新規試験法提案書

眼刺激性試験代替法
再構築ヒト角膜様上皮モデル法
(Reconstructed Human Cornea-like Epithelium Test Method: RhCE法)

平成29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 29 年 1 月 6 日
No.　2016-02

眼刺激性試験代替法　再構築ヒト角膜様上皮モデル法
(Reconstructed Human Cornea-like Epithelium Test Method: RhCE法)
に関する提案

平成 28 年 7 月 5 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： 再構築ヒト角膜様上皮モデル法（Reconstructed human Cornea-like Epithelium Test Method: RhCE 法）は、化学物質による眼刺激性を評価でき、ボトムアップ方式において UN GHS 区分外を検出する方法として、行政的利用が可能であると考える。

この提案書は、Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 492 “Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage”をもとに、眼刺激性試験資料編纂委員会により作成された「再構築ヒト角膜様上皮モデル法評価報告書」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再構築ヒト角膜様上皮モデル法の使用を提案するものである。

大野泰雄

JaCVAM 評価会議　議長

西川秋佳

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

大 野 泰 雄　（運営委員会推薦）：座長
飯 塚 尚 文　（独立行政法人　医薬品医療機器総合機構）
五十嵐良明　（国立医薬品食品衛生研究所）
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牧　 栄 二　（日本免疫毒性学会）
森 田　 健　（日本環境変異原学会）
山 田 隆 志　（独立行政法人　製品評価技術基盤機構）
横 関 博 雄　（日本皮膚アレルギー・接触皮膚炎学会）
吉 田 武 美　（日本毒性学会）
吉 村　 功　（座長推薦）

任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日

JaCVAM 評価会議

大 野 泰 雄　（運営委員会推薦）：座長
飯 塚 尚 文　（独立行政法人　医薬品医療機器総合機構）
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任期：平成 28 年 4 月 1 日～平成 30 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）

加 藤　 篤　（国立感染症研究所）

日下部哲也　（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）

平 林 容 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　毒性部）

篠 田 和 俊　（独立行政法人　医薬品医療機器総合機構）

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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）

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中 村 高 敏　（独立行政法人　医薬品医療機器総合機構）

日 田　 充　（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）

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
安全性予測評価部）

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三 澤　 馨　（厚生労働省　医薬・生活衛生局　医薬品審査管理課）

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
安全性予測評価部　第二室）：事務局

JaCVAM statement on
the Reconstructed human Cornea-like Epithelium Eye Irritation Test Method

At a meeting held on 5 July 2016 at the National Institute of Health Sciences (NIHS) in Tokyo, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Proposal: The Reconstructed human Cornea-like Epithelium (RhCE) test method is a suitable method for assessing ocular irritation potential in a regulatory context as part of a bottom-up approach for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage (No Category) under the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS).

This statement was prepared following a review of the Organisation for Economic Co-operation and Development (OECD) Test Guideline 492 “Reconstructed human Cornea-like Epithelium test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage” as well as a validation report on the RhCE test method prepared by the Ocular Irritation Testing JaCVAM Editorial Committee to acknowledge that the results of a review and study by the JaCVAM Regulatory Acceptance Board have confirmed the usefulness of this assay.
Based on the above, we propose the RhCE test method as a useful means for safety assessment by regulatory agencies.

Yasuo Ohno
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

6 January 2017

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

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

Mr. Yasuo Ohno (nominee by JaCVAM Steering Committee) : Chairperson
Mr. Naofumi Iizuka (Pharmaceuticals and Medical Devices Agency)
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Yuji Ishii (Biological Safety Research Center: BSRC, NIHS)
Ms. Yumiko Iwase (Japan Pharmaceutical Manufacturers Association)
Mr. Kazuhiro Kaneko (Japan Chemical Industry Association)
Mr. Eiji Maki (Japanese Society of Immunotoxicology)
Mr. Takeshi Morita (Japanese Environmental Mutagen Society)
Mr. Akiyoshi Nishikawa (BSRC,NIHS)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Ms. Koko Tanigawa (Japanese Society for Alternatives to Animal Experiments)
Mr. Takashi Yamada (National Institute of Technology and Evaluation)
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)
Mr. Takemi Yoshida (Japanese Society of Toxicology)
Mr. Isao Yoshimura (nominee by Chairperson)

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

Mr. Yasuo Ohno (nominee by JaCVAM Steering Committee) : Chairperson
Mr. Naofumi Iizuka (Pharmaceuticals and Medical Devices Agency)
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Noriyasu Imai (Japanese Society for Alternatives to Animal Experiments)
Mr. Tomoaki Inoue (Japanese Society of Immunotoxicology)
Mr. Yuji Ishii (BSRC, NIHS)
Ms. Yumiko Iwase (Japan Pharmaceutical Manufacturers Association)
Mr. Takeshi Morita (Japanese Environmental Mutagen Society)
Mr. Shunji Nakai (Japan Chemical Industry Association)
Ms. Ruriko Nakamura (National Institute of Technology and Evaluation)
Mr. Akiyoshi Nishikawa (BSRC, NIHS)
Mr. Satoshi Numazawa (Japanese Society of Toxicology)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)

Term: From 1st April 2016 to 31st March 2018

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

Mr. Akiyoshi Nishikawa (BSRC, NIHS): Chairperson
Mr. Toru Kawanishi (NIHS)
Mr. Mitsuru Hida (Ministry of Health, Labour and Welfare)
Ms. Yoko Hirabayashi (Division of Toxicology, BSRC, NIHS)
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)
Mr. Atsushi Kato (National Institute of Infectious Diseases)
Mr. Tetsuya Kusakabe (Ministry of Health, Labour and Welfare)
Mr. Kaoru Misawa (Ministry of Health, Labour and Welfare)
Mr. Takatoshi Nakamura (Pharmaceutical & Medical Devices Agency)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Ms. Yuko Sekino (Division of Pharmacology, BSRC, NIHS)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Mr. Atsuya Takagi (Animal Management Section of the Division of Toxicology, BSRC, NIHS)
Mr. Masaaki Tsukano (Ministry of Health, Labour and Welfare)
Mr. Hajime Kojima (Division of Risk Assessment, BSRC, NIHS): Secretary

眼刺激性試験代替法　再構築ヒト角膜様上皮モデル法

(Reconstructed Human Cornea-like Epithelium Test Method: RhCE法)

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眼刺激性試験代替法の評価会議報告書

再構築ヒト角膜様上皮モデル法
(Reconstructed Human Cornea-like Epithelium Test Method: RhCE 法)

JaCVAM 評価会議

平成 28 年（2016 年）7 月 5 日

JaCVAM 評価会議

大 野 泰 雄　（運営委員会推薦）：座長
飯 塚 尚 文　（独立行政法人　医薬品医療機器総合機構）
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任期：平成 28 年 4 月 1 日～平成 30 年 3 月 31 日

再構築ヒト角膜様上皮モデル法（Reconstructed human Cornea-like Epithelium Test Method: RhCE 法）は、ウサギを用いた Draize 眼刺激性試験法の代替法として、被験物質のヒト角膜様上皮モデル組織に対する細胞毒性を指標に用い、その物質の眼刺激性を評価する試験法である。

ボトムアップ方式において United Nations Globally Harmonized System of Classification and Labelling of Chemicals（UN GHS）区分外物質を検出する方法としてバリデーションが行われ [1)]、2015 年に OECD TG492 として採択された [2)]。JaCVAM 評価会議は、眼刺激性試験資料編纂委員会により作成された「再構築ヒト角膜様上皮モデル法評価報告書」[3)]を用いて、本試験法の妥当性について検討した。

１．試験法の定義

名称 : 再構築ヒト角膜様上皮モデル法（Reconstructed human Cornea-like Epithelium Test Method: RhCE 法）

代替する対象毒性試験：　Draize 眼刺激性試験法

試験法の概略：　RhCE 法は、再構築ヒト角膜様上皮モデル（Reconstructed human Cornea-like Epithelium:RhCE）組織を用いた眼刺激性試験である。RhCE 法の一つである EpiOcular^{TM} を用いる眼刺激性試験法（EpiOcular^{TM} EIT）では、被験物質が液体の場合は約 30 分間、固体の場合は約 6 時間、ヒト角膜様上皮構造を有する組織に曝露した後、MTT の取り込み量をもとにした細胞生存率を測定し、眼刺激性評価の指標として用いる。これは、MTT がミトコンドリアの脱水素酵素の基質となる性質を利用し、細胞内に取り込まれた MTT が脱水素酵素により還元され、生成されたホルマザン量（青色）は生存細胞数に比例することに基づいている（MTT 還元法）。

２．評価に用いた資料および評価内容の科学的妥当性

眼に異物が入った場合、眼の刺激は、神経等の特定の受容体に作用する場合を除き、一般に角膜や結膜の細胞傷害から始まる。Draize 法における眼刺激性の程度の判定は、主に角膜の初期傷害の程度に大きく影響され、それは角膜上皮細胞の細胞死の程度と相関関係にある。本試験法は、ヒトの角膜上皮様構造を有する RhCE 組織（EpiOcular^{TM}）を用いて、被験物質の細胞毒性を指標として眼刺激性を評価する試験法である。これらのことから、本試験法はウサギを用いる眼刺激性試験の代替法として科学的妥当性がある。

EpiOcular^{TM} EIT については、EURL ECVAM(European Union Reference Laboratory for Alternatives to Animal Testing)と CE(Cosmetics Europe)の共同バリデーション試験 [1)]が行われた。その試験では、正確性の評価のために必要な動物試験のデータを持っていることを前提条件に、UN GHS 区分物質・区分外物質や固体・液体等のバランスを考慮して被験物質の選択が行われた。バリデーション試験は、コード化された 107 物質について行われた。その後プロトコルの最適化を行って、新たな固体 8 物質を追加し、115 物質で評価された。

EpiOcular™ EIT は、再現性も正確性も EURL ECVAM バリデーション運営委員会の定めた基準を満たしており、さらに ESAC の第三者評価[4]を経て、UN GHS 区分外物質を検出する方法として 2015 年 7 月に OECD により試験ガイドラインに採択された（TG492）[2]。JaCVAM 眼刺激性試験資料編纂委員会は、これらの資料を用いて本試験法を評価しており、科学的に妥当であると考える。

３．本試験法の有用性と適用限界

EpiOcular™ は市販されており、米国の製造元より入手できる。RhCE 組織以外は、特殊な機材や試薬を必要とせず、特別な手技も必要ないことから技術移転性は高いと判断できる。バリデーション試験においても、参加施設の技術者に対して技術習得のためのトレーニングが行われた結果、技術移転性に問題がなかったことが確認されている。但し、EpiOcular™ EIT を実施する試験施設の技術習得を確かめるための熟達度確認物質の一覧が TG492 に定められており、それらを用いて技術移転が達成できたことを確かめる必要がある。

UN GHS 区分外物質を検出する方法としての妥当性を調べるためのバリデーション試験において施設内再現性は、UN GHS 分類判定の施設ごとの施設内再現性は EURL ECVAM-CE バリデーション運営委員会が定めた基準（85%以上）を満たしていた[1]。また、施設間再現性に関しては、本試験で得られた細胞生存率（複数回試験）の平均値をもとに UN GHS 分類判定を施設ごとに行い、その一致度を検討した（固体物質のデータを含む）。その結果、バリデーション運営委員会が定めた基準（80%以上）を満たしていた[1]。

UN GHS 区分外物質を検出する方法として試験法の正確性に関しては、物質自体の色がホルマザン定量での吸光度測定を妨げる 3 物質と、強い還元性物質 1 物質を除く 111 物質の結果を用いて評価が行われた。その結果、下記のように、液体物質のみの場合、固体物質のみの場合および液体物質・固体物質を含めた場合に分けて解析した場合のいずれにおいても、一致度、偽陰性率、偽陽性率はバリデーション運営委員会が定めた受け入れ基準[1]を満たしていた。

EpiOcular™ EIT　の正確性

	物質数	一致度(%)	偽陰性率(%)	偽陽性率(%)
液体	52	81.9	1.7	33.3
固体	59	78.0	6.5	39.3
液体・固体	111	79.7	4.3	37.0
代替法としての受け入れ基準[1]	-	≥ 75	≤ 10	≤ 40

眼刺激性代替法資料編纂委員会の報告書（表１）より引用[3]

但し、細胞生存率の算出に MTT 還元法を用いる他の代替法と同様に、被験物質が MTT を還元する物質の場合、あるいはホルマザンと同じような波長（570 nm 近辺）に吸収を持つ着色物質の場合には、吸光度補正を行う必要がある。その手順については、TG492 の本文の説明および ANNEX II のフローチャートを参照する必要がある。

また、本試験法を適用するには、試験法の性質と正確性の確保を考慮して以下の制限が設けられる。

1) バリデーション試験において被験物質に含められなかった気体（ガス）およびエアゾールは適用物質から除外される。
2) UN GHS 区分 1、区分 2（2A・2B）物質の検出には用いることはできない。

