as causing serious eye damage or as not classified for eye irritation/serious eye damage with the BCOP test method would require additional testing (*in vitro* and/or *in vivo*) to establish a definitive classification.

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 opacity and increased permeability in an isolated bovine cornea. Toxic effects to the cornea are measured by: (i) decreased light transmission (opacity), and (ii) increased passage of sodium fluorescein dye (permeability). The opacity and permeability assessments of the cornea following exposure to a test chemical are combined to derive an *In Vitro* Irritancy Score (IVIS), which is used to classify the irritancy level of the test chemical.

4. Definitions are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. This Test Guideline is based on the ICCVAM BCOP test method protocol (6)(7), which was originally developed from information obtained from the Institute for In Vitro Sciences (IIVS) protocol and INVITTOX Protocol 124 (8). The latter represents the protocol used for the European Community-sponsored prevalidation study conducted in 1997-1998. Both of these protocols were based on the BCOP test method first reported by Gautheron et al. (9).

6. The BCOP test method can be used to identify chemicals inducing serious eye damage as defined by UN GHS, i.e. chemicals to be classified as UN GHS Category 1 (4). When used for this purpose, the BCOP test method has an overall accuracy of 79% (150/191), a false positive rate of 25% (32/126), and a false negative rate of 14% (9/65), when compared to in vivo rabbit eye test method data classified according to the UN GHS classification system (3) (see Annex 2, Table 1). When test chemicals within certain chemical (i.e., alcohols, ketones) or physical (i.e., solids) classes are excluded from the database, the BCOP test method has an overall accuracy of 85% (111/131), a false positive rate of 20% (16/81), and a false negative rate of 8% (4/50) for the UN GHS classification system (3). The potential shortcomings of the BCOP test method when used to identify chemicals inducing serious eye damage (UN GHS Category 1) are based on the high false positive rates for alcohols and ketones and the high false negative rate for solids observed in the validation database (1)(2)(3). However, since not all alcohols and ketones are over-predicted by the BCOP test method and some are correctly predicted as UN GHS Category 1, these two organic functional groups are not considered to be out of the applicability domain of the test method. It is up to the user of this Test Guideline to decide if a possible over-prediction of an alcohol or ketone can be accepted or if further testing should be performed in a weight-of-evidence approach. Regarding the false negative rates for solids, 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 (10). It should also be noted that none of the false negatives identified in the ICCVAM validation database (2)(3), in the context of identifying chemicals inducing serious eye damage (UN GHS Category 1), resulted in IVIS $\leq$ 3, which is the criterion used to identify a test chemical as a UN GHS No Category. Moreover, BCOP false negatives in this context are not critical since all test chemicals that produce an 3 < IVIS $\leq$ 55 would be subsequently tested with other adequately validated in vitro tests, or as a last option in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach. Given the fact that some solid chemicals are correctly predicted by the BCOP test method as UN GHS Category 1, this physical state is also not considered to be out of the applicability domain of the test method. Investigators could consider using this test method for all types of chemicals, whereby an IVIS > 55 should be accepted as indicative of a response inducing serious eye damage that should be classified as UN GHS Category 1 without further testing. However, as already mentioned, positive results obtained with alcohols or ketones should be interpreted cautiously due to potential over-prediction.

7. The BCOP 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). When used for this purpose, the BCOP test method has an overall accuracy of 69% (135/196), a false positive rate of 69% (61/89), and a false negative rate of 0% (0/107), when compared to *in vivo* rabbit eye test method data classified according to the UN GHS classification system (3) (see Annex 2, Table 2). The false positive rate obtained (*in vivo* UN GHS No Category chemicals producing an IVIS > 3, see paragraph 47) is considerably high, but not critical in this context since all test chemicals that produce an 3 < IVIS $\leq$ 55 would be subsequently tested with other adequately validated *in vitro* tests, or as a last option in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach. The BCOP test method shows no specific shortcomings for the testing of alcohols, ketones and solids when the purpose is to identify chemicals that do not require classification for eye irritation or serious eye

damage (UN GHS No Category) (3). Investigators could consider using this test method for all types of chemicals, whereby a negative result (IVIS $\leq$ 3) should be accepted as indicative that no classification is required (UN GHS No Category). Since the BCOP test method can only identify correctly 31% of the chemicals that do not require classification for eye irritation or serious eye damage, this test method should not be the first choice to initiate a Bottom-Up approach (5), if other validated and accepted *in vitro* methods with similar high sensitivity but higher specificity are available.

8. The BCOP validation database contained 113 substances and 100 mixtures in total (2)(3). The BCOP test method is therefore considered applicable to the testing of both substances and mixtures.

9. The BCOP test method is not recommended for the identification of test chemicals that should be classified as irritating to eyes (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 (2)(3). For this purpose, further testing with another suitable method may be required.

10. All procedures with bovine eyes and bovine corneas should follow the testing facility's applicable regulations and procedures for handling animal-derived materials, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions are recommended (11).

11. Whilst the BCOP test method does not consider conjunctival and iridal injuries, it addresses corneal effects, which are the major driver of classification *in vivo* when considering the UN GHS classification. The reversibility of corneal lesions cannot be evaluated *per se* in the BCOP 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 (12). However, further scientific knowledge is required to understand how irreversible effects not linked with initial high level injury occur. Finally, the BCOP test method does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

12. 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. As outlined in OECD Guidance Document No. 160 (13), users are encouraged to preserve corneas 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.

13. For any laboratory initially establishing this test method, the proficiency chemicals provided in Annex 3 should be used. A laboratory can use these chemicals to demonstrate their technical competence in performing the BCOP test method prior to submitting BCOP test method data for regulatory hazard classification purposes.

PRINCIPLE OF THE TEST

14. The BCOP test method is an organotypic model that provides short-term maintenance of normal physiological and biochemical function of the bovine cornea *in vitro*. In this test method, damage by the test chemical is assessed by quantitative measurements of changes in corneal opacity and permeability with an opacitometer and a visible light spectrophotometer, respectively. Both measurements are used to calculate an IVIS, which is used to assign an *in vitro* irritancy hazard classification category for prediction of the *in vivo* ocular irritation potential of a test chemical (see Decision Criteria in paragraph 47).

15. The BCOP test method uses isolated corneas from the eyes of freshly slaughtered cattle. Corneal opacity is measured quantitatively as the amount of light transmission through the cornea. Permeability is measured quantitatively as the amount of sodium fluorescein dye that passes across the full thickness of the cornea, as detected in the medium in the posterior chamber. Test chemicals are applied to the epithelial surface of the cornea by addition to the anterior chamber of the corneal holder. Annex 4 provides a description and a diagram of a corneal holder used in the BCOP test method. Corneal holders can be obtained commercially from different sources or can be constructed.

Source and Age of Bovine Eyes and Selection of Animal Species

16. Cattle sent to slaughterhouses are typically killed either for human consumption or for other commercial uses. Only healthy animals considered suitable for entry into the human food chain are used as a source of corneas for use in the BCOP test method. Because cattle have a wide range of weights, depending on breed, age, and sex, there is no recommended weight for the animal at the time of slaughter.

17. Variations in corneal dimensions can result when using eyes from animals of different ages. Corneas with a horizontal diameter >30.5 mm and central corneal thickness (CCT) values ≥ 1100 µm are generally obtained from cattle older than eight years, while those with a horizontal diameter < 28.5 mm and CCT <900 µm are generally obtained from cattle less than five years old (14). For this reason, eyes from cattle greater than 60 months old are not typically used. Eyes from cattle less than 12 months of age have not traditionally been used since the eyes are still developing and the corneal thickness and corneal diameter are considerably smaller than that reported for eyes from adult cattle. However, the use of corneas from young animals (*i.e.,* 6 to 12 months old) is permissible since there are some advantages, such as increased availability, a narrow age range, and decreased hazards related to potential worker exposure to Bovine Spongiform Encephalopathy (15). As further evaluation of the effect of corneal size or thickness on responsiveness to corrosive and irritant substances would be useful, users are encouraged to report the estimated age and/or weight of the animals providing the corneas used in a study.

Collection and Transport of Eyes to the Laboratory

18. Eyes are collected by slaughterhouse employees. To minimize mechanical and other types of damage to the eyes, the eyes should be enucleated as soon as possible after death and cooled immediately after enucleation and during transport. To prevent exposure of the eyes to potentially irritant substances,

the slaughterhouse employees should not use detergent when rinsing the head of the animal.

