新規試験法提案書

再構築ヒト角膜様上皮モデル法（RhCE法）
SkinEthic™ HCE EIT

平成30年 3 月

国立医薬品食品衛生研究所

新規試験法提案書

平成 30 年 3 月 23 日
No.　2017-04

再構築ヒト角膜様上皮モデル法（RhCE法）
SkinEthic™ HCE EITに関する提案

平成 30 年 2 月 21 日に川崎、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）SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT) 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”と RhCE 法 SkinEthic™ HCE EIT バリデーション報告書等をもとに、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 Skin Ethic™ HCE/S を用いた眼刺激性試験評価報告書」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 RhCE 法 SkinEthic™ HCE EIT の使用を提案するものである。

JaCVAM 評価会議　議長

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

大 野 泰 雄　（公益財団法人　木原記念横浜生命科学振興財団）：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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仲 井 俊 司　（日本化学工業協会）
中村るりこ　（独立行政法人　製品評価技術基盤機構）
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JaCVAM 運営委員会

西川秋佳（国立医薬品食品衛生研究所　安全性生物試験研究センター）：委員長
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本間正充（国立医薬品食品衛生研究所　安全性生物試験研究センター　変異遺伝部）
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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 RhCE test method, SkinEthic™ HCE EIT

At a meeting held on 21 February 2018 at the National Institute of Health Sciences (NIHS) in Kawasaki, 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 Eye Irritation (RhCE) test method, SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT) 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, SkinEthic™ HCE EIT 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, SkinEthic™ HCE EIT as a useful means for safety assessment by regulatory agencies.

Chairperson
JaCVAM Regulatory Acceptance Board

Chairperson
JaCVAM Steering Committee

23 March 2018

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 (Kihara Memorial Yokohama Foundation for the Advancement of Life Sciences) : 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 (Biological Safety Research Center: 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)
Ms. Maki Noguchi (Pharmaceuticals and Medical Devices Agency)**
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 Cutaneous Immunology and Allergy)

Term: From 1st April 2016 to 31st March 2018
*: From 1st April 2016 to 31st March 2017
**: From 1st April 2017 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. Manabu Fuchioka (Ministry of Health, Labour and Welfare)
Ms. Yoko Hirabayashi (Division of Toxicology, BSRC, NIHS)
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)
Ms. Mitsue Hirota (Pharmaceutical & Medical Devices Agency)
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)
Mr. Yasunari Kanda (Division of Pharmacology, BSRC, NIHS)
Mr. Atsushi Kato (National Institute of Infectious Diseases)
Mr. Kouichirou Koike (Ministry of Health, Labour and Welfare)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Mr. Taku Oohara (Ministry of Health, Labour and Welfare)
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. Shinichi Watanabe (Ministry of Health, Labour and Welfare)
Mr. Hajime Kojima (Division of Risk Assessment, BSRC, NIHS): Secretary

再構築ヒト角膜様上皮モデル法（RhCE法）
SkinEthic™ HCE EIT

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評価会議報告書

再構築ヒト角膜様上皮モデル法（RhCE 法）
SkinEthic™ HCE/S を用いた眼刺激性試験（SkinEthic™ HCE EIT）

JaCVAM 評価会議

平成 30 年（2018 年）2 月 21 日

JaCVAM 評価会議

大 野 泰 雄 （公益財団法人　木原記念横浜生命科学振興財団）：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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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 眼刺激性試験の代替試験法として、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。OECD TG492 としてボトムアップ方式で United Nations Globally Harmonized System of Classification and Labelling of Chemicals（UN GHS）区分外物質を検出できる RhCE 法の一つとして、EpiOcular™ が採択されている。この度、SkinEthic™ ヒト角膜様上皮モデル（SkinEthic™ HCE/S）を用いた眼刺激性試験(SkinEthic™ HCE EIT)が RhCE 法の一つとして、改訂 TG 492[1)]に検証済み標準試験法として追加された。SkinEthic™ HCE/S を用いる RhCE 法は、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研究[2)]が行われ、プロトコルの最適化、3 施設バリデーション研究および ESAC（EURL ECVAM Scientific Advisory Committee）の第三者評価を経て、2017 年 10 月に改訂 TG492 に追記された。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(RhCE 法) Skin Ethic™ HCE/S を用いた眼刺激性試験(Skin Ethic™ HCE EIT) 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 SkinEthic™ ヒト角膜様上皮モデルを用いた眼刺激性試験）