バリデーション試験に用いられた混合物の数は限られていたが、バリデーション試験とは別に、動物試験のデータがある農薬処方（97 処方、うち区分外は 43 処方）を用いて評価が行われた[5)]。結果は一致度 82%、偽陰性率 9%、偽陽性率 28%で、これらはバリデーション運営委員会が定めた受け入れ基準を満たしていることから、混合物にも適用できる可能性がある。

以上の点から、TG492 に準拠して実施した場合、ボトムアップ方式において UN GHS 区分外物質を検出する方法として有用であると考える。

４．目的とする物質又は製品の毒性を評価する試験法としての、社会的受け入および行政上の利用の可能性

社会的受け入れ性：

本試験法は RhCE 組織に対する化学物質の細胞毒性を指標に用いて眼刺激性を評価する試験法であり、生きた動物を用いないという点で、3Rs の精神に合致している。また、EpiOcular™ の入手は容易で、短時間で安価に実施でき、特殊な機材や試薬を必要とせず、必要な手技も複雑なものでない。したがって、入手した EpiOcular™ が品質基準の許容範囲にあり、かつ実施する試験施設の技術習得がガイドラインの熟達度確認物質で確かめられていれば、基本的な細胞培養の技術と設備を有する施設であれば実施可能であり、技術移転性は高い。以上より、本試験法の社会的受け入れ性は高い。

行政上の利用性：

本試験法は、化学物質による眼刺激性を評価でき、ボトムアップ方式において UN GHS 区分外を検出する方法として、行政的利用が可能であると考える。

なお、EpiOcular™ EIT 以外の RhCE 法を用いる場合には、OECD TG492 の性能標準に記載された物質を用いて[6)]、その妥当性を確認しておく必要がある。

参考文献

1) EURL ECVAM-CE (2014) Prospective validation study of Reconstructed human Tissue-based test methods for identifying chemical not requiring classification for serious eye damage/eye irritation – Validation Study Report.

2) OECD (2015) Test Guideline 492. Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage.

3) JaCVAM　眼刺激性代替法資料編纂委員会：OECD TG492 ヒト角膜様上皮モデル法 (Reconstructed Human Cornea-like Epithelium Test Method: RhCE法)評価報告書（2016年1月25日）

4) ESAC (2014) ESAC Opinion based on the ESAC Scientific Peer Review of the ECVAM Eye Irritation Validation Study (EIVS) and a related Cosmetics Europe study on HPLC/UPLC-photometry as an alternative endpoint detection system for formazan reaction product.

5) Kolle SN et al. (2015) The EpiOcular^{TM} eye irritation test is the method of choice for the *in vitro* eye irritation testing of agrochemical formulations: Correlation analysis of EpiOcular Eye Irritation Test and BCOP test data according to UN GHS, US EPA and Brazil ANVISA classification schemes. ATLA. 43, 181-198.

6) OECD (2015) Series on Testing & Assessment 216. Performance standards for the assessment of proposed similar or modified in vitro Reconstructed human Cornea-like Epithelium (RhCE) test method for eye hazard

眼刺激性試験代替法の評価報告書

再構築ヒト角膜様上皮モデル法
(Reconstructed Human Cornea-like Epithelium Test Method: RhCE 法)

眼刺激性代替法資料編纂委員会

平成 28 年（2016 年）1 月 25 日

眼刺激性代替法資料編纂委員会

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略語

CAS: Chemical Abstracts Services

CE: Cosmetics Europe

EURL ECVAM: the European Union Reference Laboratory for Alternatives to Animal Testing

ESAC: ECVAM Scientific Advisory Committee

GHS: Globally Harmonized System of Classification and Labeling of Chemicals

JaCVAM: Japanese Center for the Validation of Alternative Methods

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OD: optical density

OECD: Organization for Economic Co-operation and Development

SOP: Standard Operating Procedures

TG: Test Guideline

UN: United Nations

要旨

再構築ヒト角膜様上皮モデル法（Reconstructed Human Cornea-like Epithelium Test Method：RhCE法）は、化学物質の RhCE 組織に対する細胞毒性を指標に用い、その物質の眼刺激性を評価する試験法である。UN GHS 区分外物質を検出する方法として EURL ECVAM と CE の共同バリデーションを経て 2015 年に OECD によりテストガイドライン TG 492 として採択された。JaCVAM 眼刺激性代替法資料編纂委員会は、TG 492 で示された RhCE 法の 1 つである EpiOcular^{TM} を用いた眼刺激性試験について、バリデーション報告書、第三者評価報告書、関連論文などをもとに試験法の概要および委員会の意見をまとめた。

従来のウサギを用いた Draize 法の結果を基準として正確を評価したとき、一致度は 79.7%、偽陽性率は 37.0%、偽陰性率は 4.3%であった。技術移転性に関して懸念される結果は得られておらず、施設内再現性および施設間再現性はどちらも 90%以上であった。これらの値は、バリデーション運営委員会が定めた基準を満たしていた。

本委員会は、RhCE 法はボトムアップ方式で UN GHS 区分外物質を検出する方法として用いることができると結論した。

1. まえおき

化学物質の眼刺激性を評価する方法として従来使用されてきたのは、Draize 法というウサギを用いた動物試験である。しかし、近年の動物福祉に対する関心の高まりや欧州における法規制改正は、その代替法の開発・バリデーションを促進した。OECD がテストガイドラインとしてすでに採択した代替法は、ウシ摘出角膜の混濁および透過性試験法（BCOP 法、TG 437）、ニワトリ摘出眼球を用いた眼刺激性試験法（ICE 法、TG 438）、およびフルオレセイン漏出試験法（FL 法、TG 460）の 3 試験法である。BCOP 法と ICE 法は食用などの目的で処分された動物より摘出した器官・組織を用いて化学物質の曝露により生じる角膜の物理的特性の変化を指標に眼刺激性を評価する試験法で、トップダウン方式において重篤な眼の傷害を起こす（すなわち、UN GHS 区分 1）物質を検出する方法として、またボトムアップ方式において眼に対する重篤な損傷性を有する物質および眼刺激性物質とは分類されない（すなわち、UN GHS 区分外）物質を検出する方法として用いられる。FL 法は尿細管上皮細胞の単層培養を用いて化学物質の曝露により生じる細胞間結合の傷害を指標に眼刺激性を評価する試験法で、トップダウン方式でのみ用いられる。

再構築ヒト角膜様上皮モデル法（Reconstructed Human Cornea-like Epithelium Test Method、以下 RhCE 法）は、被験物質のヒト角膜様上皮モデル（RhCE）組織に対する細胞毒性を指標に用い、その物質の眼刺激性を評価する試験法である。RhCE 組織に被験物質を曝露した後、陰性対照との比較により細胞生存率を算出し被験物質の眼刺激性を評価する。

RhCE 法は EURL ECVAM と CE の共同バリデーション研究（EURL ECVAM-CE, 2014）および ESAC の第三者評価（ESAC, 2014）を経て、UN GHS 区分外物質を検出する方法として 2015 年 7 月に OECD によりテストガイドラインに採択された（TG 492）。

本報告書はバリデーション報告書、第三者評価報告書、関連論文などをもとに OECD TG 492 RhCE 法の概要を説明し、本委員会の意見をまとめたものである。

2. 試験法の位置づけ

RhCE 法は、UN GHS 区分外物質（単一物質および混合物）を検出するために用いる試験法である。

3. 試験法の原理

眼刺激性は、物質が角膜を含む眼表面に接触し細胞傷害を引き起こすことから始まる。その機序は様々であるが、細胞毒性が重要な役割を担っている。また、物質の眼刺激性は主に角膜の初期傷害の深度により決定され、それは細胞死の程度と相関関係にある。RhCE 法は、再構築ヒト角膜様上皮モデルである RhCE 組織を用いて、被験物質の細胞毒性を指標として眼刺激性を評価する試験法である。

TG 492 で認められている RhCE 法は、ヒト新生児包皮細胞由来の市販の RhCE 組織である EpiOcular^{TM} を用いた眼刺激性試験（EpiOcular^{TM} EIT）である（Kaluzhny et al, 2011）。被験物質が液体の場合は約 30 分間、固体の場合は約 6 時間、RhCE 組織に曝露した後での MTT の取り込み量をもとにした細胞生存率をエンドポイントに用いる。これは、MTT が脱水素酵素の基質となる性質を利用し、細胞内に取り込まれた MTT がミトコンドリア内脱水素酵素により還元され、生成されたホルマ

ザン量（青色）が生存細胞数に比例することを基本原理としている（MTT 還元法)。細胞生存率が陰性対照と比較して 60%を超える場合、被験物質は UN GHS 分類において区分外であると判定する。

4. 試験手順

以下に示す手順は、TG 492 で認められている EpiOcular™ EIT についてのものである。EpiOcular™ はアメリカの製造元より購入できる。詳細は、TG 492 および SOP（SOP Version 9, 2015）を参照すること。

4-1. RhCE 組織の機能的条件

RhCE 組織の機能的条件には、RhCE 組織の生存率とバリア機能が含まれ、その許容範囲は以下のとおりである。

生存率：陰性対照において $0.8 < OD < 2.5$
バリア機能：0.3% Triton X-100 に対し $12.2 \leq ET_{50}\ (min) \leq 37.5$
（ET_{50}：細胞生存率を 50%低下させるのに必要な曝露時間）

これらは RhCE 組織の製造者の出荷基準としても採用される。また、製造者は組織学的検査で RhCE 組織がヒトの角膜上皮様構造を有することを保証しておく必要がある。一方、RhCE 組織の使用者は試験で得られた陽性および陰性対照の結果を背景データと比較して、再現性を確認する必要がある。

4-2. 被験物質の適用

物質あたり少なくとも 2 組織を用いる。被験物質は調製せず、原体そのものを用いる。37˚C 以下でピペットで扱えるものは液体として、それ以外は固体として試験を行う。特に、被験物質が粘性のある物質や樹脂・ワックス・ゲル状物質の場合は、37±1˚C で 15±1 分間水浴でインキュベートして液体か固体かを判断する。RhCE 組織はリン酸緩衝生理食塩水（Mg^{2+}/Ca^{2+} free）を用いて 30±2 分間標準培養条件[1]で前処理する。液体被験物質の場合、RhCE 組織に 50 µL を適用し、標準培養条件で 30±2 分間培養する。その後、リン酸緩衝生理食塩水で十分に洗浄して被験物質を除去し、12±2 分間室温で培地に浸漬後、新たな培地を用いて標準培養条件で 120±15 分間培養する。固体被験物質の場合、RhCE 組織の表面を覆うのに十分な量、約 50 mg を均一に適用し、標準培養条件で 6±0.25 時間培養する。その後、リン酸緩衝生理食塩水で十分に洗浄して被験物質を除去し、25±2 分間室温で培地に浸漬後、新たな培地を用いて標準培養条件で 18±0.25 時間培養する。

陽性対照には酢酸メチル（CAS No. 79-20-9)、陰性対照には超純水が推奨される。対照物質の適用・後処理は対照となる被験物質（液体または固体）の条件に準じる。

4-3. 細胞生存率の算出

細胞生存率算出には MTT 還元法を用いる。培地を除去し、1 mg/mL MTT 溶液 0.3 mL 中で 180±10 分間標準培養条件で反応させ、その後 2 mL イソプロパノール（または同様な溶媒）で青色のホルマザンを抽出する。液体被験物質の場合は、RhCE 組織の上部・底部両方から抽出する。固体被験物質および色の付いている液体被験物質の場合は、組織に残存する被験物質の混入を最小限に抑えるため、

[1] 暗所、37±1˚C、5±1% CO_2、高湿度

ホルマザンの抽出はRhCE組織の底部のみから行う。液体被験物質でも、洗浄しづらい場合には、底部のみから抽出を行う。同時対照物質に対しての抽出方法は被験物質と同じとする。抽出したホルマザンの定量はOD_{570nm}測定またはHPLC/UPLCで行う。

被験物質がMTT還元物質の場合、あるいはホルマザンと同じような波長（570 nm近辺）に吸収を持つ着色物質の場合、吸光度補正を行う必要がある。その手順については TG 本文の説明および ANNEX II のフローチャートを参照すること。

4-4. 試験成立の承認基準

以下の条件を満たした場合、試験の成立を承認する。

1）陰性対照のODが$0.8 < OD < 2.5$であること。

2）陽性対照の平均細胞生存率が50%未満であること。

3）被験物質および陰性・陽性対照のそれぞれにおいて、2 組織を用いた場合は細胞生存率の差が20%未満、3組織以上を用いた場合は細胞生存率の標準偏差が18%以下であること。

4-5. 刺激性の判定

刺激性判定のカットオフ値は平均細胞生存率60%である。すなわち、平均細胞生存率が60%を超えた場合、被験物質はGHS 区分外と判断される。平均細胞生存率が60%以下の場合、本試験法では偽陽性が生じえるし、また区分1と区分2を検出できないことから、他の試験法による追加試験が必要となる。