19. Eyes should be immersed completely in cooled Hanks' Balanced Salt Solution (HBSS) in a suitably sized container, and transported to the laboratory in such a manner as to minimize deterioration and/or bacterial contamination. Because the eyes are collected during the slaughter process, they might be exposed to blood and other biological substances, including bacteria and other microorganisms. Therefore, it is important to ensure that the risk of contamination is minimized (*e.g.,* by keeping the container containing the eyes on wet ice during collection and transportation and by adding antibiotics to the HBSS used to store the eyes during transport [*e.g.,* penicillin at 100 IU/mL and streptomycin at 100 µg/mL]).

20. The time interval between collection of the eyes and use of corneas in the BCOP test method should be minimized (typically collected and used on the same day) and should be demonstrated to not compromise the assay results. These results are based on the selection criteria for the eyes, as well as the positive and negative control responses. All eyes used in the assay should be from the same group of eyes collected on a specific day.

Selection Criteria for Eyes Used in the BCOP Test Method

21. The eyes, once they arrive at the laboratory, are carefully examined for defects including increased opacity, scratches, and neovascularization. Only corneas from eyes free of such defects are to be used.

22. The quality of each cornea is also evaluated at later steps in the assay. Corneas that have opacity greater than seven opacity units or equivalent for the opacitometer and cornea holders used after an initial one hour equilibration period are to be discarded (NOTE: the opacitometer should be calibrated with opacity standards that are used to establish the opacity units, see Annex 4).

23. Each treatment group (test chemical, concurrent negative and positive controls) consists of a minimum of three eyes. Three corneas should be used for the negative control corneas in the BCOP test method. Since all corneas are excised from the whole globe, and mounted in the corneal chambers, there is potential for artifacts from handling upon individual corneal opacity and permeability values (including negative control). Furthermore, the opacity and permeability values from the negative control corneas are used to correct the test chemical-treated and positive control-treated corneal opacity and permeability values in the IVIS calculations.

PROCEDURE

Preparation of the Eyes

24. Corneas, free of defects, are dissected with a 2 to 3 mm rim of sclera remaining to assist in subsequent handling, with care taken to avoid damage to the corneal epithelium and endothelium. Isolated corneas are mounted in specially designed corneal holders that consist of anterior and posterior compartments, which interface with the epithelial and endothelial sides of the cornea, respectively. Both

chambers are filled to excess with pre-warmed phenol red free Eagle's Minimum Essential Medium (EMEM) (posterior chamber first), ensuring that no bubbles are formed. The device is then equilibrated at 32 ± 1°C for at least one hour to allow the corneas to equilibrate with the medium and to achieve normal metabolic activity, to the extent possible (the approximate temperature of the corneal surface *in vivo* is 32°C).

25. Following the equilibration period, fresh pre-warmed phenol red free EMEM is added to both chambers and baseline opacity readings are taken for each cornea. Any corneas that show macroscopic tissue damage (*e.g.,* scratches, pigmentation, neovascularization) or an opacity greater than seven opacity units or equivalent for the opacitometer and cornea holders used are discarded. A minimum of three corneas are selected as negative (or solvent) control corneas. The remaining corneas are then distributed into treatment and positive control groups.

26. Because the heat capacity of water is higher than that of air, water provides more stable temperature conditions for incubation. Therefore, the use a water bath for maintaining the corneal holder and its contents at 32 ± 1ºC is recommended. However, air incubators might also be used, assuming precaution to maintain temperature stability (*e.g.,* by pre-warming of holders and media).

Application of the Test Chemical

27. Two different treatment protocols are used, one for liquids and surfactants (solids or liquids), and one for non-surfactant solids.

28. Liquids are tested undiluted. Semi-solids, creams, and waxes are typically tested as liquids. Neat surfactant substances are tested at a concentration of 10% w/v in a 0.9% sodium chloride solution, distilled water, or other solvent that has been demonstrated to have no adverse effects on the test system. Appropriate justification should be provided for alternative dilution concentrations. Mixtures containing surfactants may be tested undiluted or diluted to an appropriate concentration depending on the relevant exposure scenario *in vivo*. Appropriate justification should be provided for the concentration tested. Corneas are exposed to liquids and surfactants for 10 minutes. Use of other exposure times should be accompanied by adequate scientific rationale. Please see Annex 1 for a definition of surfactant and surfactant-containing mixture.

29. Non-surfactant solids are typically tested as solutions or suspensions at 20% w/v concentration in a 0.9% sodium chloride solution, distilled water, or other solvent that has been demonstrated to have no adverse effects on the test system. In certain circumstances and with proper scientific justification, solids may also be tested neat by direct application onto the corneal surface using the open chamber method (see paragraph 32). Corneas are exposed to solids for four hours, but as with liquids and surfactants, alternative exposure times may be used with appropriate scientific rationale.

30. Different treatment methods can be used, depending on the physical nature and chemical characteristics (*e.g.,* solids, liquids, viscous vs. non-viscous liquids) of the test chemical. The critical factor is ensuring that the test chemical adequately covers the epithelial surface and that it is

adequately removed during the rinsing steps. A closed-chamber method is typically used for non-viscous to slightly viscous liquid test chemicals, while an open-chamber method is typically used for semi-viscous and viscous liquid test chemicals and for neat solids.

31. In the closed-chamber method, sufficient test chemical (750 µL) to cover the epithelial side of the cornea is introduced into the anterior chamber through the dosing holes on the top surface of the chamber, and the holes are subsequently sealed with the chamber plugs during the exposure. It is important to ensure that each cornea is exposed to a test chemical for the appropriate time interval.

32. In the open-chamber method, the window-locking ring and glass window from the anterior chamber are removed prior to treatment. The control or test chemical (750 µL, or enough test chemical to completely cover the cornea) is applied directly to the epithelial surface of the cornea using a micro-pipet. If a test chemical is difficult to pipet, the test chemical can be pressure-loaded into a positive displacement pipet to aid in dosing. The pipet tip of the positive displacement pipet is inserted into the dispensing tip of the syringe so that the material can be loaded into the displacement tip under pressure. Simultaneously, the syringe plunger is depressed as the pipet piston is drawn upwards. If air bubbles appear in the pipet tip, the test article is removed (expelled) and the process repeated until the tip is filled without air bubbles. If necessary, a normal syringe (without a needle) can be used since it permits measuring an accurate volume of test chemical and an easier application to the epithelial surface of the cornea. After dosing, the glass window is replaced on the anterior chamber to recreate a closed system.

Post-Exposure Incubation

33. After the exposure period, the test chemical, the negative control, or the positive control substance is removed from the anterior chamber and the epithelium washed at least three times (or until no visual evidence of test chemical can be observed) with EMEM (containing phenol red). Phenol red- containing medium is used for rinsing since a color change in the phenol red may be monitored to determine the effectiveness of rinsing acidic or alkaline materials. The corneas are washed more than three times if the phenol red is still discolored (yellow or purple), or the test chemical is still visible. Once the medium is free of test chemical, the corneas are given a final rinse with EMEM (without phenol red). The EMEM (without phenol red) is used as a final rinse to ensure removal of the phenol red from the anterior chamber prior to the opacity measurement. The anterior chamber is then refilled with fresh EMEM without phenol red.

34. For liquids or surfactants, after rinsing, the corneas are incubated for an additional two hours at 32 ± 1ºC. Longer post-exposure time may be useful in certain circumstances and could be considered on a case-by-case basis. Corneas treated with solids are rinsed thoroughly at the end of the four-hour exposure period, but do not require further incubation.

35. At the end of the post-exposure incubation period for liquids and surfactants and at the end of the four-hour exposure period for non-surfactant solids, the opacity and permeability of each cornea are recorded. Also, each cornea is observed visually and pertinent observations recorded (e.g., tissue peeling, residual test chemical, non-uniform opacity patterns). These observations could be important as they may

be reflected by variations in the opacitometer readings.

Control Substances

36. Concurrent negative or solvent/vehicle controls and positive controls are included in each experiment.

37. When testing a liquid substance at 100%, a concurrent negative control (*e.g.,* 0.9% sodium chloride solution or distilled water) is included in the BCOP test method so that nonspecific changes in the test system can be detected and to provide a baseline for the assay endpoints. It also ensures that the assay conditions do not inappropriately result in an irritant response.

38. When testing a diluted liquid, surfactant, or solid, a concurrent solvent/vehicle control group is included in the BCOP test method so that nonspecific changes in the test system can be detected and to provide a baseline for the assay endpoints. Only a solvent/vehicle that has been demonstrated to have no adverse effects on the test system can be used.