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を用いた眼刺激性試験である。RhCE 法のひとつである SkinEthic™ HCE 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 4 時間被験物質を SkinEthic™ HCE/S に曝露した後、MTT の還元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）が生存細胞数に比例することに基づいている（MTT 還元法）。被験物質が液体の場合、細胞生存率が陰性対照と比較して 60%を超えると、また、被験物質が固体の場合は細胞生存率が 50%を超えると UN GHS 分類において区分外であると判定する。これらカットオフ値以下の場合は、本試験法では偽陽性が生じること、区分 1 と区分 2 を識別できないことから、他の試験法による追加試験が必要になる。

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、ヒトの角膜上皮に類似した構造を有する RhCE（SkinEthic™ HCE/S）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。

SkinEthic™ HCE EIT については、EURL ECVAM と CE の共同バリデーション研究[2)]が行われた。その後プロトコルの最適化を行って、UN GHS 区分物質・区分外物質や固体・液体のバランスを考慮して 120 物質について 3 施設でバリデーション研究が行われた。被験物質には、MTT 還元物質やホルマザンと同じような波長をもつ着色物質も含められた。また、バリデーション研究とは別に、リードラボにおいて 80 物質について追加試験が行われた[4,5)]。

SkinEthic™ HCE EIT は、施設内再現性、施設間再現性および試験法の正確性についても EURL ECVAM バリデーション運営委員会の定めた基準を満たした。さらに ESAC の第三者評価[6)]を経て、UN GHS 区分外物質を検出する方法として 2017 年 10 月に改訂 TG492 に追記された。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、科学的に妥当な評価であると考える。

３．本試験法の有用性と適用限界

RhCE 法に用いる SkinEthic™ HCE/S は市販されており、これ以外は特殊な機材や試薬を必要とせず、手技も複雑ではないことから技術移転性は高いと判断できる。但し、入手した SkinEthic™ HCE/S 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る必要がある。

UN GHS 区分外物質を検出する方法としての信頼性を調べるため、施設内再現性および施設間再現性を検討するバリデーション研究が行われている。バリデーション研究において、各施設の施設内再現性は、液体用プロトコルで 88.3-95.0%、固体用プロトコルで 95.0-96.7%であり、EURL ECVAM 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[2)]。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、液体用プロトコルで 93.3%、固体用プロトコルで 96.7%、全体では 95.0%となり、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[3)]。さらに、バリデーション研究で得られたデータによる正確性の評価では、液体プロトコルで感度 98.3%、特異度 69.4%および正確度 84.8%、固体用プロトコルで感度 92.2%、特異度 76.6%および正確度 84.4%となりバリデーション運営委員会が定めた基準（感度 90%以上、特異度 60%以上、正確度 75%以上）を満たしていた[3)]。

表．SkinEthic^{TM} HCE EIT の正確性

	物質数	感度(%)	特異度(%)	正確度(%)
液体	60	98.3	69.4	84.8
固体	60	92.2	76.6	84.4
代替法としての受け入れ基準 [1)]	-	≥ 90%	≥60%	≥75%

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、改訂 TG492 の本文の説明および ANNEX IV の試験法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) バリデーション研究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 区分の区分 1 物質と区分 2（2A/2B）物質の識別には用いることはできない。

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、社会的受け入れ性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 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、SkinEthic^{TM} HCE/S 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 SkinEthic^{TM} HCE/S 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区分外物質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、RhCE法にEpiOcular™またはSkinEthic™ HCE/S以外を用いる場合には、OECD TG492の性能標準に記載された物質を用いて、その妥当性を確認しておく必要がある[1)]。

参考文献

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2) 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.