5. バリデーション試験

EURL ECVAM と CE の協同で EpiOcular™ EIT のバリデーション試験が行われた。正確性の評価のために必要な動物試験のデータを持っていることを前提条件に、UN GHS 区分物質・区分外物質や固体・液体等のバランスを考慮して被験物質の選択が行われた（Appendix 1）。

コード化された 107 物質についてすべてを 3 施設で試験した（本試験）。試験はすべての被験物質について3回実施された。固体物質に対しては、その後プロトコールの最適化（被験物質の曝露時間を90±5分から6±0.25時間に変更）が行われたため、107物質中の固体および新たな固体8物質について、追加試験が1施設で行われた。

このバリデーション試験では吸光度でホルマザンを定量し、細胞生存率を求めている。HPLC/UPLCを利用したホルマザン定量・細胞生存率算出については、CE により皮膚刺激性試験に用いられるヒト組織モデルと合わせてバリデーションが行われている。

6. 試験法の信頼性

6-1. 技術移転性

バリデーション試験を開始する前に、バリデーション参加施設の技術者（施設当たり3名）に対して技術習得のためのトレーニングを行った。その後で、8物質を用いて技術移転の確認を行ったところ、異なる判定は1施設の技術者1名の1液体物質のみであった。EpiOcular™ EIT を実施する試験施設の技術習得を確かめるための熟達度確認物質の一覧は、Appendix 2にある。

6-2. 施設内再現性

バリデーション試験では施設ごとに 1 被験物質につき複数回の試験を行っている。本試験では、GHS 分類判定の施設ごとの施設内再現性は 94.2–96.2%であった（固体物質のデータを含む)。1 施設で行われた追加試験での施設内再現性は 96.6%であった。これらの値は EURL ECVAM-CE バリデーション運営委員会が定めた基準（85%以上）を満たした。

6-3. 施設間再現性

本試験で得られた細胞生存率（複数回試験）の平均値をもとに GHS 分類判定を施設ごとに行い、その一致度を検討した（固体物質のデータを含む)。その結果、GHS 分類判定の施設間再現性は 93.2%であった。この値はバリデーション運営委員会が定めた基準（80%以上）を満たした。プロトコル最適化後の固体物質に対しての GHS 分類判定の施設間再現性のデータは、追加試験を 1 施設のみで行ったため存在しない。バリデーション運営委員会は、施設内再現性は本試験・追加試験とも高い値であり、また、同時にバリデーションを行った別の RhCE 法において曝露時間を変更した際に、変更前と比較して変更後でも同等の高い施設間再現性が得られていることから、プロトコル最適化後の施設間再現性の確認は必要ないと判断している。ESAC の第三者評価では、プロトコル最適化後の施設間再現性がない点が大きな支障になるとは考えていない。

7. 試験法の正確性

バリデーション報告書での正確性の評価には、バリデーション試験で用いられた 115 物質のうち物質自体の色がホルマザン定量での OD 測定を妨げる 3 物質と強い還元性物質 1 物質を除く 111 物質の結果が用いられた。成立した全試験のデータを用いて求めた結果は表 1 の通りである。EpiOcular™ EIT の正確性はバリデーション運営委員会が定めた受け入れ基準を満たしている。

表 1. EpiOcular™ EIT の正確性－全試験データ

	物質数	一致度度(%)	偽陰性率(%)	偽陽性率(%)
液体	52	81.9	1.7	33.3
固体	59	78.0	6.5	39.3
液体・固体	111	79.7	4.3	37.0
代替法としての受け入れ基準	-	≥ 75	≤ 10	≤ 40

ESAC の第三者評価では、被験物質ごとに判定を最高スコア（Best-case、得られた試験データのうち一つでも動物試験のデータと一致した判定があればそれを採用）と最低スコア（Worst-case、得られた試験データのうち一つでも動物試験のデータと一致しなかった判定があればそれを採用）の 2 パターンで確定し、その結果を用いて正確性の検討をしている。その結果は表 2 の通りである。最低スコアでは偽陽性率については受け入れ基準を満たさなかったが、それ以外の正確性の数値はすべて受け入れ基準を満たした。本試験法の予測精度は最低スコアと最高スコアの間になることを考慮すると、

EpiOcular™ EIT の正確性はバリデーション運営委員会が定めた受け入れ基準を満たすと第三者評価は結論した。なお、第三者評価では、バリデーションレポートで除外された強い還元性物質も組み入れられている。

表 2. EpiOcular™ EIT の正確性－物質ごとのデータ

	物質数	一致度度(%)	偽陰性率(%)	偽陽性率(%)
最低スコア				
液体	53	75.5	7.7	40.7
固体	59	76.3	6.5	42.9
液体・固体	112	76.1	7.0	41.1
最高スコア				
液体	53	84.9	0.0	29.6
固体	59	79.7	6.5	35.7
液体・固体	112	82.1	3.5	32.7
代替法としての受け入れ基準	-	≥ 75	≤ 10	≤ 40

バリデーションに用いられた混合物の数は限られていた。バリデーション試験とは別に、動物試験のデータがある農薬処方（97 処方、うち区分外は 43 処方）を用いて評価を行った報告では、正確度 82%、偽陰性率 9%、偽陽性率 28%であった（Kolle et al, 2015）。

8. 試験法の適用範囲

TG 492 は、試験法の性質と正確性の確保を考慮して RhCE 法の適用に以下の制限を設けている。

1) バリデーション試験において被験物質に含められなかった気体（ガス）およびエアゾールは適用物質から除外される。
2) GHS 区分 1、区分 2（2A・2B）物質の検出には用いることはできない。

9. 本委員会の結論

EpiOcular™ EIT は再現性も正確性もバリデーション運営委員会の定めた基準を満たしており、本委員会はバリデーション運営委員会の定めた基準は妥当であると考える。EpiOcular™ EIT に用いる RhCE 組織は市販されており、アメリカの製造元より入手できる。RhCE 組織以外は、特殊な機材や試薬を必要とせず、必要な手技も複雑なものでない。入手した RhCE 組織が機能的条件の許容範囲にあり、かつ実施する試験施設の技術習得がガイドラインの熟達度確認物質で確かめられていれば、EpiOcular™ EIT は UN GHS 区分外物質を検出する方法として用いることができる。

EpiOcualr™ EIT は RhCE 法を代表するものと考えられるので、TG 492 に準拠して実施した場合、RhCE 法はボトムアップ方式で UN GHS 区分外物質を検出する方法として用いることができる、と本委員会は考える。

10. 文献

EpiOcular™ EIT SOP, Version 9 (April 6, 2015). EpiOcluar™ EIT for the prediction of acute oculatr irritation of chemicals. Available at: http:// https://eurl-ecvam.jrc.ec.europa.eu/ (as of Nov. 2015)

ESAC (2014) ESAC Opinion based on the ESAC Scientific Peer Review of the ECVAM Eye Irritation Validation Study (EIVS) and a related Cosmetics Europe study on HPLC/UPLC-photometry as an alternative endpoint detection system for formazan reaction product.

EURL ECVAM-CE (2014) Prospective validation study of Reconstructed human Tissue-based test methods for identifying chemical not requiring classification for serious eye damage/eye irritation – Validation Study Report.

Kaluzhny Y et al (2011) Development of the EpiOcular™ Eye Irritation Test for hazard identification and labelling of eye irritating chemicals in response to the requirements of the EU cosmetics directive and REACH legislation. ATLA. 39, 339-64.

Kolle SN et al (2015). The EpiOcular™ Eye Irritation Test is the method of choice for *in vitro* eye irritation testing of agrochemical formulations: Correlation analysis of EpiOcular Eye Irritation Test and BCOP test data according to UN GHS, US EPA and Brazil ANVISA classification schemes. ATLA. 43, 181-198.

OECD (2012) Test Guideline 437. 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.

OECD (2012) Test Guideline 438. Isolated Chicken Eye test method for identifying i) chemicals inducing serious eye damage and ii) chemicals not requiring classification for eye irritation or serious eye damage.

OECD (2012) Test Guideline 460. Fluorescein Leakage test method for identifying ocular corrosives and severe irritants.

OECD (2015) Test Guideline 492. Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage.

OECD (2015) Series on Testing & Assessment 216. Performance standards for the assessment of proposed similar or modified in vitro Reconstructed human Cornea-like Epithelium (RhCE) test method for eye hazard

Appendix 1

バリデーションに用いられた物質のカテゴリーごとの数

全体	115
眼刺激性 GHS 分類	
区分 1	30
区分 2A	16
区分 2B	14
区分外	55
物質の特性	
液体	53
固体	62
Chemical Class	
Ether	25
Alcohol	19
Aryl	18
Carboxylic acid ester	16
Carboxylic acid	14
Alkoxy	13
Aromatic heterocyclic halide	10
Aryl halide	10
Alkyl halide	9
Phenol	9
Allyl	6
Ammonium salt	6
Benzyl	6
Aliphatic Amine, tertiary	5
Alkane, branched with tertiary carbon	5
Aromatic amine	5
Dihydroxyl group	5
Guanidine	5
Saturated heterocyclic fragment	5
Sulfate	5

注）バリデーション報告書をもとに作成。Chemical Class は物質数の多いものを抽出した。物質によっては複数の Chemical Class にまたがっている場合もある。

Appendix 2

RhCE 法の熟達度確認物質

物質名	CAS 番号	性状	GHS 分類
Methylthioglycolate	2365-48-2	液体	区分 1
Tetraethylene glycol diacrylate	17831-71-9	液体	区分 1
2,5-Dimethyl-2,5-hexanediol	110-03-2	固体	区分 1
Sodium oxalate	62-76-0	固体	区分 1
2,4,11,13-Tetraazatetradecane-diimidamine, N,N''-bis(4-chlorophenyl)- 3,12-diimino-,di-D-glucose (20%, aqueous)	18472-51-0	液体	区分 2A
1,5-Naphthalenediol	83-56-7	固体	区分 2A
Diethyl toluamide	134-62-3	液体	区分 2B
2,2-Dimethyl-3-methylenebicyclo[2.2.1]heptane	79-92-5	固体	区分 2B
Dipropyl disulphide	629-19-6	液体	区分外
Piperonyl butoxide	51-03-6	液体	区分外
1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-7	液体	区分外
Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	粘性物	区分外
Potassium tetrafluoroborate	14075-53-7	固体	区分外
1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl)urea	101-20-2	固体	区分外
2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4- (1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	固体	区分外

Adopted:
28 July 2015

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage

INTRODUCTION

1. *Serious eye damage* refers to 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, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). Also according to UN GHS, *eye irritation* refers to the 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. Test chemicals inducing serious eye damage are classified as UN GHS Category 1, while those inducing eye irritation are classified as UN GHS Category 2. Test 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.

2. The assessment of serious eye damage/eye irritation has typically involved the use of laboratory animals (OECD Test Guideline (TG) 405; adopted in 1981 and revised in 1987, 2002 and 2012) (2). In relation to animal welfare concerns, TG 405 recommends the use of a sequential testing strategy for the determination of the serious eye damage/eye irritation potential of chemicals. This testing strategy is described in a Supplement to the Guideline and includes the use of validated, scientifically valid and accepted *in vitro* test methods, thus decreasing or avoiding pain and suffering of animals (2).

3. This Test Guideline describes an *in vitro* procedure allowing the identification of chemicals (substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage in accordance with UN GHS. It makes use of reconstructed human cornea-like epithelium (RhCE) which closely mimics the histological, morphological, biochemical and physiological properties of the human corneal epithelium. Four other *in vitro* test methods have been validated, considered scientifically valid and adopted as OECD Test Guidelines (TGs) 437 (3), 438 (4), 460 (5) and 491 (32) to address the human health endpoint serious eye damage/eye irritation.

4. The only *in vitro* test method currently covered by this Test Guideline is the EpiOcular™ Eye Irritation Test (EIT), which makes use of a commercially available RhCE tissue construct as test system.

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The EpiOcular™ Eye Irritation Test (EIT) was validated by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and Cosmetics Europe between 2008 and 2013 (6)(7)(8)(9)(10). From this validation study and its independent peer review (11) it was concluded that the EpiOcular™ EIT is able to correctly identify chemicals (both substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage according to UN GHS (1), and the test method was recommended as scientifically valid for that purpose (11). The EpiOcular™ EIT is thus referred to as the Validated Reference Method (VRM) in the present Test Guideline.