39. A substance known to induce a positive response is included as a concurrent positive control in each experiment to verify the integrity of the test system and its correct conduct. However, to ensure that variability in the positive control response across time can be assessed, the magnitude of irritant response should not be excessive.

40. Examples of positive controls for liquid test chemicals are 100% ethanol or 100% dimethylformamide. An example of a positive control for solid test chemicals is 20% w/v imidazole in 0.9% sodium chloride solution.

41. 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

42. Opacity is determined by the amount of light transmission through the cornea. Corneal opacity is measured quantitatively with the aid of an opacitometer, resulting in opacity values measured on a continuous scale.

43. Permeability is determined by the amount of sodium fluorescein dye that penetrates all corneal cell layers (*i.e.,* the epithelium on the outer cornea surface through the endothelium on the inner cornea surface). One mL sodium fluorescein solution (4 or 5 mg/mL when testing liquids and surfactants or non- surfactant solids, respectively) is added to the anterior chamber of the corneal holder, which interfaces with the epithelial side of the cornea, while the posterior chamber, which interfaces with the endothelial side of the cornea, is filled with fresh EMEM. The holder is then incubated in a horizontal position for 90 ± 5 min at 32 ± 1 ºC. The amount of sodium fluorescein that crosses into the posterior chamber is

quantitatively measured with the aid of UV/VIS spectrophotometry. Spectrophotometric measurements evaluated at 490 nm are recorded as optical density (OD_{490}) or absorbance values, which are measured on a continuous scale. The fluorescein permeability values are determined using OD_{490} values based upon a visible light spectrophotometer using a standard 1 cm path length.

44. Alternatively, a 96-well microtiter plate reader may be used provided that; (i) the linear range of the plate reader for determining fluorescein OD_{490} values can be established; and (ii), the correct volume of fluorescein samples are used in the 96-well plate to result in OD_{490} values equivalent to the standard 1 cm path length (this could require a completely full well [usually 360µL]).

DATA AND REPORTING

Data Evaluation

45. Once the opacity and mean permeability (OD_{490}) values have been corrected for background opacity and the negative control permeability OD_{490} values, the mean opacity and permeability OD_{490} values for each treatment group should be combined in an empirically-derived formula to calculate an *in vitro* irritancy score (IVIS) for each treatment group as follows:

$$IVIS = \text{mean opacity value} + (15 \times \text{mean permeability } OD_{490} \text{ value})$$

46. Sina *et al*. (16) reported that this formula was derived during in-house and inter-laboratory studies. The data generated for a series of 36 compounds in a multi-laboratory study were subjected to a multivariate analysis to determine the equation of best fit between *in vivo* and *in vitro* data. Scientists at two separate companies performed this analysis and derived nearly identical equations.

47. The opacity and permeability values should also be evaluated independently to determine whether a test chemical induced corrosivity or severe irritation through only one of the two endpoints (see Decision Criteria).

Decision Criteria

48. The IVIS cut-off values for identifying test chemicals as inducing serious eye damage (UN GHS Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) are given hereafter:

IVIS	UN GHS
≤ 3	No Category
> 3; ≤ 55	No prediction can be made
> 55	Category 1

Study Acceptance Criteria

49. A test is considered acceptable if the positive control gives an IVIS that falls within two standard deviations of the current historical mean, which is to be updated at least every three months, or each time an acceptable test is conducted in laboratories where tests are conducted infrequently (*i.e.,* less than once a month). The negative or solvent/vehicle control responses should result in opacity and permeability values that are less than the established upper limits for background opacity and permeability values for bovine corneas treated with the respective negative or solvent/vehicle control. A single testing run composed of at least three corneas should be sufficient for a test chemical when the resulting classification is unequivocal. However, in cases of borderline results in the first testing run, a second testing run should be considered (but not necessarily required), as well as a third one in case of discordant mean IVIS results between the first two testing runs. In this context, a result in the first testing run is considered borderline if the predictions from the 3 corneas were non-concordant, such that:

- 2 of the 3 corneas gave discordant predictions from the mean of all 3 corneas, OR,
- 1 of the 3 corneas gave a discordant prediction from the mean of all 3 corneas, AND the discordant result was >10 IVIS units from the cut-off threshold of 55.
- If the repeat testing run corroborates the prediction of the initial testing run (based upon the mean IVIS value), then a final decision can be taken without further testing. If the repeat testing run results in a non-concordant prediction from the initial testing run (based upon the mean IVIS value), then a third and final testing run should be conducted to resolve equivocal predictions, and to classify the test chemical. It may be permissible to waive further testing for classification and labeling in the event any testing run results in a UN GHS Category 1 prediction.

Test Report

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

Test 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/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/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.

Test Method Conditions

- Opacitometer used (*e.g.*, model and specifications) and instrument settings;
- Calibration information for devices used for measuring opacity and permeability (*e.g.*, opacitometer and spectrophotometer) to ensure linearity of measurements;
- Type of corneal holders used (*e.g.*, model and specifications);
- Description of other equipment used;
- 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).

Criteria for an Acceptable Test

- Acceptable concurrent positive and negative control ranges based on historical data;
- If applicable, acceptable concurrent benchmark control ranges based on historical data.

Eyes Collection and Preparation

- Identification of the source of the eyes (*i.e.*, the facility from which they were collected);
- Corneal diameter as a measure of age of the source animal and suitability for the assay;
- Storage and transport conditions of eyes (*e.g.,* date and time of eye collection, time interval prior to initiating testing, transport media and temperature conditions, any antibiotics used);
- Preparation & mounting of the bovine corneas including statements regarding their quality, temperature of corneal holders, and criteria for selection of corneas 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 concentration(s), application, exposure time and post-exposure incubation time used;
- Description of evaluation and decision criteria used;
- Description of study acceptance criteria used;
- Description of any modifications of the test procedure;
- Description of decision criteria used.

Results

- Tabulation of data from individual test samples (*e.g.*, opacity and OD490 values and calculated IVIS for the test chemical and the positive, negative, and benchmark controls [if included], reported in tabular form, including data from replicate repeat experiments as appropriate, and means ± the standard deviation for each experiment);
- Description of other effects observed;
- The derived in vitro UN GHS classification, if applicable.

Discussion of the Results

Conclusion

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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. Opacity can be evaluated subjectively as done in the Draize rabbit eye test, or objectively with an instrument such as an "opacitometer."

Corneal permeability: Quantitative measurement of damage to the corneal epithelium by a determination of the amount of sodium fluorescein dye that passes through all corneal cell layers.

Eye irritation: Production of changes in the eye following the application of a 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.

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.

***In Vitro* Irritancy Score (IVIS):** An empirically-derived formula used in the BCOP test method whereby the mean opacity and mean permeability values for each treatment group are combined into a single *in vitro* score for each treatment group. The *IVIS* = mean opacity value + (15 x mean permeability value).

Irreversible effects on the eye: See "Serious eye damage".

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: Chemicals that are not classified for Eye irritation (UN GHS Category 2, 2A, or 2B) or Serious eye damage (UN GHS Category 1). Interchangeable with "UN GHS No Category".

Opacitometer: An instrument used to measure "corneal opacity" by quantitatively evaluating light transmission through the cornea. The typical instrument has two compartments, each with its own light source and photocell. One compartment is used for the treated cornea, while the other is used to calibrate and zero the instrument. Light from a halogen lamp is sent through a control compartment (empty chamber without windows or liquid) to a photocell and compared to the light sent through the experimental compartment, which houses the chamber containing the cornea, to a photocell. The difference in light transmission from the photocells is compared and a numeric opacity value is presented on a digital display.

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 positive response should not be excessive.

Reversible effects on the eye: See "Eye irritation".

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.

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).

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 samples 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.

Surfactant-containing mixture: In the context of this Test Guideline, it is a mixture containing one or more surfactants at a final concentration of > 5%.

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 eye damage”.

UN GHS Category 2: See “Eye irritation”.

UN GHS No Category: Chemicals 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 test chemical.