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4) Alépée N., et al. (2016) Multi-laboratory validation of SkinEthic HCE test method for testing serious eye damage/eye irritation using liquid chemicals. Toxicol In Vitro 31, 43-53.

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6) ESAC (2016) ESAC Opinion on the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT). ESAC Opinion No. 2016-02 of 24 June 2016.

評価報告書

再構築ヒト角膜様上皮モデル法（RhCE 法）
SkinEthic™ HCE/S を用いた眼刺激性試験（SkinEthic™ HCE EIT）

眼刺激性試験資料編纂委員会

平成 29 年（2017 年）12 月 4 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

EIT: Eye Irritation Test

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

HCE: Human Corneal Epithelium

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

SDS: sodium dodecyl sulphate

SOP: Standard Operating Procedures

TG: Test Guideline

UN: United Nations

VRM: Validated Reference Method

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り、OECD TG 492 として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採択されている。SkinEthic™ ヒト角膜上皮モデルを用いた眼刺激性試験（SkinEthic™ Human Corneal Eithelium Eye Irritation Test: SkinEthic™ HCE EIT）は RhCE 法の一つで、改訂 TG 492 に追加された検証済み標準試験法である。本報告では、SkinEthic™ HCE/S EIT のバリデーション研究論文、第三者評価報告書、関連論文などをもとに試験法の概要を説明し JaCVAM 眼刺激性代替法資料編纂委員会の意見をまとめた。

SkinEthic™ HCE EIT の信頼性・正確性を確認するため、液体物質および固体物質それぞれ 60 物質を用いた 3 施設によるバリデーション研究が行われた。各施設で 1 物質あたり 3 回の試験を行った。この 3 施設バリデーション研究における SkinEthic™ HCE/S EIT の正確性は、液体物質および固体物質において、それぞれ感度 98.3%および 92.2%、特異度 69.4%および 76.6%、正確度 84.8%および 84.4%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 85%以上であった。これらの正確性および再現性の値は、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、SkinEthic™ HCE/S EIT 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）、フルオレセイン漏出試験法（FL 法、TG 460）、in vitro 短時間曝露法（STE 法、TG 491）、そして、再構築ヒト角膜様上皮モデル法（RhCE 法、TG 492）の 5 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 UN GHS 区分 1（重篤な眼の傷害を起こす）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 UN GHS 区分外（眼に対する重篤な損傷性および眼刺激性を有する物質とは分類されない）物質を検出する方法として用いられる。FL 法は単層培養した尿細管上皮細胞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、トップダウン方式においてのみ用いられる。STE 法は単層培養した角膜細胞を用いて物質の曝露による細胞毒性（細胞生存率）を指標に眼刺激性を評価する方法で、トップダウン方式およびボトムアップ方式において用いられる。

RhCE 法は、再構築ヒト角膜様上皮モデル（RhCE）組織に対する被験物質の細胞毒性を指標とし、ボトムアップ方式により UN GHS 区分外物質を検出することでその物質の眼刺激性を評価する試験法である。RhCE 法である EpiOcular™ 眼刺激性試験（EpiOcular™ Eye Irritation Test: EpiOcular™ EIT）および SkinEthic™ ヒト角膜上皮モデル眼刺激性試験（SkinEthic™ HCE EIT）の 2 試験法について EURL ECVAM と CE の共同バリデーション研究が行われた。EpiOcular™ EIT は、プロトコルの最適化、追加試験、ESAC の第三者評価を経て、RhCE 法の検証済み標準試験法（Validated Reference Method: VRM）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）。その後、SkinEthic™ HCE EIT もプロトコルの最適化、3 施設バリデーション研究および ESAC の第三者評価を経て、2 つ目の VRM（VMR2）として 2017 年 10 月に改訂 TG 492 に追加された。