5. It is currently generally accepted that, in the foreseeable future, no single *in vitro* test method will be able to fully replace the *in vivo* Draize eye test (2)(12) to predict across the full range of serious eye damage/eye irritation responses for different chemical classes. However, strategic combinations of several alternative test methods within (tiered) testing strategies such as the Bottom-Up/Top-Down approach may be able to fully replace the Draize eye test (13). The Bottom-Up approach (13) is designed to be used when, based on existing information, a chemical is expected not to cause sufficient eye irritation to require a classification, while the Top-Down approach (13) is designed to be used when, based on existing information, a chemical is expected to cause serious eye damage. The EpiOcular™ EIT is recommended to identify chemicals that do not require classification for eye irritation or serious eye damage according to UN GHS (UN GHS No Category) (1) without further testing, within a testing strategy such as the Bottom-Up/Top-Down approach suggested by Scott *et al.* e.g., as an initial step in a Bottom-Up approach or as one of the last steps in a Top-Down approach (13). However, the EpiOcular™ EIT is not intended to differentiate between UN GHS Category 1 (serious eye damage) and UN GHS Category 2 (eye irritation). This differentiation will need to be addressed by another tier of a test strategy (13). A chemical that is identified as requiring classification for eye irritation/serious eye damage with EpiOcular™ EIT will thus require additional testing (*in vitro* and/or *in vivo*) to establish a definitive classification, using e.g., TG 437, 438, 460 or 491.

6. The purpose of this Test Guideline is to describe the procedures used to evaluate the eye hazard potential of a test chemical based on its ability to induce cytotoxicity in a RhCE tissue construct, as measured by the MTT assay (14) (see paragraph 20). The viability of the RhCE tissue following exposure to a test chemical is determined in comparison to tissues treated with the negative control substance (% viability), and is then used to predict the eye hazard potential of the test chemical.

7. Performance Standards (15) are available to facilitate the validation of new or modified *in vitro* RhCE-based test methods similar to EpiOcular™ EIT, in accordance with the principles of Guidance Document No. 34 (16), and allow for timely amendment of this Test Guideline for their inclusion. Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to the Performance Standards, if these test methods have been reviewed and included in this Test Guideline by the OECD.

DEFINITIONS

8. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS AND LIMITATIONS

9. This Test Guideline is based on a commercial three-dimensional RhCE tissue construct that is produced using primary human epidermal keratinocytes i.e., EpiOcular™ OCL-200. The EpiOcular™ OCL-200 RhCE tissue construct is similar to the *in vivo* corneal epithelium three-dimensional structure and is produced using cells from the species of interest (17). Moreover, this Test Guideline directly covers an important mechanistic step determining the overall *in vivo* serious eye damage/eye irritation response of a chemical upon ocular exposure, i.e., penetration of the chemical through the cornea and production of cell and tissue damage. Cell damage can occur by several modes of action (see paragraph 19), but cytotoxicity

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plays an important, if not the primary, mechanistic role in determining the overall serious eye damage/eye irritation response of a chemical, manifested *in vivo* mainly by corneal opacity, iritis, conjunctival redness and/or conjunctival chemosis, regardless of the physicochemical processes underlying tissue damage.

10. A wide range of chemicals, covering a large variety of chemical types, chemical classes, molecular weights, LogPs, chemical structures, etc., have been tested in the validation study underlying this Test Guideline. The EpiOcular™ EIT validation database contained 113 chemicals in total, covering 95 different organic functional groups according to an OECD QSAR toolbox analysis (7)(8). The majority of these chemicals represented mono-constituent substances, but several multi-constituent substances (including 3 homopolymers, 5 copolymers and 10 quasi polymers) were also included in the study. In terms of physical state and UN GHS Categories, the 113 tested chemicals were distributed as follows: 13 Category 1 liquids, 15 Category 1 solids, 6 Category 2A liquids, 10 Category 2A solids, 7 Category 2B liquids, 7 Category 2B solids, 27 No Category liquids and 28 No Category solids (7)(8).

11. This Test Guideline is applicable to substances and mixtures, and to solids, liquids, semi-solids and waxes. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be ground to a fine powder before application; no other pre-treatment of the sample is required. Gases and aerosols have not been assessed in a validation study. While it is conceivable that these can be tested using RhCE technology, the current Test Guideline does not allow testing of gases and aerosols.

12. Test chemicals absorbing light in the same range as MTT formazan (naturally or after treatment) and test chemicals able to directly reduce the vital dye MTT (to MTT formazan) may interfere with the tissue viability measurements and need the use of adapted controls for corrections. The type of adapted controls that may be required will vary depending on the type of interference produced by the test chemical and the procedure used to quantify MTT formazan (see paragraphs 35-41).

13. Results generated in pre-validation (18) and full validation (7)(9)(10) studies have demonstrated that EpiOcular™ EIT is transferable to laboratories considered to be naïve in the conduct of the assay and also to be reproducible within- and between laboratories. Based on these studies, the level of reproducibility in terms of concordance of predictions that can be expected from EpiOcular™ EIT is in the order of 95% within laboratories and 93% between laboratories.

14. The EpiOcular™ EIT can be used to identify chemicals that do not require classification for eye irritation or serious eye damage according to the UN GHS classification system (1). Considering the data obtained in the validation study (7), the EpiOcular™ EIT has an overall accuracy of 80% (based on 112 chemicals), sensitivity of 96% (based on 57 chemicals), false negative rate of 4% (based on 57 chemicals), specificity of 63% (based on 55 chemicals) and false positive rate of 37% (based on 55 chemicals), when compared to reference *in vivo* rabbit eye test data (OECD TG 405) (2)(12) classified according to the UN GHS classification system (1). A study where 97 liquid agrochemical formulations were tested with EpiOcular™ EIT demonstrated a similar performance of the test method for this type of mixtures as obtained in the validation study (19). The 97 formulations were distributed as follows: 21 Category 1, 19 Category 2A, 14 Category 2B and 43 No Category, classified according to the UN GHS classification system (1) based on reference *in vivo* rabbit eye test data (OECD TG 405) (2)(12). An overall accuracy of 82% (based on 97 formulations), sensitivity of 91% (based on 54 formulations), false negative rate of 9% (based on 54 formulations), specificity of 72% (based on 43 formulations) and false positive rate of 28% (based on 43 formulations) were obtained (19). Increasing the cut-off to distinguish classified from non-classified chemicals from 60%, as proposed in this TG (see paragraph 43), to 65% leads to an increase in sensitivity to 94% and a decrease in specificity to 67%, with overall accuracy being maintained at 82%. The false negative rate obtained with EpiOcular™ EIT with either substances or mixtures (i.e., *in vivo* UN GHS Category 2 chemicals producing a mean percent tissue viability > 60%, which are therefore predicted by EpiOcular™ EIT as not requiring classification and labelling; see paragraph 43) is within the overall

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probability of at least 12% that chemicals identified as UN GHS Category 2 by the *in vivo* Draize eye test are equally identified as UN GHS No Category in a repeated test due to the method's inherent within-test variability (20). The false positive rate obtained with EpiOcular™ EIT with either substances or mixtures (i.e., *in vivo* UN GHS No Category chemicals producing a mean percent tissue viability ≤ 60%, which are therefore predicted by EpiOcular™ EIT as requiring classification and labelling; see paragraph 43) is not critical in the context of this Test Guideline since all test chemicals that produce a tissue viability ≤ 60% will require further testing with other adequately valid *in vitro* test methods, or as a last option in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach. This test method can be used for all types of chemicals, whereby a negative result (tissue viability > 60%) should be accepted for not classifying a chemical for eye irritation and serious eye damage (UN GHS No Category). The appropriate regulatory authorities should be consulted before using the EpiOcular™ EIT under classification schemes other than UN GHS.

15. A limitation of this Test Guideline is that it does not allow discrimination between eye irritation/reversible effects on the eye (Category 2) and serious eye damage/irreversible effects on the eye (Category 1), nor between eye irritants (optional Category 2A) and mild eye irritants (optional Category 2B), as defined by UN GHS (1). For these purposes, further testing with other suitable test methods is required.

16. The validation study demonstrated that EpiOcular™ EIT is able to correctly predict chemicals requiring classification for serious eye damage/eye irritation independently of the types of ocular effects observed *in vivo* (i.e., corneal, iridal and conjunctival injuries) (7)(8)(9)(10). In this respect, it should be noted that effects on the iris are of lesser importance for classification of chemicals according to UN GHS (20). In fact, iritis on its own rarely drives the UN GHS classification of chemicals *in vivo* (both Category 1 and Category 2) (1.8-3.1% of the chemicals) since the great majority of the test chemicals that cause classifiable effects to the iris also cause classifiable corneal opacity (20).

17. The term "test chemical" is used in this Test Guideline to refer to what is being tested[1] and is not related to the applicability of the RhCE test method to the testing of substances and/or mixtures.

PRINCIPLE OF THE TEST

18. The test chemical is applied topically to a minimum of two three-dimensional RhCE tissue constructs and tissue viability is measured following exposure and a post-treatment incubation period. The RhCE tissues are reconstructed from primary human cells, which have been cultured for several days to form a stratified, highly differentiated squamous epithelium morphologically similar to that found in the human cornea. The EpiOcular™ RhCE tissue construct consists of at least 3 viable layers of cells and a non-keratinized surface, showing a cornea-like structure analogous to that found *in vivo*.

19. Chemical-induced serious eye damage/eye irritation, manifested *in vivo* mainly by corneal opacity, iritis, conjunctival redness and/or conjunctival chemosis, is the result of a cascade of events beginning with penetration of the chemical through the cornea and/or conjunctiva and production of damage to the cells. Cell damage can occur by several modes of action, including: cell membrane lysis (e.g., by surfactants, organic solvents); coagulation of macromolecules (particularly proteins) (e.g., by surfactants, organic solvents, alkalis and acids); saponification of lipids (e.g., by alkalis); and alkylation or other covalent interactions with macromolecules (e.g., by bleaches, peroxides and alkylators) (13)(21)(22). However, it has been shown that cytotoxicity plays an important, if not the primary, mechanistic role in determining the overall serious eye damage/eye irritation response of a chemical regardless of the physicochemical processes underlying tissue damage (23)(24). Moreover, the serious eye damage/eye

[1] In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term "test chemical" describing what is being tested should now be applied in new and updated Test Guidelines.

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irritation potential of a chemical is principally determined by the extent of initial injury (25), which correlates with the extent of cell death (23) and with the extent of the subsequent responses and eventual outcomes (26). Thus, slight irritants generally only affect the superficial corneal epithelium, the mild and moderate irritants damage principally the epithelium and superficial stroma and the severe irritants damage the epithelium, deep stroma and at times the corneal endothelium (24)(27). The measurement of viability of the EpiOcular™ RhCE tissue construct after topical exposure to a test chemical to discriminate chemicals not requiring classification for serious eye damage/eye irritancy (UN GHS No Category) from those requiring classification and labelling (UN GHS Categories 1 and 2) is based on the assumption that all chemicals inducing serious eye damage or eye irritation will induce cytotoxicity in the corneal epithelium and/or conjunctiva.

20. RhCE tissue viability in EpiOcular™ EIT is measured by enzymatic conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide; CAS number 298-93-1] by the viable cells of the tissue into a blue MTT formazan salt that is quantitatively measured after extraction from tissues (14). Chemicals not requiring classification and labelling according to UN GHS are identified as those that do not decrease tissue viability below a defined threshold (i.e., tissue viability > 60%, for UN GHS No Category).

DEMONSTRATION OF PROFICIENCY

21. Prior to routine use of EpiOcular™ EIT for regulatory purposes, laboratories should demonstrate technical proficiency by correctly predicting the fifteen proficiency chemicals listed in Table 1. These chemicals were selected from the chemicals used in the EURL ECVAM/Cosmetics Europe Eye Irritation Validation Study (EIVS) (7)(8). The selection includes, to the extent possible, chemicals that: (i) cover different physical states; (ii) cover the full range of *in vivo* serious eye damage/eye irritation responses based on high quality results obtained in the reference *in vivo* rabbit eye test (OECD TG 405) (2)(12) and the UN GHS classification system (i.e., Categories 1, 2A, 2B, or No Category) (1); (iii) cover the various *in vivo* drivers of classification as reported by Adriaens *et al.* (20); (iv) are representative of the chemical classes used in the validation study (8); (v) cover a good and wide representation of organic functional groups (8); (vi) have chemical structures that are well-defined (8); (vii) are coloured and/or direct MTT reducers; (viii) produced reproducible results in EpiOcular™ EIT during its validation; (ix) were correctly predicted by EpiOcular™ EIT during its validation; (x) cover the full range of *in vitro* responses based on high quality EpiOcular™ EIT data (0 to 100% viability); (xi) are commercially available; and (xii) are not associated with prohibitive acquisition and/or disposal costs. In situations where a listed chemical is unavailable or cannot be used for other justified reasons, another chemical fulfilling the criteria described above, e.g. from the chemicals used in the validation of the VRM, could be used. Such deviations should however be justified.