ANNEX 2

PREDICTIVE CAPACITY OF THE BCOP TEST METHOD

Table 1: Predictive Capacity of BCOP for identifying chemicals inducing serious eye damage [UN GHS/ EU CLP Cat 1 vs Not Cat 1 (Cat 2 + No Cat); US EPA Cat I vs Not Cat I (Cat II + Cat III + Cat IV)]

Classification System	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
UN GHS EU CLP	191	78.53	150/191	86.15	56/65	13.85	9/65	74.60	94/126	25.40	32/126
US EPA	190	78.95	150/190	85.71	54/63	14.29	9/63	75.59	96/127	24.41	31/127

Table 2: Predictive Capacity of BCOP for identifying chemicals not requiring classification for eye irritation or serious eye damage ("non-irritants") [UN GHS/ EU CLP No Cat vs Not No Cat (Cat 1 + Cat 2); US EPA Cat IV vs Not Cat IV (Cat I + Cat II + Cat III)]

Classification System	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
UN GHS EU CLP	196	68.88	135/196	100	107/107	0	0/107	31.46	28/89	68.54	61/89
US EPA	190	82.11	156/190	93.15	136/146	6.85	10/146	45.45	20/44	54.55	24/44

ANNEX 3

PROFICIENCY CHEMICALS FOR THE BCOP 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 in the *in vivo* rabbit eye test (TG 405) (17) and the UN GHS classification system (*i.e.,* Categories 1, 2A, 2B, or Not Classified) (4). Other selection criteria were that substances are commercially available, that there are high quality *in vivo* reference data available, and that there are high quality *in vitro* data available from the BCOP test method. Reference data are available in the Streamlined Summary Document (3) and in the ICCVAM Background Review Document for the BCOP test method (2)(18).

Table 1: Recommended substances for demonstrating technical proficiency with the BCOP Test Method

Chemical	CASRN	Chemical Class[1]	Physical Form	*In Vivo* Classification[2]	BCOP Classification
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-Dichlorobenzoyl chloride	4659-45-4	Acyl halide	Liquid	Category 2A	No accurate/reliable prediction
Ethyl-2-methylacetoacetate	609-14-3	Ketone, Ester	Liquid	Category 2B	No accurate/reliable prediction
Ammonium nitrate	6484-52-2	Inorganic salt	Solid	Category 2[3]	No accurate/reliable
EDTA, di-potassium salt	25102-12-9	Amine, Carboxylic acid (salt)	Solid	Not Classified	Not Classified
Tween 20	9005-64-5	Ester, Polyether	Liquid	Not Classified	Not Classified
2-Mercaptopyrimidine	1450-85-7	Acyl halide	Solid	Not Classified	Not Classified
Phenylbutazone	50-33-9	Heterocyclic	Solid	Not Classified	Not Classified
Polyoxyethylene 23 lauryl ether (BRIJ-35) (10%)	9002-92-0	Alcohol	Liquid	Not Classified	Not Classified

Abbreviations: CASRN = Chemical Abstracts Service Registry Number

[1]Chemical classes were assigned to each test chemical 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).

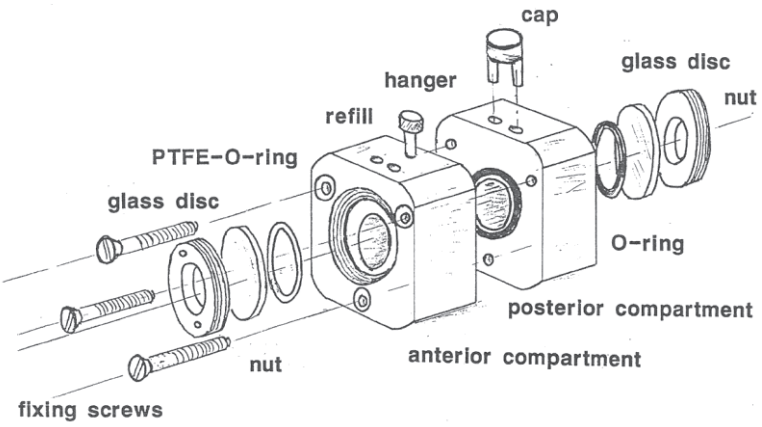
[2]Based on results from the *in vivo* rabbit eye test (OECD TG 405) (17) and using the UN GHS (4).

[3]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 4

THE BCOP CORNEAL HOLDER

1. The BCOP corneal holders are made of an inert material (*e.g.,* polypropylene). The holders are comprised of two halves (an anterior and posterior chamber), and have two similar cylindrical internal chambers. Each chamber is designed to hold a volume of about 5 mL and terminates in a glass window, through which opacity measurements are recorded. Each of the inner chambers is 1.7 cm in diameter and 2.2 cm in depth[1]. An o-ring located on the posterior chamber is used to prevent leaks. The corneas are placed endothelial side down on the o-ring of the posterior chambers and the anterior chambers are placed on the epithelial side of the corneas. The chambers are maintained in place by three stainless steel screws located on the outer edges of the chamber. The end of each chamber houses a glass window, which can be removed for easy access to the cornea. An o-ring is also located between the glass window and the chamber to prevent leaks. Two holes on the top of each chamber permit introduction and removal of medium and test compounds. They are closed with rubber caps during the treatment and incubation periods. The light transmission through corneal holders can potentially change as the effects of wear and tear or accumulation of specific chemical residues on the internal chamber bores or on the glass windows may affect light scatter or reflectance. The consequence could be increases or decreases in baseline light transmission (and conversely the baseline opacity readings) through the corneal holders, and may be evident as notable changes in the expected baseline initial corneal opacity measurements in individual chambers (i.e., the initial corneal opacity values in specific individual corneal holders may routinely differ by more than 2 or 3 opacity units from the expected baseline values). Each laboratory should consider establishing a program for evaluating for changes in the light transmission through the corneal holders, depending upon the nature of the chemistries tested and the frequency of use of the chambers. To establish baseline values, corneal holders may be checked before routine use by measuring the baseline opacity values (or light transmission) of chambers filled with complete medium, without corneas. The corneal holders are then periodically checked for changes in light transmission during periods of use. Each laboratory can establish the frequency for checking the corneal holders, based upon the chemistries tested, the frequency of use, and observations of changes in the baseline corneal opacity values. If notable changes in the light transmission through the corneal holders are observed, appropriate cleaning and/or polishing procedures of the interior surface of the cornea holders or replacement have to be considered.



[1]The dimensions provided are based on a corneal holder that is used for cows ranging in age from 12 to 60 months old. In the event that animals 6 to 12 months are being used, the holder would instead need to be designed such that each chamber holds a volume of 4 mL, and each of the inner chambers is 1.5 cm in diameter and 2.2 cm in depth. With any newly designed corneal holder, it is very important that the ratio of exposed corneal surface area to posterior chamber volume should be the same as the ratio in the traditional corneal holder. This is necessary to assure that permeability values are correctly determined for the calculation of the IVIS by the proposed formula.

ANNEX 5

THE OPACITOMETER

2. The opacitometer is a light transmission measuring device. For example, for the OP-KIT equipment from Electro Design (Riom, France) used in the validation of the BCOP test method, light from a halogen lamp is sent through a control compartment (empty chamber without windows or liquid) to a photocell and compared to the light sent through the experimental compartment, which houses the chamber containing the cornea, to a photocell. The difference in light transmission from the photocells is compared and a numeric opacity value is presented on a digital display. The opacity units are established. Other types of opacitometers with a different setup (e.g., not requiring the parallel measurements of the control and experimental compartments) may be used if proven to give similar results to the validated equipment.

3. The opacitometer should provide a linear response through a range of opacity readings covering the cut-offs used for the different classifications described by the Prediction Model (*i.e.,* up to the cut-off determining corrosiveness/severe irritancy). To ensure linear and accurate readings up to 75-80 opacity units, it is necessary to calibrate the opacitometer using a series of calibrators. Calibrators are placed into the calibration chamber (a corneal chamber designed to hold the calibrators) and read on the opacitometer. The calibration chamber is designed to hold the calibrators at approximately the same distance between the light and photocell that the corneas would be placed during the opacity measurements. Reference values and initial set point depend on the type of equipment used. Linearity of opacity measurements should be ensured by appropriate (instrument specific) procedures. For example, for the OP-KIT equipment from Electro Design (Riom, France), the opacitometer is first calibrated to 0 opacity units using the calibration chamber without a calibrator. Three different calibrators are then placed into the calibration chamber one by one and the opacities are measured. Calibrators 1, 2 and 3 should result in opacity readings equal to their set values of 75, 150, and 225 opacity units, respectively, ±5%.