本報告書は改訂 TG 492 の VRM2 である SkinEthic™ HCE EIT のバリデーション研究論文、第三者評価報告書、その他関連論文などをもとに本試験法の概要を説明し、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。

改訂 TG 492 で追加された VRM は、市販のヒト不死化角膜上皮細胞由来の RhCE 組織である SkinEthic™ HCE/S を用いた眼刺激性試験（SkinEthic™ HCE EIT）である。被験物質が液体の場合は約 30 分間、固体の場合は約 4 時間、SkinEthic™ HCE/S に曝露した後、MTT 試験より算出した細胞生存率をエンドポイントに用いる。これは、細胞内に取り込まれた MTT の還元により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。被験物質が液体の場合、細胞生存率が陰性対照と比較して 60%を超えると、被験物質は UN GHS 分類において区分外であると判定する。被験物質が固体の場合は細胞生存率が 50%を超えると UN GHS 分類において区分外であると判定する。

4. 試験手順

SkinEthic™ HCE EIT の手順を以下に示す。詳細は、改訂 TG 492 を参照する。SkinEthic™ HCE/S は EPISKIN 社（フランス）より購入できる。

4-1. RhCE 組織の機能的条件

RhCE 組織の機能的条件は、以下のとおりである。

生存率：陰性対照において $1.0 < OD \leq 2.5$
（使用時の基準であり、製造者の出荷基準は異なる場合もある）
バリア機能：SDS の 30 分間曝露において $1.0 \leq IC_{50}\ (mg/mL) \leq 3.2$
（IC_{50}：細胞生存率を 50%低下させるのに必要な濃度）
形態：少なくとも 3 層の上皮細胞があり、その表面は角化していない
再現性：陽性および陰性対照の結果を背景データをもとに設定した許容値内にある

これらは SkinEthic™ HCE/S の製造者の出荷基準として採用される。一方、SkinEthic™ HCE/S の使用者は生存率と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 RhCE 組織を用いる。被験物質は評価対象物質そのものを用いる。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。液体被験物質の場合、SkinEthic™ HCE/S に 30±2 µL を適用し、標準培養条件[1]で 30±2 分間培養する。その後、リン酸緩衝生理食塩水 20 mL で洗浄して被験物質を除去し、30±2 分間標準培養条件で培地に浸漬する。固体被験物質の場合、30±2 mg を均一に適用し、標準培養条件で 4±0.1 時間培養する。その後、リン酸緩衝生理食塩水 25 mL で洗浄して被験物質を除去し、30±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±0.5 時間培養する。

同時陽性対照には酢酸メチル（CAS No. 79-20-9）、同時陰性対照にはリン酸緩衝生理食塩水が推奨される。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±15

[1] 37±2°C、5±1% CO_2、≥95% 湿度

分間標準培養条件で反応させ、その後 1.5 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無色透明でない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、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、洗浄が困難な場合には、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様に行う。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、細胞生存率の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 IV のフローチャートを参照する。

4-4. 試験成立の承認基準

以下の条件をすべて満たした場合、試験の成立を承認する。

1）陰性対照の平均 OD が $1.0 < OD \leq 2.5$ であること。

2）陽性対照の平均細胞生存率が液体被験物質では 30%以下、固体被験物質では 20%以下であること。

3）被験物質および陰性・陽性対照のそれぞれにおいて 2 RhCE 組織の細胞生存率の差が 20%未満であること。3 RhCE 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。なお、複数の被験物質を同時に試験した場合は、この条件を満たさない被験物質のみ不成立とする。

4-5. 刺激性の判定

液体被験物質では平均細胞生存率が 60%を超えた場合、被験物質は GHS 区分外と判断される。固体被験物質では平均細胞生存率が 50%を超えた場合、被験物質は GHS 区分外と判断される。これらのカットオフ値以下の場合は、本試験法では偽陽性が生じえるし、また区分 1 と区分 2 物質を識別できないことから、他の試験法による追加試験が必要となる。