Table 1: List of proficiency chemicals

Chemical Name	CASRN	Organic Functional Group[1]	Physical State	VRM viability (%)[2]	VRM Prediction	MTT Reducer	Colour interf.
***In Vivo* Category 1[3]**							
Methylthioglycolate	2365-48-2	Carboxylic acid ester; Thioalcohol	L	10.9±6.4	Cat 2 / Cat 1	Y (strong)	N
Tetraethylene glycol diacrylate	17831-71-9	Acrylate; Ether	L	34.9±15.3	Cat 2 / Cat 1	N	N
2,5-Dimethyl-2,5-hexanediol	110-03-2	Alcohol	S	2.3±0.2	Cat 2 / Cat 1	N	N

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Chemical Name	CASRN	Organic Functional Group[1]	Physical State	VRM viability (%)[2]	VRM Prediction	MTT Reducer	Colour interf.
Sodium oxalate	62-76-0	Oxocarboxylic acid	S	29.0±1.2	Cat 2 / Cat 1	N	N
***In Vivo* Category 2A[3]**							
2,4,11,13-Tetraazatetradecane-diimidamide, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate (20%, aqueous) [4]	18472-51-0	Aromatic heterocyclic halide; Aryl halide; Dihydroxyl group; Guanidine	L	4.0±1.1	Cat 2 / Cat 1	N	Y (weak)
1,5-Naphthalenediol	83-56-7	Fused carbocyclic aromatic; Naphthalene; Phenol	S	21.0±7.4	Cat 2 / Cat 1	Y (medium)	N
***In Vivo* Category 2B[3]**							
Diethyl toluamide	134-62-3	Benzamide	L	15.6±6.3	Cat 2 / Cat 1	N	N
2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	79-92-5	Alkane, branched with tertiary carbon; Alkene; Bicycloheptane; Bridged-ring carbocycles; Cycloalkane	S	4.7±1.5	Cat 2 / Cat 1	N	N
***In Vivo* No Category[3]**							
1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	Alkoxy; Ammonium salt; Aryl; Imidazole; Sulphate	L	79.9±6.4	No Cat	N	N
Dipropyl disulphide	629-19-6	Disulfide	L	81.7±6.4	No Cat	N	N
Piperonyl butoxide	51-03-6	Alkoxy; Benzodioxole; Benzyl; Ether	L	104.2±4.2	No Cat	N	N
Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	Acylal; Alcohol; Allyl; Ether	Viscous	77.6±5.4	No Cat	N	N
1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	Aromatic heterocyclic halide; Aryl halide; Urea derivatives	S	106.7±5.3	No Cat	N	N
2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	Alkane branched with quaternary carbon; Fused carbocyclic aromatic; Fused saturated heterocycles; Precursors quinoid compounds; tert-Butyl	S	102.7±13.4	No Cat	N	N

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Chemical Name	CASRN	Organic Functional Group[1]	Physical State	VRM viability (%)[2]	VRM Prediction	MTT Reducer	Colour interf.
Potassium tetrafluoroborate	14075-53-7	Inorganic Salt	S	88.6±3.3	No Cat	N	N

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals (1); VRM = Validated Reference Method, i.e., EpiOcular™ EIT; Colour interf. = colour interference with the standard absorbance (Optical Density (OD)) measurement of MTT formazan.

[1]Organic functional group assigned according to an OECD Toolbox 3.1 nested analysis (8).

[2]Based on results obtained in the EURL ECVAM/Cosmetics Europe Eye Irritation Validation Study (EIVS) (7)(9)(10).

[3]Based on results from the *in vivo* rabbit eye test (OECD TG 405) (2)(12) and using the UN GHS (1).

[4]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 corneal opacity 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 corneal opacity score of 1 (at day 7) that fully recovered at day 9.

22. As part of the proficiency testing, it is recommended that users verify the barrier properties of the tissues after receipt as specified by the RhCE tissue construct producer (see paragraphs 24, 26 and 29). This is particularly important if tissues are shipped over long distance / time periods. Once a test method has been successfully established and proficiency in its use has been acquired and demonstrated, such verification will not be necessary on a routine basis. However, when using a test method routinely, it is recommended to continue to assess the barrier properties at regular intervals.

PROCEDURE

23. The only test method currently covered by this Test Guideline is the scientifically valid EpiOcular™ EIT (7)(11), referred to as the Validated Reference Method (VRM). The Standard Operating Procedures (SOP) for the EpiOcular™ EIT are available and should be employed when implementing and using the test method in a laboratory (28). The following paragraphs describe the main components and procedures of the EpiOcular™ EIT.

RHCE TEST METHOD COMPONENTS

General conditions

24. Relevant human-derived cells should be used to reconstruct the cornea-like epithelium three-dimensional tissue, which should be composed of progressively stratified but not cornified cells. The RhCE tissue construct is prepared in inserts with a porous synthetic membrane through which nutrients can pass to the cells. Multiple layers of viable, non-keratinized epithelial cells should be present in the reconstructed cornea-like epithelium. The RhCE tissue construct should have the epithelial surface in direct contact with air so as to allow for direct topical exposure of test chemicals in a fashion similar to how the corneal epithelium would be exposed *in vivo*. The RhCE tissue construct should form a functional barrier with sufficient robustness to resist rapid penetration of cytotoxic benchmark substances, e.g., Triton X-100. The barrier function should be demonstrated and may be assessed by determination of the exposure time required to reduce tissue viability by 50% (ET_{50}) upon application of the benchmark substance at a specified, fixed concentration (e.g., 100 µL of 0.3% (v/v) Triton X-100) (see paragraph 29). The containment properties of the RhCE tissue construct should prevent the passage of test chemical around the edge of the viable tissue, which could lead to poor modelling of corneal exposure. The RhCE tissue construct should be free of contamination by bacteria, viruses, mycoplasma, and fungi.

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Functional conditions

Viability

25. The assay used for quantifying tissue viability is the MTT assay (14). Viable cells of the RhCE tissue construct reduce the vital dye MTT into a blue MTT formazan precipitate, which is then extracted from the tissue using isopropanol (or a similar solvent). The extracted MTT formazan may be quantified using either a standard absorbance (Optical Density (OD)) measurement or an HPLC/UPLC-spectrophotometry procedure (29). The OD of the extraction solvent alone should be sufficiently small, i.e., OD < 0.1. The RhCE tissue construct users should ensure that each batch of the RhCE tissue construct used meets defined criteria for the negative control. An acceptability range (upper and lower limit) for the negative control OD values (in the test method conditions) should be established by the RhCE tissue construct developer/supplier. Acceptability ranges for the negative control OD values for the VRM are given in Table 2 (30). An HPLC/UPLC-spectrophotometry user should use the negative control OD ranges provided in Table 2 as the acceptance criterion for the negative control. It should be documented that the tissues treated with the negative control substance are stable in culture (provide similar tissue viability measurements) for the duration of the test exposure period.

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Table 2: Acceptability ranges for negative control OD values

Test Method	Lower acceptance limit	Upper acceptance limit
EpiOcular™ EIT (OCL-200) – VRM (for both the liquids and the solids protocols)	> 0.8[1]	< 2.5

[1]This acceptance limit considers the possibility of extended shipping/storage time (e.g., > 4 days), which has been shown not to impact on the performance of the test method (30).

Barrier function

26. The RhCE tissue construct should be sufficiently thick and robust to resist the rapid penetration of cytotoxic benchmark substances, e.g., Triton X-100, as estimated by e.g., ET_{50} (Table 3). The barrier function of each batch of the RhCE tissue construct used should be demonstrated by the RhCE tissue construct developer/vendor upon supply of the tissues to the end user (see paragraph 29).

Morphology

27. Histological examination of the RhCE tissue construct should demonstrate human cornea-like epithelium structure (including at least 3 layers of viable epithelial cells and a non-keratinized surface), but is not required for each tissue batch used. For the VRM, appropriate morphology has been established and therefore does not need to be demonstrated again by a test method user for each tissue batch used.

Reproducibility

28. The results of the positive and negative controls of the test method should demonstrate reproducibility over time.

Quality control (QC)

29. The RhCE tissue construct should only be used if the developer/supplier demonstrates that each batch of the RhCE tissue construct used meets defined production release criteria, among which those for viability (see paragraph 25) and barrier function (see paragraph 26) are the most relevant. An acceptability range (upper and lower limit) for the barrier function as measured by the ET_{50} (see paragraphs 24 and 26) should be established by the RhCE tissue construct developer/supplier. The ET_{50} acceptability range used as QC batch release criterion by the developer/supplier of the EpiOcular™ OCL-200 tissue construct (used in the VRM) is given in Table 3. Data demonstrating compliance with all production release criteria should be provided by the RhCE tissue construct developer/supplier to the test method users so that they are able to include this information in the test report. Only results produced with tissues fulfilling all of these production release criteria can be accepted for reliable prediction of chemicals not requiring classification and labelling for eye irritation or serious eye damage in accordance with UN GHS.

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Table 3: QC batch release criterion

Test Method	Lower acceptance limit	Upper acceptance limit
EpiOcular™ EIT (OCL-200) - VRM (100 µL of 0.3% (v/v) Triton X-100)	ET_{50} = 12.2 min	ET_{50} = 37.5 min

Application of the Test Chemical and Control Substances

30. At least two tissue replicates should be used for each test chemical and each control substance in each run. Before exposure to test chemicals or a control substance, the tissue surface of the VRM is pre-treated with 20 µL of calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (Ca^{2+}/Mg^{2+}-free DPBS) and incubated in the dark at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air (standard culture conditions) for 30±2 minutes to mimic the wet conditions of human eye. After this 30±2 minutes pre-treatment, treatment of the tissues is initiated with exposure to the test chemical(s) and control substances. Two different treatment protocols are used, one for liquid test chemicals and one for solid test chemicals (28). In either case, a sufficient amount of test chemical or control substance should be applied to uniformly cover the epithelial surface while avoiding an infinite dose (see paragraphs 31 and 32). According to the test procedure, it is essential that the tissue viability measurements are not made immediately after exposure to the test chemical, but rather after a sufficiently long post-exposure incubation period (in fresh medium) after the test chemical has been rinsed from the tissue. This period allows both for recovery from weak cytotoxic effects and for appearance of clear cytotoxic effects.

31. Test chemicals that can be pipetted at 37°C or lower temperatures (using a positive displacement pipette, if needed) are treated as liquids in the VRM. Viscous, waxy, resinous and gel-like test chemicals with unclear physical state should be incubated at 37±1°C for 15±1 minutes before deciding which treatment protocol to use. If such test chemicals become pipettable after this incubation period (using a positive displacement pipette, if necessary), they should be treated as liquids and should be applied to the tissues directly from the water bath (at 37±1°C), otherwise they should be treated as solids (see paragraph 32). In the VRM, 50 µL of liquid test chemical are evenly spread over the 0.6 cm^2 of the tissue surface (83.3 $\mu L/cm^2$ application). Tissues treated with liquid test chemicals and with control substances tested concurrently to liquid test chemicals are incubated for 30±2 minutes at standard culture conditions. At the end of the exposure period, the test chemical and control substances should be carefully removed from the tissue surface by extensive rinsing with Ca^{2+}/Mg^{2+}-free DPBS at room temperature. This rinsing step is followed by a 12±2 minutes post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) and a 120±15 minutes post-exposure incubation in fresh medium at standard culture conditions, prior to performing the MTT assay (28).

32. Test chemicals that cannot be pipetted at temperatures up to 37°C are treated as solids in the VRM. In the VRM, approximately 50 mg of solid test chemical are evenly applied over the 0.6 cm^2 of the tissue surface using a calibrated tool (e.g., a levelled spoonful calibrated to hold 50 mg of sodium chloride) (approximately 83.3 mg/cm^2 application). The amount of test chemical applied should be sufficient to cover the entire surface of the tissue. Whenever possible, solids should be tested as a fine powder. Tissues treated with solid test chemicals and with control substances tested concurrently to solid test chemicals are incubated for 6±0.25 hours at standard culture conditions. At the end of the exposure period, the test chemical and control substances should be carefully removed from the tissue surface by extensive rinsing with Ca^{2+}/Mg^{2+}-free DPBS at room temperature. This rinsing step is followed by a 25±2 minutes post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) and an 18±0.25 hours post-exposure incubation in fresh medium at standard culture conditions, prior to performing the MTT assay (28).