Organisation de Coopération et de Développement Économiques
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THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

STREAMLINED SUMMARY DOCUMENT SUPPORTING OECD GUIDELINE 437 ON THE BOVINE CORNEAL OPACITY AND PERMEABILITY FOR EYE IRRITATION/CORROSION

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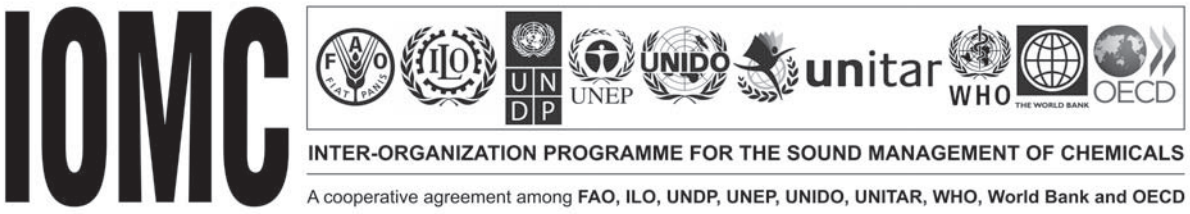

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ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2013

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FOREWORD

This streamlined summary document (SSD) was developed to provide summary information in support of OECD Test Guideline 437 on the Bovine Corneal Opacity and Permeability 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 437 (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- and between-laboratory reproducibility of the method.

The SSD was approved by the WNT with a few changes to paragraph 12, including additional references 23, 24, 25 and 26, 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.

Description of applicability domain and performance based on the retrospective validation studies of the Bovine Corneal Opacity and Permeability (BCOP) Test Method (Test Guideline 437) 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 Bovine Corneal Opacity and Permeability (BCOP) 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 161 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 BCOP 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 in the EU at that time). On the basis of all the collected data and information the BCOP 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 (Test Guideline 405) (8), which is not (or not expected to be) fully reversible within 21 days of application. Following these recommendations, the OECD officially adopted the BCOP as OECD Test Guideline (TG) 437 for identifying chemicals inducing serious eye damage in September 2009 (9).

The 2006-2009 Validation Studies

3. Following the 2003-2006 retrospective validation study NICEATM/ICCVAM, in collaboration with ECVAM and JaCVAM, further evaluated between 2006 and 2009 the usefulness and limitations of the BCOP test method for the additional identification of chemicals (substances and mixtures) 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 in the EU at that time), *i.e.*, its usefulness and limitations for initiating a Bottom-Up approach (1).

4. The updated BCOP validation database comprises 238 studies on 213 chemicals (107 substances and 106 mixtures) (UN GHS / EU CLP: 65 Cat 1, 32 Cat 2A, 3 Cat 2B, 1 Cat 2, 6 Cat 1/Cat 2, 1 No Cat/Cat 2A, 89 No Cat, 5 SCNM, 11 without *in vivo* data; US EPA: 63 Cat I, 23 Cat II, 1 Cat II/Cat III, 59 Cat III, 44 Cat IV, 13 SCNM, 11 without *in vivo* data), collected from seven individual studies (Gautheron *et al.* 1994 (10), Balls *et al.* 1995 (11), Swanson *et al.* 1995 (12), Southee 1998 (13), Swanson and Harbell 2000 (14), Bailey et al. 2004 (15), Antimicrobial Cleaning Products (AMCP) BRD (16)), which were used to determine the predictive capacity of the BCOP test method (for a comprehensive list see Annex 1). Another 54 mixtures and 13 surfactant substances reported in Casterton *et al.* 1996 (17), Gettings *et al.* 1996 (18), Swanson and Harbell 2000 (14) and in

the AMCP BRD (16) were considered by NICEATM-ICCVAM in the retrospective validation of BCOP, but were not used to calculate the predictive capacity of the test method due to incomplete *in vitro* and/or *in vivo* data (2) (3) (19). The components of each of the mixtures tested in the BCOP test method can be found in Annexes 2-5 (copied from reference 19).

5. In May 2009, NICEATM/ICCVAM convened a public meeting of an independent international scientific peer review panel (PRP) on alternative ocular safety testing methods, composed of members from several EU Member States, USA, Japan and Canada. The PRP was charged with reviewing the data compiled in view of evaluating the validation status of the BCOP test method for identifying chemicals ***not classified for eye irritation or serious eye damage*** (UN GHS No Category; EU DSD Not Classified; US EPA Category IV). The PRP concluded that the usefulness of the BCOP for the identification of chemicals not classified for eye irritation or serious eye damage depended on the intended purpose (*i.e.*, the classification system) and that the BCOP can be used for the identification of chemicals not classified for eye irritation or serious eye damage (*i.e.*, to initiate a Bottom-Up approach (1)) under the UN GHS and EU DSD classification systems for which no false negatives were identified. Since the BCOP showed 6% false negatives for the US EPA classification system, the PRP did not recommend the BCOP for this classification system (20). The final ICCVAM conclusions and recommendations on the use BCOP to identify chemicals not requiring classification for eye irritation or serious eye damage were published in a Test Method Evaluation Report (TMER), to which, amongst other documents, a revised BRD and the PRP Report were annexed (19).

BCOP Issue Paper Supporting the Adoption of TG 437 for the Identification of Chemicals Not Classified for Eye Irritation or Serious Eye Damage

6. In April 2011, following the conclusions of the 2006-2009 retrospective validation study, an OECD project for updating TG 437 (9) was included in the work plan of the OECD Test Guidelines Programme. The aim of the project was to address a possible update of TG 437 to allow its use also for the identification of chemicals not requiring classification for eye irritation or serious eye damage under UN GHS, EU CLP and EU DSD classification systems. An Issue Paper was prepared by a consultant to the OECD Secretariat with the aim to review existing BCOP data and make a recommendation on the use of TG 437 for the purpose described above (Annex 6). This document was tabled for discussion by the eye irritation/corrosion expert group at an OECD expert meeting that was held on 6-7 December 2012. The BCOP Issue Paper reviewed the BCOP data presented in the ICCVAM BCOP BRD (19) as well as other newly published data in connection with the use of BCOP to identify chemicals inducing serious eye damage as well as chemicals not requiring classification for eye irritation or serious eye damage. The BCOP Issue Paper further attempted to analyse the added value of BCOP to identify chemicals inducing serious eye damage as well as chemicals not requiring classification for eye irritation or serious eye damage, when used together with other test methods in a testing strategy. All information regarding the predictive capacity of BCOP included in this SSD is based on the data reviewed in the BCOP Issue Paper and on the analysis presented in that paper.

7. In 2013, the OECD officially adopted an updated version of TG 437 allowing the use of BCOP 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 BCOP Test Method

8. The BCOP test method (TG 437) is an organotypic *ex vivo* assay that uses living bovine corneas isolated from the eyes of freshly slaughtered animals killed for human consumption. In this test method, the irritation potential of a test substance is assessed by quantitative measurements of changes in opacity and permeability of the isolated exposed corneas, using an opacitometer and a visible light spectrophotometer, respectively (9). Both permeability and opacity are used to calculate an *in vitro* irritancy score (IVIS), which is used to assign an *in vitro* irritancy hazard classification for prediction of the *in vivo* ocular irritation potential of a test substance.

9. The prediction model used by NICEATM/ICCVAM in their evaluations of BCOP predictive capacity was based on the prediction model originally proposed by Gautheron *et al*. (1994) (10) and later modified by Balls *et al*. (1995) (11) and Southee (1998) (13). This prediction model allows for the categorisation of chemicals to four regulatory irritant categories, with cut-offs at IVIS values of 3, 25 and 55. In OECD TG 437 updated in 2013, an IIVS $\leq$ 3 is used to identify a chemical as not requiring classification for eye irritation or serious eye damage (UN GHS No Category), an IVIS > 55 is used to identify a chemical as inducing serious eye damage (UN GHS Category 1), and no prediction can be made if an 3 < IVIS $\leq$ 55 is obtained. Finally, it has also been recommended that chemicals producing permeability values equal or greater than 0.6 be identified as inducing serious eye damage (UN GHS Category 1). This may be especially important for chemicals that do not produce appreciable opacity in the isolated bovine cornea, but that can damage the epithelium and increase permeability, *e.g.*, surfactant-based personal care formulations (2) (3).

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

10. In the BCOP test method, damage to the isolated cornea is assessed by measuring corneal opacity and permeability in a short-term test that typically takes less than 8 hours to perform. The two endpoints are measured quantitatively with an opacitometer and an ultraviolet/visible (UV/VIS) spectrophotometer, respectively, after ten minutes exposure plus two hours post-exposure incubation for liquid and surfactant chemicals or after four hours of exposure to solid chemicals (2).