平均細胞生存率がカットオフ値近辺の場合（±5%）は 2 回目の試験を実施する。1 回目と 2 回目の試験で結果が一致しない場合は 3 回目の試験を実施する。

5. バリデーション研究

開発者の L'Oréal 社をリードラボとして、さらに 2 施設（Charles River Laboratories、Flemish Institute for Technological Research）を加え、合計 3 施設で SkinEthic™ HCE EIT のバリデーション研究が行われた。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）。MTT 還元物質やホルマザンと同じような波長に吸収を持つ着色物質も含められた。

コード化された 120 物質すべてを 3 施設で試験した。試験はすべての被験物質について 3 回実施された。

また、この 3 施設バリデーション研究とは別に、リードラボにおいて、80 物質について追加試験が行われた（Appendix 1）。試験はすべての被験物質について 3 回実施された。

6. 試験法の信頼性

6-1. 技術移転性

バリデーション研究を開始する前に、リードラボにより、参加 2 施設の技術者に対して SkinEthic™ HCE EIT の液体・固体用プロトールを用いてトレーニングが行われた。その後、液体物質および固体物質それぞれ 9 物質（コード化）を用いて技術移転の確認を行った。1 物質あたり 3 回試験を実施したところ、良好な結果が得られた。

なお、RhCE 法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改訂 TG 492 に提示されている（Appendix 2）。

6-2. 施設内再現性

バリデーション研究では施設ごとに 1 被験物質につき複数回の試験を行っている。GHS 分類判定の施設ごとの施設内再現性は、液体用プロトコルでは 88.3 - 95.0%、固体用プロトコルでは 95.0 - 96.7%であった。リードラボで行われた追加試験での施設内再現性は液体用プロトコルでは 91.1%、固体用プロトコルでは 97.1%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（85%以上）を満たした。

6-3. 施設間再現性

施設ごとに複数回の試験の細胞生存率を平均して GHS 分類判定を行い、施設間再現性を検討した。その結果、3 施設間の再現性は液体用プロトコルでは 93.3%、固体用プロトコルでは 96.7%であり、全体では 95.0%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。

7. 試験法の正確性

正確性の評価には、バリデーション研究として 3 施設で行われた全試験のデータを用いた。結果は表 1 の通りである。

表 1. SkinEthic™ HCE EIT の正確性（3 施設バリデーション）

評価項目 （受け入れ基準）	液体	固体
感度（≥90%）	98.3%	92.2%
特異度（≥60%）	69.4%	76.6%
正確度（≥75%）	84.8%	84.4%

SkinEthic™ HCE EIT の正確性はバリデーション運営委員会が定めた基準（感度 90%以上、特異度 60%以上、正確度 75%以上）を満たした。

なお、バリデーション研究においてリードラボで得られたデータとリードラボにおいて実施された追加試験の 80 物質（45 液体物質、35 固体物質）のデータを合わせてリードラボにおける正確性を評価した場合、液体物質では感度 100%、特異度 65.3%、正確度 83.5%、固体物質では感度 89.7%、特異度 73.6%、正確度 80.7%であった。バリデーション研究の正確性とほぼ同様な結果が得られた。

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) バリデーション研究において被験物質に含まれなかった気体（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

SkinEthic™ HCE EIT の 3 施設によるバリデーション研究の結果、本試験法は再現性および正確性についてバリデーション運営委員会の定めた基準を満たしていた。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。

SkinEthic™ HCE EIT に用いる RhCE 組織は市販されており、また、RhCE 組織以外は、特殊な機材や試薬を必要とせず、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として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、SkinEthic™ HCE EIT 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

1) Alépée N et al (2016) Multi-laboratory validation of SkinEthic HCE test method for testing serious eye damage/eye irritation using liquid chemicals. Toxicol In Vitro 31, 43-53.

2) Alépée N et al (2016) Multi-laboratory evaluation of SkinEthic HCE test method for testing serious eye damage/eye irritation using solid chemicals and overall performance of the test method with regard to solid and liquid chemicals testing. Toxicol In Vitro 34, 55-70.