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33. Concurrent negative and positive controls should be included in each run to demonstrate that the viability (determined with the negative control) and the sensitivity (determined with the positive control) of the tissues are within acceptance ranges defined based on historical data. The concurrent negative control also provides the baseline (100% tissue viability) to calculate the relative percent viability of the tissues treated with the test chemical ($\%Viability_{test}$). The recommended positive control substance to be used with the VRM is neat methyl acetate (CAS No. 79-20-9; Sigma-Aldrich, Cat# 186325; liquid). The recommended negative control substance to be used with the VRM is ultrapure H_2O. These were the control substances used in the pre-validation and validation studies of the VRM and are those for which most historical data exist. The use of suitable alternative positive or negative control substances should be scientifically and adequately justified. Separate negative and positive controls are needed for liquid and solid test chemicals. For controls performed concurrently to liquid test chemicals, 50 µL of the negative control and positive control substances should be applied to the tissues exactly as for the liquid test chemicals followed by a 30±2 minutes exposure at standard culture conditions, rinsing, a 12±2 minutes post-exposure immersion in fresh medium at room temperature and a 120±15 minutes post-exposure incubation in fresh medium at standard culture conditions, prior to performing the MTT assay. For controls performed concurrently to solid test chemicals, 50 µL of the negative control and positive control substances should be applied to the tissues (as described for the liquid test chemicals) followed by a 6±0.25 hours exposure at standard culture conditions, rinsing, a 25±2 minutes post-exposure immersion in fresh medium at room temperature and an 18±0.25 hours post-exposure incubation in fresh medium at standard culture conditions, prior to performing the MTT assay (28). One single set of negative and positive controls is sufficient for all test chemicals of the same physical state (liquids or solids) included in the same run.

Tissue Viability Measurements

34. The MTT assay is a standardised quantitative method (14) that should be used to measure tissue viability under this Test Guideline. It is compatible with use in a three-dimensional tissue construct. The MTT assay is performed immediately following the post-exposure incubation period. In the VRM, the RhCE tissue construct sample is placed in 0.3 mL of MTT solution at 1 mg/mL for 180±10 minutes at standard culture conditions. The vital dye MTT is reduced into a blue MTT formazan precipitate by the viable cells of the RhCE tissue construct. The precipitated blue MTT formazan product is then extracted from the tissue using 2 mL of isopropanol (or a similar solvent). Tissues tested with liquid test chemicals should be extracted from both the top and the bottom of the tissues, while tissues tested with solid test chemicals and coloured liquids should be extracted from the bottom of the tissue only (to minimise any potential contamination of the isopropanol extraction solution with any test chemical that may have remained on the tissue). Tissues tested with liquid test chemicals that are not readily washed off may also be extracted from the bottom of the tissue only. The concurrently tested negative and positive control substances should be treated similarly to the tested chemical. The extracted MTT formazan may be quantified either by a standard absorbance (OD) measurement at 570 nm using a filter band pass of maximum ± 30 nm or by using an HPLC/UPLC-spectrophotometry procedure (see paragraph 41) (29).

35. Optical properties of the test chemical or its chemical action on MTT may interfere with the measurement of MTT formazan leading to a false estimate of tissue viability. Test chemicals may interfere with the measurement of MTT formazan by direct reduction of the MTT into blue MTT formazan and/or by colour interference if the test chemical absorbs, naturally or due to treatment procedures, in the same OD range as MTT formazan (i.e., around 570 nm). Pre-checks should be performed before testing to allow identification of potential direct MTT reducers and/or colour interfering chemicals and additional controls should be used to detect and correct for potential interference from such test chemicals (see paragraphs 36-40). This is especially important when a specific test chemical is not completely removed from the RhCE tissue construct by rinsing or when it penetrates the cornea-like epithelium and is therefore present in the RhCE tissue constructs when the MTT assay is performed. For test chemicals absorbing light in the same range as MTT formazan (naturally or after treatment), which are not compatible with the standard absorbance (OD) measurement of MTT formazan due to too strong interference, i.e., strong absorption at

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570±30 nm, an HPLC/UPLC-spectrophotometry procedure to measure MTT formazan may be employed (see paragraphs 40 and 41) (29). A detailed description of how to detect and correct for direct MTT reduction and interferences by colouring agents is available in the VRM SOP (28). An illustrative flowchart providing guidance on how to identify and handle direct MTT-reducers and/or colour interfering chemicals is also provided in Annex II.

36. To identify potential interference by test chemicals absorbing light in the same range as MTT formazan (naturally or after treatment) and decide on the need for additional controls, spectral analysis of a test chemical in water (environment during exposure) and/or isopropanol (extraction solvent) should be performed. In the VMR, 50 µL or 50 mg of test chemical are added to (i) 1 mL of water and incubated for approximately 1 hour at standard culture conditions and/or (ii) 2 mL of isopropanol and incubated for 2-3 hours at room temperature (28). If the test chemical in water and/or isopropanol absorbs sufficient light in the range of 570±30 nm (for the VRM, if the OD of the test chemical solution is > 0.08 after subtraction of the OD for isopropanol or water, which corresponds to approximately 5% of the mean OD of the negative control), the test chemical is presumed to interfere with the standard absorbance (OD) measurement of MTT formazan and further colorant controls should be performed or, alternatively, an HPLC/UPLC-spectrophotometry procedure should be used in which case these controls are not required (see paragraphs 40 and 41 and Annex II). When performing the standard absorbance (OD) measurement, each interfering test chemical should be applied on at least two viable tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step, to generate a non-specific colour in living tissues (NSC_{living}) control (28). The NSC_{living} control needs to be performed concurrently to the testing of the coloured test chemical and, in case of multiple testing, an independent NSC_{living} control needs to be conducted with each test performed (in each run) due to the inherent biological variability of living tissues. True tissue viability is calculated as: the percent tissue viability obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution ($\%Viability_{test}$) minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT, run concurrently to the test being corrected ($\%NSC_{living}$), i.e., True tissue viability = [$\%Viability_{test}$] - [$\%NSC_{living}$].

37. To identify direct MTT reducers, each test chemical should be added to freshly prepared MTT solution. In the VRM, 50 µL or 50 mg of test chemical are added to 1 mL of 1 mg/mL MTT solution and the mixture is incubated for approximately 3 hours at standard culture conditions (28). 50 µL of sterile deionized water in MTT solution is used as negative control. If the MTT mixture containing the test chemical (or suspension for insoluble test chemicals) turns blue/purple, the test chemical is presumed to directly reduce MTT and a further functional check on non-viable RhCE tissue constructs should be performed, independently of using the standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure (see Annex II). This additional functional check employs killed tissues that possess only residual metabolic activity but absorb and retain the test chemical in a similar way as viable tissues. In the VRM, killed tissues are prepared by exposure to low temperature ("freeze-killed"). Each MTT reducing test chemical is applied on at least two killed tissue replicates, which undergo the entire testing procedure, to generate a non-specific MTT reduction (NSMTT) control (28). A single NSMTT control is sufficient per test chemical regardless of the number of independent tests/runs performed. True tissue viability is calculated as: the percent tissue viability obtained with living tissues exposed to the MTT reducer ($\%Viability_{test}$) minus the percent non-specific MTT reduction obtained with the killed tissues exposed to the same MTT reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT), i.e., True tissue viability = [$\%Viability_{test}$] - [%NSMTT].

38. Test chemicals that are identified as producing both colour interference (see paragraph 36) and direct MTT reduction (see paragraph 37) will also require a third set of controls when performing the standard absorbance (OD) measurement, apart from the NSMTT and NSC_{living} controls described in the previous paragraphs. This is usually the case with darkly coloured test chemicals absorbing light in the range of 570±30 nm (e.g., blue, purple, black) because their intrinsic colour impedes the assessment of their

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capacity to directly reduce MTT as described in paragraph 37. This forces the use of NSMTT controls, by default, together with the NSC_{living} controls. Test chemicals for which both NSMTT and NSC_{living} controls are performed may be absorbed and retained by both living and killed tissues. Therefore, in this case, the NSMTT control may not only correct for potential direct MTT reduction by the test chemical, but also for colour interference arising from the absorption and retention of the test chemical by killed tissues. This could lead to double correction for colour interference since the NSC_{living} control already corrects for colour interference arising from the absorption and retention of the test chemical by living tissues. To avoid a possible double correction for colour interference, a third control for non-specific colour in killed tissues (NSC_{killed}) needs to be performed (see Annex II). In this additional control, the test chemical is applied on at least two killed tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step. A single NSC_{killed} control is sufficient per test chemical regardless of the number of independent tests/runs performed, but should be performed concurrently to the NSMTT control and with the same tissue batch. True tissue viability is calculated as: the percent tissue viability obtained with living tissues exposed to the test chemical ($\%Viability_{test}$) minus %NSMTT minus $\%NSC_{living}$ plus the percent non-specific colour obtained with killed tissues exposed to the interfering test chemical and incubated with medium without MTT, calculated relative to the negative control ran concurrently to the test being corrected ($\%NSC_{killed}$), i.e., True tissue viability = $[\%Viability_{test}]$ - [%NSMTT] - $[\%NSC_{living}]$ + $[\%NSC_{killed}]$.

39. It is important to note that non-specific MTT reduction and non-specific colour interferences may increase the OD (when performing standard absorbance measurements) of the tissue extract above the linearity range of the spectrophotometer and that non-specific MTT reduction can also increase the MTT formazan peak area (when performing HPLC/UPLC-spectrophotometry measurements) of the tissue extract above the linearity range of the spectrophotometer. On this basis, it is important for each laboratory to determine the OD/peak area linearity range of their spectrophotometer with e.g., MTT formazan (CAS # 57360-69-7), commercially available from e.g., Sigma-Aldrich (Cat# M2003), before initiating the testing of test chemicals for regulatory purposes.

40. The standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess direct MTT-reducers and colour interfering test chemicals, when the observed interference with the measurement of MTT formazan is not too strong (i.e., the ODs of the tissue extracts obtained with the test chemical without any correction for direct MTT reduction and/or colour interference are within the linear range of the spectrophotometer). Nevertheless, results for test chemicals producing %NSMTT and/or $\%NSC_{living} \geq 60\%$ of the negative control should be taken with caution as this is the cut-off used in EpiOcular™ EIT to distinguish classified from not classified chemicals (see paragraph 43). Standard absorbance (OD) can however not be measured when the interference with the measurement of MTT formazan is too strong (i.e., leading to uncorrected ODs of the test tissue extracts falling outside of the linear range of the spectrophotometer). Coloured test chemicals or test chemicals that become coloured in contact with water or isopropanol that interfere too strongly with the standard absorbance (OD) measurement of MTT formazan may still be assessed using HPLC/UPLC-spectrophotometry (see Annex II). This is because the HPLC/UPLC system allows for the separation of the MTT formazan from the chemical before its quantification (29). For this reason, NSC_{living} or NSC_{killed} controls are never required when using HPLC/UPLC-spectrophotometry, independently of the chemical being tested. NSMTT controls should nevertheless be used if the test chemical is suspected to directly reduce MTT (following the procedure described in paragraph 37). NSMTT controls should also be used with test chemicals having a colour (intrinsic or appearing when in water) that impedes the assessment of their capacity to directly reduce MTT as described in paragraph 37. When using HPLC/UPLC-spectrophotometry to measure MTT formazan, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. For test chemicals able to directly reduce MTT, true tissue viability is calculated as: $\%Viability_{test}$ minus %NSMTT, as described in the last sentence of paragraph 37. Finally, it should be noted that direct MTT-reducers or direct MTT-reducers that are also colour interfering, which are retained

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in the tissues after treatment and reduce MTT so strongly that they lead to ODs (using standard OD measurement) or peak areas (using UPLC/HPLC-spectrophotometry) of the tested tissue extracts that fall outside of the linearity range of the spectrophotometer cannot be assessed with EpiOcular™ EIT, although these are expected to occur in only very rare situations.

41. HPLC/UPLC-spectrophotometry may be used with all types of test chemicals (coloured, non-coloured, MTT-reducers and non-MTT reducers) for measurement of MTT formazan (29). Due to the diversity of HPLC/UPLC-spectrophotometry systems, it is not feasible for each user to establish the exact same system conditions. As such, qualification of the HPLC/UPLC-spectrophotometry system should be demonstrated before its use to quantify MTT formazan from tissue extracts by meeting the acceptance criteria for a set of standard qualification parameters based on those described in the U.S. Food and Drug Administration guidance for industry on bioanalytical method validation (29)(31). These key parameters and their acceptance criteria are shown in Annex III. Once the acceptance criteria defined in Annex III have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure MTT formazan under the experimental conditions described in this Test Guideline.

Acceptance Criteria

42. For each run using EpiOcular™ tissue batches that met the quality control (see paragraph 29), tissues treated with the negative control substance should exhibit OD reflecting the quality of the tissues that followed shipment, receipt steps and all protocol processes and should not be outside the historically established boundaries described in Table 2 (see paragraph 25). Similarly, tissues treated with the positive control substance, i.e., methyl acetate, should show a mean tissue viability < 50% (relative to the negative control) with either the liquids' or the solids' protocols, thus reflecting the ability of the tissues to respond to an irritant test chemical under the conditions of the test method (28). The variability between tissue replicates of test chemicals and control substances should fall within the accepted limits (i.e., the difference of viability between two tissue replicates should be less than 20% or the SD between three tissue replicates should not exceed 18%). If either the negative control or positive control included in a run is outside of the accepted ranges, the run is considered "non-qualified" and should be repeated. If the variability between tissue replicates of a test chemical is outside of the accepted range, the test must be considered "non-qualified" and the test chemical should be re-tested.