11. Depending on the physicochemical properties of the test substance, post-exposure measurements may be extended to 24 hours (*e.g.,* for chemicals with delayed responses). In contrast, the *in vivo* rabbit eye test involves a qualitative visual evaluation 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 BCOP, liquids, semi-solids, creams and waxes are usually applied undiluted for 10 minutes, then rinsed off the cornea, followed by a 2-hour post-exposure incubation of the cornea in assay medium. Neat surfactant substances are typically tested at a concentration of 10% w/v, while mixtures containing surfactants may be tested undiluted or diluted to an appropriate concentration depending on the relevant exposure scenario *in vivo*. The same exposure and post-exposure regime used for liquids is followed for surfactant substances and mixtures containing surfactants. Solids are usually applied as a suspension or solution (20%) for four hours, then rinsed off the cornea before opacity and permeability measurements are performed. Post-exposure incubation is not required for solids. Whether the test substance is a liquid or a solid, the entire cornea is exposed for a specified duration. In the *in vivo* rabbit eye test, liquid and solid test substances are applied to the conjunctival sac, usually in an undiluted form. Because the rabbit eye can 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 BCOP. 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. Protective mechanisms for the eye (*e.g.,* blinking, tear film) are built into *in vivo* testing, but are absent in *in vitro* / *ex vivo* testing. However, note that for some test substances (*e.g.,* solids), blinking can also induce mechanical damage *in vivo*, contributing to a higher degree of irritation. Thus, the BCOP test method differs from the *in vivo* rabbit eye test method in the following significant ways (2):

- The BCOP 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 in the BCOP assay, while they are assessed with qualitative observations in the *in vivo* rabbit eye test.

- Corneal exposure conditions, including test substance concentration and exposure duration, are well controlled in the BCOP assay, but subject to potentially greater variation *in vivo*,

due in part to the blink response and natural tearing of the eye in a live animal. Moreover, 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 (21).

- The observation period of the BCOP assay is typically less than 24 hours, whereas ocular effects are typically evaluated in the *in vivo* rabbit eye test for a minimum of 72 hours and can extend up to 21 days.

- Reversibility/irreversibility of corneal effects induced by a test substance cannot be observed in the BCOP assay *per se*, but histological evaluation of the exposed cornea 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 (22). However, 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, are built into *in vivo* testing, but are absent in *in vitro* / *ex vivo* testing.

- The BCOP 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

12. The potential shortcomings of the BCOP test method when used to identify chemicals inducing serious eye damage (UN GHS Category 1) are based on the high false positive rates for alcohols and ketones and the high false negative rate for solids observed in the validation database (2) (3) (19). When substances within these chemical and physical classes are excluded from the database, the accuracy of the BCOP test method is substantially improved (2) (3) (19) (see Table 7 below). However, since not all alcohols and ketones are over-predicted by the BCOP test method and some are correctly predicted as UN GHS Category 1, these two organic functional groups are not considered to be out of the applicability domain of the test method. Positive results obtained with alcohols or ketones should nevertheless be interpreted cautiously due to potential over-prediction. Moreover, given the fact that some solids are also correctly predicted by the BCOP test method as UN GHS Category 1, and that all solids in the validation database that were underpredicted by BCOP showed an $3 < \text{IVIS} \leq 55$ (*i.e.*, no prediction can be made) and would therefore need to be subsequently tested with other suitable test method(s) in a sequential testing strategy (8) (Annex 6), this physical state is also not considered to be out of the applicability domain of the BCOP 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 (23). 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 (24)(25)(26). 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.

13. When used to identify chemicals not requiring classification for eye irritation or serious eye damage under the UN GHS classification system, the BCOP test method has a considerably high false positive rate (*in vivo* UN GHS No Category chemicals producing an IVIS > 3) (19). This is however not considered critical since all test substances that produce an $3 < \text{IVIS} \leq 55$ (*i.e.*, no prediction can be made) would need to be subsequently tested with other suitable test method(s) in a sequential

testing strategy. The BCOP test method shows no specific shortcomings for the testing of alcohols, ketones and solids when the purpose is to identify chemicals that do not require classification for eye irritation or serious eye damage (UN GHS No Category) (see Table 8 below).

14. Another limitation of the test method is that, although it 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 BCOP directly addresses corneal effects, which are the major driver of classification *in vivo* when considering the UN GHS classification system (Annex 6). Also, although the reversibility of corneal lesions cannot be evaluated *per se* in the BCOP 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 (22). However, further scientific knowledge is required to understand how irreversible effects not linked with initial high level injury occur. Finally, the BCOP does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

15. The BCOP test method is not recommended for the identification of test substances that should be classified as irritating to eyes (UN GHS Category 2 or Category 2A) or test substances 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 (19). For this purpose, further testing with another suitable method may be required.

Table 1: Physicochemical properties and compatibility with the BCOP

Physicochemical property	Is a material with this property compatible with the BCOP assay?
Fixative	Unknown
Solvent	Yes
Extreme pH	Yes
Gases	No
Liquids	Yes
Solid materials	Yes
Emulsions	Yes
Granular materials	No
Suspensions	Yes
Coloured materials	Yes
Diluted concentrations of chemicals	Yes
Highly viscous materials	Yes
Volatile materials	Yes
Reactive chemistries	Yes[1]
Hydrophobic/lipophilic chemicals	Yes
Neat concentrations of chemicals	Yes

[1]Reactive chemistries may require the use of extended post-treatment incubations, up to 24 hours, prior to the opacity and permeability endpoints for expression of effects. Histopathology is highly recommended for such studies.

Applicability Domain

16. The Test Guideline can be used for testing all types of substances and mixtures, including those outside the chemical classes defined by the chemicals used in the validation study, provided there is no evidence that the method is not valid for the chemical tested.

Categories of Irritancy

17. Based on the conclusions of the 2003-2006 and 2006-2009 retrospective validation studies (3) (19), TG 437 was approved for classification of chemicals inducing serious eye damage (UN GHS Category 1) (in 2009), as well as 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, in 2013.

Potential Role in an ITS

18. The BCOP can be used as a validated *ex vivo* test method in a tiered testing approach as recommended in the supplement 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) (Annex 6). Due to its low specificity when used to identify chemicals not requiring classification for eye irritation or serious eye damage, the BCOP should not be the first choice to initiate a Bottom-Up approach (1), if other validated and accepted *in vitro* methods with similar high sensitivity but higher specificity are available.

19. Two studies have looked at the combination of BCOP with other *in vitro* test methods in testing strategies for the prediction of ocular hazards. Kolle et al. (2011) (27) combined BCOP with EpiOcular™ EIT (the latter can only be used to identify UN GHS No Category) in a two-tier Bottom-up/Top-Down test strategy and Hayashi et al. (2012) (28) combined BCOP with EpiOcular™ EIT, the Short Time Exposure (STE)[1] test method and the Hen's Egg Test - Chorioallantoic Membrane (HET-CAM)[2] in a two-stage Bottom-Up tiered approach. In the data published by Kolle et al. (2011), it can be seen that chemical 10/0229-1, a UN GHS No Category ("non-irritant") solid organophosphate, is overpredicted by EpiOcular™ EIT but correctly predicted by BCOP. By using BCOP to identify both UN GHS Category 1 and UN GHS No Category chemicals, this chemical can be correctly identified as "non-irritant" using either a Bottom-Up testing strategy (EpiOcular™ EIT first followed by BCOP) or a Top-Down testing strategy (BCOP first followed by EpiOcular™ EIT) (Annex 6) (27). Hayashi et al. tested 26 UN GHS No Category chemicals from the BCOP validation database in EpiOcular™ EIT, STE and HET-CAM. Of these, 13 were overpredicted as irritant by EpiOcular™ EIT (50% false positives). Two of the 13 EpiOcular™ EIT false positives (EDTA di-potassium salt, CAS # 25102-12-9; triethanolamine, CAS # 102-71-6) are correctly identified by BCOP as not requiring hazard classification and labelling (UN GHS No Category). HET-CAM also correctly identifies two other

[1]The STE has undergone a full validation study in Japan, coordinated by JaCVAM, and an international peer-review of the study is currently being organised by ICCVAM. The STE was also submitted by Japan to the OECD for the development of a new Test Guideline, and was included in the OECD work plan for the Test Guidelines Programme in 2011.

[2]The HET-CAM was evaluated by ICCVAM together with BCOP and ICE in the retrospective validation studies of 2003-2006 (3) and 2006-2009 (19). ICCVAM considered that the HET-CAM might be useful for identifying chemicals not requiring hazard classification and labeling for eye irritation but did not recommend the test method due to lack of data in the mild/moderate irritancy range. ICCVAM recommended that additional data be collected on mild and moderate irritants to more adequately characterise the usefulness of HET-CAM. Following this recommendation, an international workshop on HET-CAM was recently held at the BfR in Berlin to help advance regulatory acceptance of the test method.