3) ESAC (2016) ESAC Opinion on the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT). ESAC Opinion No. 2016-02 of 24 June 2016.

4) OECD (2017) 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.

Appendix 1

バリデーションに用いられた物質

	3 施設 バリデーション	リードラボ 追加データ
全体	120	80
眼刺激性 GHS 分類		
区分 1	32	19
区分 2A	17	12
区分 2B	13	4
区分外	58	45
物質の特性		
液体	60	45
固体	60	35
Functional Group Class		
Aromatic	21	20
Alcohol	19	17
Ester	17	12
Heterocyclic	13	12
Halogenated	9	14
Carboxylic acid	11	7
Amine	3	10
Phenol	12	1
Acrylate	6	2
Ether	0	8
Salt	6	2
Polyether	6	1
Nitrile	2	4
Onium Compound	0	6
Silicium	4	2
Ketone	3	2
Pyrimidine	1	4
Thioether	4	1

注）バリデーション研究論文（Alépée et al., 2016a, 2016b）をもとに作成。Functional Group Class は物質数の多いものを抽出。物質によっては複数の Functional Group 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
Hydroxyethyl acrylate	818-61-1	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-diimidamide, N,N''-bis(4-chlorophenyl)- 3,12-diimino-,di-D-gluconate (20%, aqueous)	18472-51-0	液体	区分 2A
Sodium benzoate	532-32-1	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
1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-7	液体	区分外
Dicaprylyl ether	629-82-3	液体	区分外
Piperonyl butoxide	51-03-6	液体	区分外
Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	粘性物	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
Potassium tetrafluoroborate	14075-53-7	固体	区分外

OECD/OCDE 492

Adopted:
9 October 2017

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, 2012 and 2017) (2). The choice of the most appropriate test method and the use of this Test Guideline should be seen in the context of the OECD Guidance Document on an Integrated Approaches on Testing and Assessment for Serious Eye Damage and Eye irritation (39).

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 (6) to address the human health endpoint serious eye damage/eye irritation.

This Guideline was adopted by the OECD Council by written procedure on 9 October 2017 [C(2017)97].

4. Two validated test methods using commercially available RhCE models are included in this Test Guideline. Validation studies for assessing eye irritation/serious eye damage have been conducted (7)(8)(9)(10)(11)(12)(13) using the EpiOcular™ Eye Irritation Test (EIT) and the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT). Each of these methods makes use of commercially available RhCE tissue constructs as test system, which are referred to in the following text as the Validated Reference Methods – VRM 1 and VRM2, respectively. From these validation studies and their independent peer review (9)(12) it was concluded that the EpiOcular™ EIT and SkinEthic™ HCE EIT are 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 methods were recommended as scientifically valid for that purpose (13).

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)(14) 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 (15). The Bottom-Up approach (15) 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 (15) is designed to be used when, based on existing information, a chemical is expected to cause serious eye damage. The EpiOcular™ EIT and SkinEthic™ HCE EIT are 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 (15). However, the EpiOcular™ EIT and SkinEthic™ HCE EIT are 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 (15). A test chemical that is identified as requiring classification for eye irritation/serious eye damage with EpiOcular™ EIT or SkinEthic™ HCE EIT will thus require additional testing (*in vitro* and/or *in vivo*) to reach a definitive conclusion (UN GHS No Category, Category 2 or Category 1), using e.g., TG 437, 438, 460 or 491.