Interpretation of Results and Prediction Model

43. The OD values/peak areas obtained with the replicate tissue extracts for each test chemical should be used to calculate the mean percent tissue viability (mean between tissue replicates) normalised to the negative control, which is set at 100%. The percentage tissue viability cut-off value distinguishing classified from non-classified test chemicals is 60%. Results should thus be interpreted as follows:

- The test chemical is identified as not requiring classification and labelling according to UN GHS (No Category) if the mean percent tissue viability after exposure and post-exposure incubation is more than (>) 60%. In this case no further testing in other test methods is required.

- The test chemical is identified as potentially requiring classification and labelling according to UN GHS (Category 2 or Category 1) if the mean percent tissue viability after exposure and post-exposure incubation is less than or equal (≤) to 60%. When the final mean percent tissue viability is less than or equal (≤) to 60% further testing with other test methods will be required because EpiOcular™ EIT shows a certain number of false positive results (see paragraph 14) and cannot resolve between UN GHS Categories 1 and 2 (see paragraph 15).

44. A single test composed of at least two tissue replicates should be sufficient for a test chemical when the result is unequivocal. However, in cases of borderline results, such as non-concordant replicate

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measurements and/or mean percent tissue viability equal to 60±5%, a second test should be considered, as well as a third one in case of discordant results between the first two tests.

45. Different percentage tissue viability cut-off values distinguishing classified from non-classified test chemicals may be considered for specific types of mixtures, where appropriate and justifiable, in order to increase the overall performance of the test method for those types of mixtures (see paragraph 14). Benchmark chemicals may be useful for evaluating the serious eye damage/eye irritation potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative ocular toxicity potential of a classified chemical within a specific range of positive responses.

DATA AND REPORTING

Data

46. Data from individual replicate tissues in a run (e.g., OD values/MTT formazan peak areas and calculated percent tissue viability data for the test chemical and controls, and the final EpiOcular™ EIT prediction) should be reported in tabular form for each test chemical, including data from repeat tests, as appropriate. In addition mean percent tissue viability and Diff (if n=2 replicate tissues) or SD (if n≥3 replicate tissues) for each individual test chemical and control should be reported. Any observed interferences of a test chemical with the measurement of MTT formazan through direct MTT reduction and/or coloured interference should be reported for each tested chemical.

Test Report

47. The test report should include the following information:

Test Chemical

- Mono-constituent substance
  - Chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers;
  - Physical state, volatility, pH, LogP, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
  - Treatment prior to testing, if applicable (e.g., warming, grinding);
  - Storage conditions and stability to the extent available.
- Multi-constituent substance, UVCB and mixture
  - Characterisation as far as possible by e.g., chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
  - Physical state, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
  - Treatment prior to testing, if applicable (e.g., warming, grinding);
  - Storage conditions and stability to the extent available.

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Positive and Negative Control Substances

- Chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers;
- Physical state, volatility, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available;
- Purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Treatment prior to testing, if applicable (e.g., warming, grinding);
- Storage conditions and stability to the extent available;
- Justification for the use of a different negative control than ultrapure H_2O, if applicable;
- Justification for the use of a different positive control than neat methyl acetate, if applicable;
- Reference to historical positive and negative control results demonstrating suitable run acceptance criteria.

Information Concerning the Sponsor and the Test Facility

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

RhCE Tisue Costruct and Protocol Used (providing rationale for the choices, if applicable)

Test Method Conditions

- RhCE tissue construct used, including batch number;
- Wavelength and band pass (if applicable) used for quantifying MTT formazan, and linearity range of measuring device (e.g., spectrophotometer);
- Description of the method used to quantify MTT formazan;
- Description of the qualification of the HPLC/UPLC-spectrophotometry system, if applicable;
- Complete supporting information for the specific RhCE tissue construct used including its performance. This should include, but is not limited to:
  - i) Viability;
  - ii) Barrier function;
  - iii) Morphology, if available;
  - iv) Reproducibility and predictive capacity;
  - v) Quality controls (QC) of the RhCE tissue construct;
- Reference to historical data of the RhCE tissue construct. This should include, but is not limited to:
  - i) Acceptability of the QC data with reference to historical batch data;
- Demonstration of proficiency in performing the test method before routine use by testing of the proficiency chemicals;

Run and Test Acceptance Criteria

- Positive and negative control means and acceptance ranges based on historical data;

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- Acceptable variability between tissue replicates for positive and negative controls;
- Acceptable variability between tissue replicates for the test chemical;

Test Procedure

- Details of the test procedure used;
- Doses of test chemical and control substances used;
- Duration and temperature of exposure, post-exposure immersion and post-exposure incubation periods (where applicable);
- Description of any modifications to the test procedure;
- Indication of controls used for direct MTT-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (positive control, negative control, NSMTT, NSC_{living} and NSC_{killed}, if applicable);
- Description of evaluation criteria used including the justification for the selection of the cut-off point for the prediction model;

Results

- Tabulation of data from individual test chemicals and control substances for each run (including repeat experiments where applicable) and each replicate measurement, including OD or MTT formazan peak area, percent tissue viability, mean percent tissue viability, Difference between tissue replicates or SD, and final prediction;
- If applicable, results of controls used for direct MTT-reducers and/or coloured test chemicals, including OD or MTT formazan peak area, %NSMTT, $\%NSC_{living}$, $\%NSC_{killed}$, Difference between tissue replicates or SD, final correct percent tissue viability, and final prediction;
- Results obtained with the test chemical(s) and control substances in relation to the define run and test acceptance criteria;
- Description of other effects observed, e.g., coloration of the tissues by a coloured test chemical;

Discussion of the Results

Conclusion

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LITERATURE

1) UN. (2013). United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS). ST/SG/AC.10/30, Fifth Revised Edition, New York and Geneva: United Nations. Available at: [http://www.unece.org/fileadmin/DAM/trans/danger/publi/ghs/ghs_rev05/English/ST-SG-AC10-30-Rev5e.pdf].

2) OECD. (2012). Acute Eye Irritation/Corrosion Guideline for Testing of Chemicals No. 405. Organisation for Economic Cooperation and Development, Paris.

3) OECD. (2013 Guidelines for Testing of Chemicals (No.437.): Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage. Organisation for Economic Cooperation and Development, Paris.

4) OECD. (2013).Guideline for Testing of Chemicals (No. 438): Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification. Organisation for Economic Cooperation and Development, Paris.

5) OECD (2012). Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants. OECD Guideline for Testing of Chemicals No. 460. Organisation for Economic Co-operation and Development, Paris.

6) Freeman, S.J., Alépée N., Barroso, J., Cole, T., Compagnoni, A., Rubingh, C., Eskes, C., Lammers, J., McNamee, P., Pfannenbecker, U., et Zuang, V. (2010). Prospective Validation Study of Reconstructed Human Tissue Models for Eye Irritation Testing. *ALTEX* **27**, Special Issue 2010, 261-266.

7) EC EURL ECVAM. (2014). Validation Study Report on the EURL ECVAM - Cosmetics Europe Prospective Validation Study of Reconstructed Human Corneal Epithelium-Based Test Methods for Identifying Chemicals not Requiring Classification and Labelling for Serious Eye Damage/Eye Irritation Testing.

8) EC EURL ECVAM. (2014). Eye Irritation *In Vitro* Assay Validation: Selection of Test Item Chemicals (EpiOcular™ Eye Irritation Test and SkinEthic™ Human Cornea Epithelium). Validation Management Group report.

9) TNO. (2014). Eye Irritation Validation Study on Human Tissue Models: Statistical Analysis and Reporting on the EpiOcular™ EIT. TNO Report TNO2013 R10396 Final, pp. 165.(Manuscript in Preparation).

10) EC EURL ECVAM. (2014). Eye Irritation Validation Study (EIVS): Statistical Analysis of the Data Generated Under SOP ver 8.0 of EpiOcular™ EIT (Solid Test Substances, Laboratory Beiersdorf), pp. 21.

11) EC EURL-ECVAM. (2014). Recommendation on the Use of the EpiOcular™ Eye Irritation Test (EIT)

for Identifying Chemicals not Requiring Classification and Labelling for Serious Eye Damage/Eye Irritation According to UN GHS.

12) Draize J.H., Woodard G., and Calvery H.O. (1944). Methods for the Study of Irritation and Toxicity of Substances Applied Topically to the Skin and Mucous Membranes. Journal of *Pharmacol*. and Exp. Therapeutics 82, 377-390.

13) Scott, L., Eskes, C., Hoffmann, S., Adriaens, E., Alépée, N., Bufo, M., Clothier, R., Facchini, D., Faller, C., Guest, R., Harbell, J., Hartung, T., Kamp, H., Le Varlet, B., Meloni, M., McNamee, P., Osborne, R., Pape, W., Pfannenbecker, U., Prinsen, M., Seaman, C., Spielman, H., Stokes, W., Trouba, K., Van den Berghe, C., Van Goethem, F., Vassallo, M., Vinardell, P., et Zuang, V. (2010). A Proposed Eye Irritation Testing Strategy to Reduce and Replace *In Vivo* Studies Using Bottom-Up and Top-Down Approaches. *Toxicol. in Vitro* **24**, 1-9.

14) Mosmann T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol*. Methods 65, 55-63.

15) OECD. (2014). Performance Standards for the Assessment of Proposed Similar or Modified *In Vitro* Reconstructed Human Cornea-Like Epithelium (RhCE) Test Methods for Identifying Chemicals not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage, Based on the Validated Reference Method EpiOcular™ EIT described in TG 492. Series on Testing and Assessment No. 216, OECD, Paris. Organisation for Economic Cooperation and Development, Paris. (Manuscript in Preparation).

16) OECD (2005). Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Environmental, Health and Safety Publications, Series on Testing and Assessment (No. 34.). Organisation for Economic Cooperation and Development, Paris

17) Kaluzhny Y., Kandárová, H., Hayden, P., Kubilus, J., d'Argembeau-Thornton, L., et Klausner, M. (2011). Development of the EpiOcular™ Eye Irritation Test for Hazard Identification and Labelling of Eye Irritating Chemicals in Response to the Requirements of the EU Cosmetics Directive and REACH Legislation. *Altern. Lab. Anim.* **39**, 339-64.

18) Pfannenbecker, U., Bessou-Touya, S., Faller, C., Harbell, J., Jacob, T., Raabe, H., Tailhardat, M., Alépée, N., De Smedt, A., De Wever, B., Jones, P., Kaluzhny, Y., Le Varlet, B., McNamee, P., Marrec-Fairley, M., et Van Goethem, F. (2013). Cosmetics Europe multi-laboratory pre-validation of the EpiOcular™ reconstituted Human Tissue Test Method for the Prediction of Eye Irritation. *Toxicol. in Vitro* **27**, 619-626.

19) Kolle S.N., Moreno, M.C.R., Mayer, W., van Cott, A., van Ravenzwaay, B., et Landsiedel, R. (2015). The EpiOcular™ Eye Irritation Test is the Method of Choice for *In Vitro* Eye Irritation Testing of Agrochemical Formulations: Correlation Analysis of EpiOcular™ Eye Irritation Test and BCOP Test Data to UN GHS, US EPA and Brazil ANIVSA Classifications. ATLA 43, 1-18.

20) Adriaens, E., Barroso, J., Eskes, C., Hoffmann, S., McNamee, P., Alépée, N., Bessou-Touya, S., De Smedt, A., De Wever, B., Pfannenbecker, U., Tailhardat, M., et Zuang, V. (2014). Retrospective Analysis of the Draize Test for Serious Eye Damage/Eye Irritation: Importance of Understanding the *In Vivo* Endpoints Under UN GHS/EU CLP for the Development and Evaluation of *In Vitro* Test Methods. *Arch. Toxicol.* **88**, 701-23.

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21) Hackett R.B., and McDonald T.O. (1991). Eye Irritation. In *Advances in Modern Toxicology: Dermatoxicology* Marzulli F.N.and Maibach H.I. (Eds.), 4th Edition, pp. 749–815. Washington, DC, USA: Hemisphere Publishing Corporation.

22) Fox D.A., and Boyes W.K. (2008). Toxic Responses of the Ocular and Visual System. In Cassaret and Doull's Toxicology: The Basic Science of Poisons Klaassen C.D.(Ed.), 7th Edition, pp. 665–697. Withby, ON, Canada: McGraw-Hill Ryerson.

23) Jester J.V., Li, H.F., Petroll, W.M., Parker, R.D., Cavanagh, H.D., Carr, G.J., Smith, B., et Maurer, J.K. (1998). Area and Depth of Surfactant Induced Corneal Injury Correlates with Cell Death. *Invest. Ophthalmol. Vis. Sci.* **39**, 922–936.

24) Maurer J.K., Parker R.D., and Jester J.V. (2002). Extent of Corneal Injury as the Mechanistic Basis for Ocular Irritation: Key Findings and Recommendations for the Development of Alternative Assays. Reg. *Tox. Pharmacol.,* 36, 106-117.