EpiOcular™ EIT false positives as "non-irritant" (1-nitropropane, CAS # 108-03-2; 1-phenyl-3-pyrazolidinone, CAS # 92-43-3), but in this case at the expense of 1 UN GHS Category 2A chemical being underclassified as No Category (4-carboxybenzaldehyde, CAS # 619-66-9). However, if a Top-Down approach would be used to test this chemical, starting with BCOP, it would be overclassified as UN GHS Category 1 instead of underclassified as UN GHS No Category. Finally, STE correctly identified 8 of 10 EpiOcular™ EIT false positives tested as "non-irritant" (including both chemicals correctly identified by BCOP but not those identified by HET-CAM), but at the same it underpredicted 3 UN GHS Category 2A and 1 Category 2B as "non-irritants". If a Top-Down approach would be used to test these four chemicals, starting with BCOP, two of them (ethanol, CAS # 64-17-5, and isopropanol, CAS # 67-63-0) would be overclassified as UN GHS Category 1 but the other two (methyl acetate, CAS # 79-20-9; ammonium nitrate, 6484-52-2) would still be underclassified as UN GHS No Category since for these, BCOP produced an IVIS between 3 and 55 (*i.e.*, no prediction can be made) (24). Methyl acetate produced a mean IVIS of 54.9 in the EC-HO validation study, which is borderline for the cut-off of 55 used to identify UN GHS Category 1. In fact, the same chemical was predicted as UN GHS Cat 1 by BCOP at BASF with an IVIS of 58.1 (27). By using BCOP to identify both UN GHS Category 1 and UN GHS No Category chemicals in combination with EpiOcular™ EIT, the false positive rate for chemicals not requiring classification for eye irritation or serious eye damage would be reduced by 7.7%, from 13/26 (50%) to 11/26 (42.3%). If HET-CAM is further integrated in the strategy, the false positive rate would be further reduced to 34.6% (9/26). If STE would also be integrated in the test strategy despite its higher false negative rate, the false positive rate would decrease to only 11.5% (3/26) (Annex 6) (28).

Mode of Action (MoA)

20. 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.*, alkylation, oxidative attack on macromolecules such as essential proteins or nucleic acids). The BCOP 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 2: Summary of events involved in chemical-induced eye irritation *in vivo* (text in italics represents irreversible responses) with information on whether they are modelled by the BCOP test method or not.

Events involved in chemical-induced eye irritation	Modelled by the BCOP 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	Unknown
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 (corneal epithelium); No (limbus)
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.	No[1]
Recovery from response, *i.e.*, length of time for cell responses to return to control levels	No

[1]In OECD TG 437, a time course is not specified. However, a time course for exposures, as well as a time course for post-exposure expression of changes, up to 24 hours, can be designed into the assay.

Chemical Classes

21. A total of 213 individual chemicals (107 substances and 106 mixtures) were evaluated in the 2006-2009 test method evaluation (19), including representatives from a number of chemical and product classes that have been evaluated using *in vivo* rabbit eye data. Chemicals with a wide range of individual responses have been evaluated. Test data were collected from Gautheron *et al.* (1994) (10), Balls *et al.* (1995) (11), Swanson *et al.* (1995) (12), Southee (1998) (13), Swanson and Harbell (2000) (14), Bailey *et al.* (2004) (15) and the AMCP BRD (16) (Annex 1). The ICCVAM PRP (20) concluded that "*the chemical database appeared adequate, however, additional chemicals in certain chemical classes will provide a more robust statistical inference as these data become available*", however, the PRP did not specifically identify any chemicals or product classes as being of special concern. For a complete list of chemical and product classes see Annex 1. The components of each of the mixtures tested in the BCOP test method can be found in Annexes 2-5 (copied from reference 19).

22. Tables 3 and 4 show the chemical and product classes for the chemicals included in the BCOP validation database. Information, including chemical name, Chemical Abstracts Service

Registry Number (CASRN), chemical and/or product class, concentration(s) tested, purity, supplier or source, and literature reference using the chemical are provided in Annex 1.

Table 3: Chemical classes tested in the BCOP test method.

Chemical Class	# of Chemicals	Chemical Class	# of Chemicals
Acyl halide	3	Imide	2
Alcohol	22	Inorganic salt	6
Aldehyde	1	Ketone	12
Alkali	3	Lactone	3
Aluminum compound	1	Nitrile compound	1
Amide	2	Nitro compound	2
Amidine	6	Oil	1
Amine	10	Onium compound	12
Amino acid	4	Organic salt	3
Boron compound	1	Organic sulfur compound	5
Carboxylic acid	17	Organophosphate	1
Ester	12	Organosilicon compound	1
Ether/Polyether	9	Phenol	1
Formulation	69	Polycyclic compound	3
Heterocyclic compound	12	Terpene	1
Hydrocarbon	18	Wax	1

Table 4: Product classes tested in the BCOP test method.

Product Class	# of Chemicals	Product Class	# of Chemicals
Adhesive	1	Fertilizer	1
Agricultural chemical	2	Flame retardant	1
Antifreeze agent	1	Flavor ingredient	3
Antimicrobial cleaning product	66	Food additive	1
Bactericide/Fungicide/ Disinfectant/Germicide	11	Herbicide	3
Beverage	1	Insect repellant	8
Bleach	3	Lubricant/lubricant additive	6
Chelating agent	2	Paint, lacquer, varnish (component)	1
Chemical/synthetic intermediate	28	Pesticide	8
Cleaner	15	Petroleum product	16
Cleanser (personal care)	13	Photographic chemical/ developing agent	2
Coupling agent	1	Plant growth regulator	2
Cutting fluid	2	Plasticizer	4
Degreaser	1	Preservative	2
Dessicant	1	Reagent	5
Detergent	11	Shampoo (hair)	14
Drug/Pharmaceutical/ Therapeutic agent and/or metabolite	17	Soap	3

Dry cleaning preparation	1	Solvent	34
Dye, in manufacture of	3	Surfactant	39
Emulsifier	1	Anionic surfactant	3
Etching and/or electroplating	2	Cationic surfactant	6
Explosive	1	Nonionic surfactant	5
Fabric softener	1	Thermometer fluid	1

Sensitivity, Specificity and Accuracy:

23. The updated BCOP validation database contains a total of 213 chemical, most of which had sufficient *in vivo* data to be assigned an ocular irritancy classification according to the UN GHS classification system (4), sufficient for the calculation of accuracy to identify UN GHS Category 1 (Top-Down) (191 chemicals: 65 Cat 1, 32 Cat 2A, 3 Cat 2B, 1 Cat 2, 1 No Cat/Cat 2A and 89 No Cat) or UN GHS No Category (Bottom-Up) (196 chemicals: 65 Cat 1, 32 Cat 2A, 3 Cat 2B, 1 Cat 2, 6 Cat 1/Cat 2 and 89 No Cat). The difference in the number of chemicals used in the two calculations (191 vs. 196) derives from (i) the use of 1 chemical (Tetraaminopyrimidine sulphate) that has two *in vivo* studies, one indicating UN GHS No Category (ECETOC) and the other UN GHS Category 2A (Gautheron et al. 1996 (10)), for the Top-Down calculations but not for the Bottom-Up calculations; and (ii) the use of 6 chemicals with *in vivo* Study Criteria Not Met (SCNM), but for which one can be certain they are either UN GHS Category 2 or UN GHS Category 1, for the Bottom-Up calculations but not for the Top-Down calculations (see Annex 1). In order to calculate the appropriate ocular irritancy hazard classification, detailed *in vivo* data consisting of cornea, iris, and conjunctiva scores for each animal at 24, 48, and 72 hours following test substance administration and/or assessment of the presence or absence of lesions at 7, 14, and 21 days were evaluated. Some of the test substances had insufficient *in vivo* data to assign a hazard classification (16 chemicals: 5 with study criteria not met, 11 without *in vivo* data). These chemicals were therefore not used to evaluate test method accuracy.

Table 5: False positive and false negative rates of the BCOP test method, by chemical class and properties of interest, for the UN GHS[1] classification system for distinguishing chemicals (substances and mixtures) inducing serious eye damage (UN GHS Category 1) from all other categories.