6. The purpose of this Test Guideline is to describe the procedure 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 (16) (see paragraph 21). 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 (17) are available to facilitate the validation of new or modified *in vitro* RhCE-based test methods similar to EpiOcular™ EIT and SkinEthic™ HCE EIT, in accordance with the principles of Guidance Document No. 34 (18), 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 commercial three-dimensional RhCE tissue constructs that are produced using either primary human epidermal keratinocytes (i.e., EpiOcular™ OCL-200) or human immortalized corneal epithelial cells (i.e., SkinEthic™ HCE/S). The EpiOcular™ OCL-200 and SkinEthic™ HCE/S RhCE tissue constructs are similar to the *in vivo* corneal epithelium three-dimensional structure and are produced using cells from the species of interest (19)(20). Moreover, the test methods directly measure cytotoxicity resulting from penetration of the chemical through the cornea and production of cell and tissue damage; the cytotoxic response then determines the overall *in vivo* serious eye damage/eye irritation outcome. Cell damage can occur by several modes of action (see paragraph 20), but cytotoxicity 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 (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 (8). The SkinEthic™ HCE EIT validation database contained 200 chemicals in total, covering 165 different organic functional groups (8)(10)(11). The majority of these chemicals represented mono-constituent substances, but several multi-constituent substances (including 10 polymers) were also included in the study. In terms of physical state and UN GHS Categories, the 200 tested chemicals were distributed as follows: 27 Category 1 liquids, 24 Category 1 solids, 19 Category 2A liquids, 10 Category 2A solids, 9 Category 2B liquids, 8 Category 2B solids, 50 No Category liquids and 53 No Category solids (10)(11).

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 36-42).

13. Results generated in pre-validation (21)(22) and full validation (8)(10)(11) studies have demonstrated that both EpiOcular™ EIT and SkinEthic™ HCE EIT are transferable to laboratories considered to be naïve in the conduct of the assays 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 from data on 113 chemicals is in the order of 95% within

laboratories and 93% between laboratories. The level of reproducibility in terms of concordance of predictions that can be expected from SkinEthic™ HCE EIT from data on 120 chemicals is in the order of 92% within laboratories and 95% 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 (8), 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)(14) 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 (23). 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)(14). 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 (23).

15. The SkinEthic™ HCE 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 (10)(11), the SkinEthic™ HCE EIT has an overall accuracy of 84% (based on 200 chemicals), sensitivity of 95% (based on 97 chemicals), false negative rate of 5% (based on 97 chemicals), specificity of 72% (based on 103 chemicals) and false positive rate of 28% (based on 103 chemicals), when compared to reference *in vivo* rabbit eye test data (OECD TG 405) (2)(14) classified according to the UN GHS classification system (1).

16. The false negative rates obtained with both RhCE test methods, with either substances or mixtures, fall within the 12% overall probability that chemicals are identified as either UN GHS Category 2 or UN GHS No Category by the *in vivo* Draize eye test, in repeated tests; this is due to the method's inherent within-test variability (24). The false positive rates obtained with both RhCE test methods with either substances or mixtures are not critical in the context of this Test Guideline since all test chemicals that produce a tissue viability equal or lower than the established cut-offs (see paragraph 44) will require further testing with other *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. These test methods can be used for all types of chemicals, whereby a negative result 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 and SkinEthic™ HCE EIT under classification schemes other than UN GHS.

17. 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 *in vitro* test methods is required.

18. 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

19. 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 epidermal keratinocytes or human immortalized corneal epithelial 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*. The SkinEthic™ HCE RhCE tissue construct consists of at least 4 viable layers of cells including columnar basal cells, transitional wing cells and superficial squamous cells similar to that of the normal human corneal epithelium (20)(26).

20. 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) (15)(27)(28). 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 (29)(30). Moreover, the serious eye damage/eye irritation potential of a chemical is principally determined by the extent of initial injury (31), which correlates with the extent of cell death (29) and with the extent of the subsequent responses and eventual outcomes (32). 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 (30)(33). The measurement of viability of the RhCE tissue construct after topical exposure to a test chemical to identify chemicals not requiring classification for serious eye damage/eye irritancy (UN GHS No Category) 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.

21. RhCE tissue viability is classically 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 (16). Chemicals not requiring classification and labelling according to UN GHS (No Category) are identified as those that do not decrease tissue viability below a defined threshold (i.e., tissue viability > 60%, in EpiOcular™ EIT and SkinEthic™ HCE EITL[2], or > 50%, in SkinEthic™ HCE EITS[3]) (see paragraph 44).