25) Jester J.V., Li, L., Molai, A., et Maurer, J.K. (2001). Extent of Corneal Injury as a Mechanistic Basis for Alternative Eye Irritation Tests. *Toxicol. in Vitro* **15**, 115–130.

26) Jester J.V., Petroll, W.M., Bean, J., Parker, R.D., Carr, G.J., Cavanagh, H.D., et Maurer, J.K. (1998). Area and Depth of Surfactant-Induced Corneal Injury Predicts Extent of Subsequent Ocular Responses. *Invest. Ophthalmol. Vis. Sci.* **39**, 2610–2625.

27) Jester J.V. (2006). Extent of Corneal Injury as a Biomarker for Hazard Assessment and the Development of Alternative Models to the Draize Rabbit Eye Test. *Cutan. Ocul. Toxicol.,* 25, 41–54.

28) EpiOcular™ EIT SOP, Version 8. (March 05, 2013). EpiOcular™ EIT for the Prediction of Acute Ocular Irritation of Chemicals. Available at: [http://www.ecvam-dbalm.jrc.ec.europa.eu/].

29) Alépée, N., Barroso, J., De Smedt, A., De Wever, B., Hibatallah, J., Klaric, M., Mewes, K.R., Millet, M., Pfannenbecker, U., Tailhardat, M., Templier, M., and McNamee, P. (2015) Use of HPLC/UPLC-Spectrophotometry for Detection of Formazan in *In Vitro* Reconstructed Human Tissue (RhT)-Based Test Methods Employing the MTT-Reduction Assay to Expand their Applicability to Strongly Coloured Test Chemicals. Toxicol *In Vitro,* 29(4). Pp 741-61.

30) Kaluzhny, Y., Kandárová, H., Handa, Y., DeLuca, J., Truong, T., Hunter, A., Kearney, P., d'Argembeau-Thornton, L., et Klausner, M. (2014). EpiOcular™ Eye Irritation Test (EIT) for Hazard Identification and Labeling of Eye Irritating Chemicals: Protocol Optimization for Solid Materials and Extended Shipment Times. Altern Lab Anim, 3(2), pp.101-27.

31) US FDA (2001). Guidance for Industry: Bioanalytical Method Validation. U.S. Department of Health and Human Services, Food and Drug Administration. May 2001. Available at: [http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf].

32) OECD (2015). Short Time Exposure *In Vitro* Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage. OECD Guideline for Testing of Chemicals No. 491. Organisation for Economic Co-operation and Development, Paris

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ANNEX I

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 (16).

Benchmark chemical: A chemical used as a standard for comparison to a test chemical. A benchmark chemical should have the following properties: (i) consistent and reliable source(s); (ii) structural, functional and/or chemical or product class similarity to the chemical(s) being tested; (iii) known physicochemical 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 test chemical suspected of not requiring classification and labelling for eye irritation or serious eye damage, which starts with the determination of chemicals not requiring classification and labelling (negative outcome) from other chemicals (positive outcome).

Chemical: A substance or mixture.

Concordance: See "Accuracy".

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

CV: Coefficient of Variation.

Dev: Deviation.

EIT: Eye Irritation Test.

EURL ECVAM: European Union Reference Laboratory for Alternatives to Animal Testing.

Eye irritation: Production of changes in the eye following the application of a test substance 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" (1).

ET_{50}: Exposure time required to reduce tissue viability by 50% upon application of a benchmark chemical at a specified, fixed concentration.

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.

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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.

HPLC: High Performance Liquid Chromatography.

Infinite dose: Amount of test chemical applied to the RhCE tissue construct exceeding the amount required to completely and uniformly cover the epithelial surface.

Irreversible effects on the eye: See “Serious eye damage”.

LLOQ: Lower Limit of Quantification.

LogP: Logarithm of the octanol-water partitioning coefficient

ME: Matrix Effect.

Mixture: A mixture or a solution composed of two or more substances in which they do not react (1).

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration ≥ 10% (w/w) and < 80% (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide.

Negative control: A sample containing all components of a test system and treated with a substance known not to induce a positive response in the test system. This sample is processed with test chemical-treated samples and other control samples and is used to determine 100% tissue viability.

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”.

NSC_{killed}: Non-Specific Colour in killed tissues.

NSC_{living}: Non-Specific Colour in living tissues.

NSMTT: Non-Specific MTT reduction.

OD: Optical Density.

Performance standards: Standards, based on a validated test method which was considered scientifically valid, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are: (i) essential test method components; (ii) a minimum list of Reference Chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the comparable levels of accuracy and

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reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals (16).

Positive control: A sample containing all components of a test system and treated with a substance known to induce a positive response in the test system. This sample is processed with test chemical-treated samples and other control samples. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (16).

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 (16).

Replacement test: A test which is designed to substitute for a test that is in routine use and accepted for hazard identification and/or risk assessment, and which has been determined to provide equivalent or improved protection of human or animal health or the environment, as applicable, compared to the accepted test, for all possible testing situations and chemicals (16).

Reproducibility: The agreement among results obtained from repeated testing of the same test chemical using the same test protocol (See "Reliability") (16).

Reversible effects on the eye: See “Eye irritation”.

RhCE: Reconstructed human Cornea-like Epithelium.

Run: A run consists of one or more test chemicals tested concurrently with a negative control and with a positive control.

SD: Standard Deviation.

Sensitivity: The proportion of all positive/active test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (16).

Serious eye damage: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance 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” (1).

Standard Operating Procedures (SOP): Formal, written procedures that describe in detail how specific routine, and test-specific, laboratory operations should be performed. They are required by GLP.

Specificity: The proportion of all negative/inactive test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (16).

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 (1).

Test: A single test chemical concurrently tested in a minimum of two tissue replicates as defined in the corresponding SOP.

Tissue viability: Parameter measuring total activity of a cell population in a reconstructed tissue as their ability to reduce the vital dye MTT, which, depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

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: The term "test chemical" is used to refer to what is being tested.

Tiered testing strategy: A stepwise testing strategy, which uses test methods in a sequential manner. All existing information on a test chemical is reviewed at each tier, using a weight-of-evidence process, to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier in the strategy. If the hazard potential/potency of a test chemical can be assigned based on the existing information at a given tier, no additional testing is required (16).

ULOQ: Upper Limit of Quantification.

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 standardised 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 (1).

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”.

UPLC: Ultra-High Performance Liquid Chromatography.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

Valid test method: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (16).

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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 (16).

VRM: Validated Reference Method.

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 substance.

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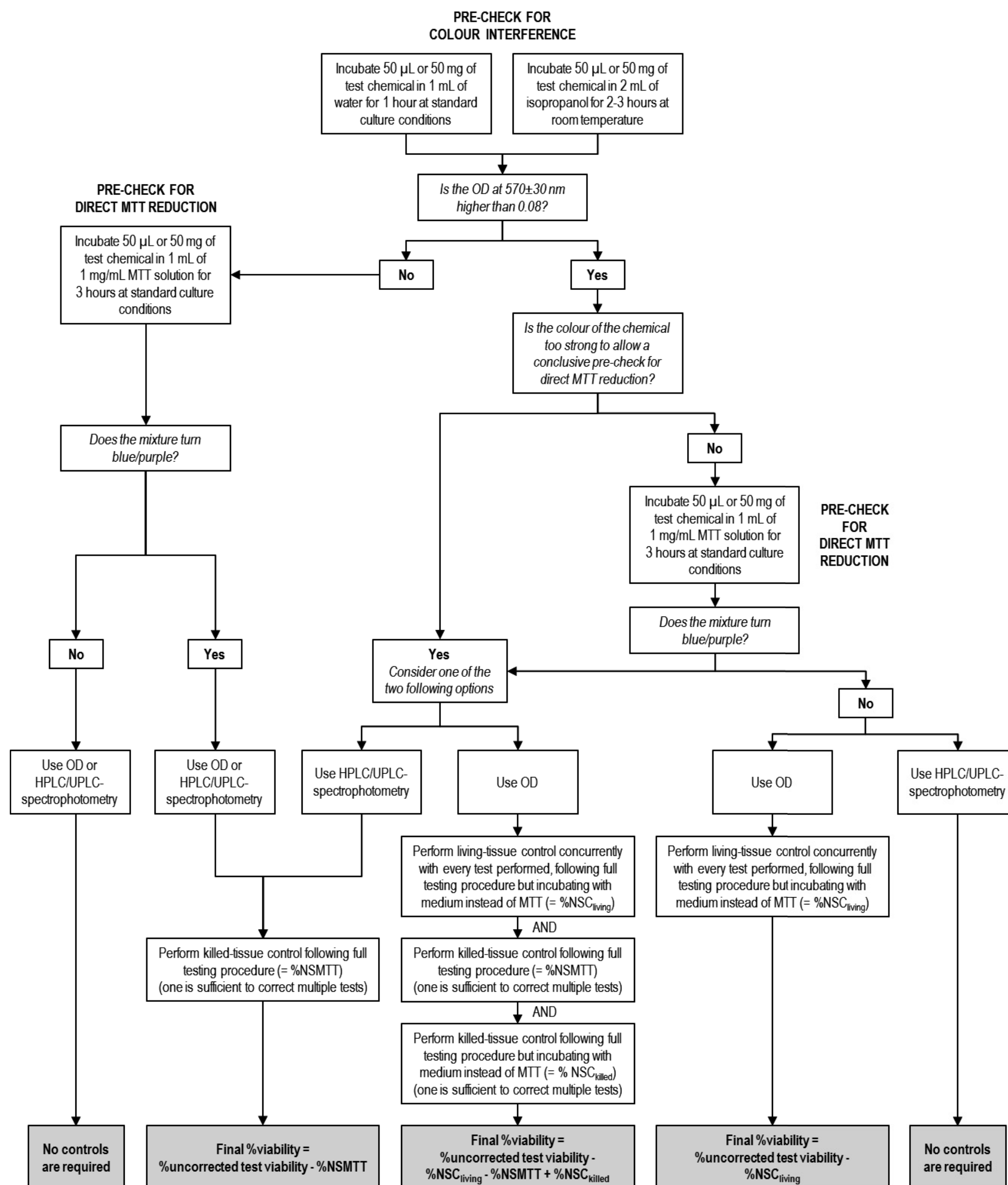
ANNEX II

ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT MTT-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE VRM SOP



ANNEX III

KEY PARAMETERS AND ACCEPTANCE CRITERIA FOR QUALIFICATION OF AN HPLC/UPLC-SPECTROPHOTOMETRY SYSTEM FOR MEASUREMENT OF MTT FORMAZAN EXTRACTED FROM RhCE TISSUE CONSTRUCTS

Parameter	Protocol Derived from FDA Guidance (29)(31)	Acceptance Criteria
Selectivity	Analysis of isopropanol, living blank (isopropanol extract from living RhCE tissue constructs without any treatment), dead blank (isopropanol extract from killed RhCE tissue constructs without any treatment), and of a dye (e.g., methylene blue)	$Area_{interference} \leq 20\%$ of $Area_{LLOQ}$[1]
Precision	Quality Controls (i.e., MTT formazan at 1.6 µg/mL, 16 µg/mL and 160 µg/mL) in isopropanol (n=5)	CV ≤ 15% or ≤ 20% for the LLOQ
Accuracy	Quality Controls in isopropanol (n=5)	%Dev ≤ 15% or ≤ 20% for LLOQ
Matrix Effect	Quality Controls in living blank (n=5)	85% ≤ %Matrix Effect ≤ 115%
Carryover	Analysis of isopropanol after an ULOQ[2] standard	$Area_{interference} \leq 20\%$ of $Area_{LLOQ}$
Reproducibility (intra-day)	3 independent calibration curves (based on 6 consecutive 1/3 dilutions of MTT formazan in isopropanol starting at ULOQ, i.e., 200 µg/mL); Quality Controls in isopropanol (n=5)	Calibration Curves: %Dev ≤ 15% or ≤ 20% for LLOQ Quality Controls: %Dev ≤ 15% and CV ≤ 15%
Reproducibility (inter-day)	Day 1: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 2: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 3: 1 calibration curve and Quality Controls in isopropanol (n=3)	
Short Term Stability of MTT Formazan in RhCE Tissue Extract	Quality Controls in living blank (n=3) analysed the day of the preparation and after 24 hours of storage at room temperature	%Dev ≤ 15%
Long Term Stability of MTT Formazan in RhCE Tissue Extract, if required	Quality Controls in living blank (n=3) analysed the day of the preparation and after several days of storage at -20°C	%Dev ≤ 15%

[1]LLOQ: Lower Limit of Quantification, defined to cover 1-2% tissue viability, i.e., 0.8 µg/mL.
[2]ULOQ: Upper Limit of Quantification, defined to be at least two times higher than the highest expected MTT formazan concentration in isopropanol extracts from negative controls (~70 µg/mL in the VRM), i.e., 200 µg/mL.

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