Category	N[2]	False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]
Overall	191	25	32/126	14	9/65
Chemical Class[4]					
Alcohol	17	50	7/14	67	2/3
Amine/Amidine	7	0	0/4	0	0/3
Carboxylic acid	14	37	3/8	33	2/6
Ester	10	12	1/8	0	0/2
Ether/Polyether	6	40	2/5	0	0/1
Heterocycle	13	14	1/7	17	1/6
Hydrocarbon	11	9	1/11	-	0/0
Inorganic salt	7	0	0/3	0	0/4
Ketone	9	44	4/9	-	0/0
Onium compound	11	0	0/3	12	1/8
Properties of Interest					
Liquids[5]	150	26	27/102	8	4/48
Solids[5]	33	15	3/20	38	5/13
Pesticide	8	66	2/3	40	2/5
Surfactant – Total[6]	75	17	6/35	7	3/40
-nonionic	25	21	3/14	9	1/11
-anionic	22	0	0/12	10	1/10
-cationic	7	0	0/1	0	0/6
pH – Total[7]	34	21	4/19	13	2/15
- acidic (pH < 7.0)	13	27	3/11	0	0/2
- basic (pH > 7.0)	16	0	0/3	15	2/13
- equals 7	3	33	1/3	-	0/0
Category 1 Subgroup[8] - Total	52[9]	-	-	17	9/52
- 4 (CO=4 at any time)	25	-	-	16	4/25
- 3 (severity/persistence)	1	-	-	0	0/1
- 2 (severity)	3	-	-	33	1/3
- 2-4 combined[10]	29	-	-	17	5/29
- 1 (persistence)	23	-	-	17	4/23

[1]GHS = Globally Harmonized System (UN 2011) (4).
[2]N = Number of chemicals.
[3]Data used to calculate the percentage.
[4]Chemical classes included in this table are represented by at least five substances tested in the BCOP test method and assignments are based on the MeSH categories (www.nlm.nih.gov/mesh).
[5]Physical form (*i.e.*, solid or liquid) not known for some chemicals, and therefore the overall number does not equal the sum of the solid and liquid chemicals.
[6]Combines single substances labelled as surfactants along with surfactant-containing mixtures.
[7]Total number of chemicals for which pH information was obtained.
[8]NICEATM-defined subgroups assigned based on the lesions that drove classification of a UN GHS Category 1 chemical. 1: based on lesions that are persistent; 2: based on lesions that are severe (not

including Corneal Opacity [CO]=4); 3: based on lesions that are severe (not including CO=4) and persistent; 4: CO = 4 at any time.

[9]The number of chemicals evaluated in the Category 1 subgroup analysis may be less than the total number of *in vivo* Category 1 chemicals evaluated, since some chemicals could not be classified into the subgroups used in the evaluation.

[10]Subcategories 2 to 4 combined to allow for a direct comparison of UN GHS Category 1 chemicals classified *in vivo* based on some lesion severity component and those classified based on persistent lesions alone.

Table 6: False positive and false negative rates of the BCOP test method, by chemical class and properties of interest, for the UN GHS[1] classification system for distinguishing chemicals (substances and mixtures) not requiring classification for eye irritation or serious eye damage (UN GHS No Category) from all other irritant categories.

Category	N[2]	False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]
Overall	196	69	61/89	0	0/107
Chemical Class[4]					
Alcohol	17	0	3/7	0	0/10
Amine/Amidine	7	25	1/4	0	0/3
Carboxylic acid	14	83	5/6	0	0/8
Ester	10	75	3/4	0	0/6
Ether/Polyether	6	25	1/4	0	0/2
Heterocycle	13	67	4/6	0	0/7
Hydrocarbon	11	73	8/11	-	0/0
Inorganic salt	7	50	1/2	0	0/5
Ketone	9	67	4/6	0	0/3
Onium compound	11	100	2/2	0	0/9
Properties of Interest					
Liquids[5]	154	75	54/72	0	0/82
Solids[5]	34	38	6/16	0	0/18
Pesticide	8	100	2/2	0	0/6
Surfactant – Total[6]	75	80	24/30	0	0/45
-nonionic	25	69	9/13	0	0/12
-anionic	23	82	9/11	0	0/12
-cationic	7	100	1/1	0	0/6
pH – Total[7]	34	78	7/9	0	0/25
- acidic (pH < 7.0)	13	75	3/4	0	0/9
- basic (pH > 7.0)	16	100	3/3	0	0/13
- equals 7	3	-	0/0	0	0/3

[1]GHS = Globally Harmonized System (UN 2011) (4).

[2]N = Number of chemicals.
[3]Data used to calculate the percentage.
[4]Chemical classes included in this table are represented by at least five substances tested in the BCOP test method and assignments are based on the MeSH categories (www.nlm.nih.gov/mesh).
[5]Physical form (*i.e.*, solid or liquid) not known for some chemicals, and therefore the overall number does not equal the sum of the solid and liquid chemicals.
[6]Combines single substances labelled as surfactants along with surfactant-containing mixtures.
[7]Total number of chemicals for which pH information was obtained.

Overall Predictive Capacity

Table 7: Predictive capacity of the BCOP test method for distinguishing chemicals (substances and mixtures) inducing serious eye damage from all other categories, as defined by the UN GHS classification system[1] (based on Table 2 in the BCOP Issue Paper; Annex 6).

Chemicals	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
All	191	79	150/191	86	56/65	14	9/65	75	94/126	25	32/126
Substances	100	73	73/100	77	20/26	23	6/26	72	53/74	28	21/74
Mixtures	91	85	77/91	92	36/39	8	3/39	79	41/52	21	11/52
All without alcohols, ketones and solids	131	85	111/131	92	46/50	8	4/50	80	65/81	20	16/81
Substances without alcohols, ketones and solids	42	83	35/42	83	10/12	17	2/12	83	25/30	17	5/30
Mixtures without alcohols, ketones and solids	89	85	76/89	95	36/38	5	2/38	78	40/51	22	11/51

Abbreviations: No. = data used to calculate the percentage.
[1]UN GHS classification system (UN 2011) (4): Category 1 vs. No Category/Category 2A/2B.

Table 8: Predictive capacity of the BCOP test method for distinguishing chemicals (substances and mixtures) not requiring classification for eye irritation or serious eye damage from all other irritant categories, as defined by the UN GHS classification system[1] (based on Table 3 in the BCOP Issue Paper; Annex 6).

Chemicals	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
All	196	69	135/196	100	107/107	0	0/107	31	28/89	69	61/89
Substances	104	72	75/104	100	55/55	0	0/55	41	20/49	59	29/49
Mixtures	92	65	60/92	100	52/52	0	0/52	20	8/40	80	32/40
All without alcohols, ketones and solids	135	64	87/135	100	75/75	0	0/75	20	12/60	80	48/60
Substances without alcohols, ketones and solids	45	64	29/45	100	24/24	0	0/24	24	5/21	76	16/21
Mixtures without alcohols, ketones and solids	90	64	58/90	100	51/51	0	0/51	18	7/39	82	32/39

Abbreviations: No. = data used to calculate the percentage.
[1]UN GHS classification system (UN 2011) (4): No Category vs. Category 1/2A/2B.

Within- and Between-Laboratory Reproducibility:

24. A thorough evaluation of the BCOP reliability was conducted in the 2003-2006 retrospective validation study concerning the use of BCOP for identifying chemicals inducing serious eye damage (2) (3). This evaluation showed a median coefficient of variation (CV) for IVIS for replicate corneas (n=3) within individual experiments ranging from 11.8% to 14.2% in one study, and mean and median CV values for IVIS for replicate corneas (n=4) within individual experiments of 71% and 35%, respectively, in a second study. The between experiment mean CV values of IVIS for 16 chemicals tested two or more times in three laboratories ranged from 12.6% to 14.8%, while the median CV values ranged from 6.7% to 12.4%.

25. Additional analyses of between-laboratory reproducibility were also conducted in the second retrospective validation study (2006-2009) to evaluate the extent of agreement of BCOP hazard

classifications among the laboratories that participated in three different inter-laboratory validation studies (10) (11) (13) (19). These analyses showed nearly 100% agreement of classification among the different laboratories in each study, confirming the results that had been obtained in the first evaluation. The extent of agreement was 100% when distinguishing chemicals inducing serious eye damage from all other ocular hazard categories for 72% (91/127) of the chemicals according to the UN GHS classification system. When distinguishing chemicals not requiring classification for eye irritation or serious eye damage from all other ocular hazard categories, the extent of agreement was 100% for 80% (103/128) of the chemicals according to the UN GHS classification system (19).

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