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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.

2 EITL: EIT for liquids in the case of SkinEthic™ HCE

3 EITS: EIT for solids in the case of SkinEthic™ HCE

DEMONSTRATION OF PROFICIENCY

22. Prior to routine use of RhCE test methods 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 validation studies of the VRMs (8)(10)(11). 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)(14) 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 (24)(25); (iv) are representative of the chemical classes used in the validation study (8)(10)(11); (v) cover a good and wide representation of organic functional groups (8)(10)(11); (vi) have chemical structures that are well-defined (8)(10)(11); (vii) are coloured and/or direct MTT reducers; (viii) produced reproducible results in RhCE test methods during their validations; (ix) were correctly predicted by RhCE test methods during their validation studies; (x) cover the full range of *in vitro* responses based on high quality RhCE test methods 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	VRM1 viability (%)[2]	VRM2 viability (%)[3]	VRM Prediction	MTT Reducer	Colour interf.
***In Vivo* Category 1[4]**								
Methylthioglycolate	2365-48-2	Carboxylic acid ester; Thioalcohol	L	10.9±6.4	5.5±7.4	No prediction can be made	Y (strong)	N
Hydroxyethyl acrylate	818-61-1	Acrylate; Alcohol	L	7.5±4.7[5]	1.6±1.0	No prediction can be made	N	N
2,5-Dimethyl-2,5-hexanediol	110-03-2	Alcohol	S	2.3±0.2	0.2±0.1	No prediction can be made	N	N
Sodium oxalate	62-76-0	Oxocarboxylic acid	S	29.0±1.2	5.3±4.1	No prediction can be made	N	N
***In Vivo* Category 2A[4]**								
2,4,11,13-Tetraazatetradecane-diimidamide, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate (20%, aqueous) [6]	18472-51-0	Aromatic heterocyclic halide; Aryl halide; Dihydroxyl group; Guanidine	L	4.0±1.1	1.3±0.6	No prediction can be made	N	Y (weak)
Sodium benzoate	532-32-1	Aryl; Carboxylic acid	S	3.5±2.6	0.6±0.1	No prediction can be made	N	N
***In Vivo* Category 2B[4]**								

Chemical Name	CASRN	Organic Functional Group[1]	Physical State	VRM1 viability (%)[2]	VRM2 viability (%)[3]	VRM Prediction	MTT Reducer	Colour interf.
Diethyl toluamide	134-62-3	Benzamide	L	15.6±6.3	2.8±0.9	No prediction can be made	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	15.8±1.1	No prediction can be made	N	N
***In Vivo* No Category[4]**								
1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	Alkoxy; Ammonium salt; Aryl; Imidazole; Sulphate	L	79.9±6.4	79.4±6.2	No Cat	N	N
Dicaprylyl ether	629-82-3	Alkoxy; Ether	L	97.8±4.3	95.2±3.0	No Cat	N	N
Piperonyl butoxide	51-03-6	Alkoxy; Benzodioxole; Benzyl; Ether	L	104.2±4.2	96.5±3.5	No Cat	N	N
Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	Acylal; Alcohol; Allyl; Ether	Viscous	77.6±5.4	89.1±2.9	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	101.9±6.6	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	97.7±5.6	No Cat	N	N
Potassium tetrafluoroborate	14075-53-7	Inorganic Salt	S	88.6±3.3	92.9±5.1	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); VRM1 = Validated Reference Method, EpiOcular™ EIT; VRM2 = Validated Reference Method, SkinEthic™ HCE 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 with EpiOcular™ EIT in the EURL ECVAM/Cosmetics Europe Eye Irritation Validation Study (EIVS) (8).

[3] Based on results obtained with SkinEthic™ HCE EIT in the validation study (10)(11).

[4]Based on results from the *in vivo* rabbit eye test (OECD TG 405) (2)(14) and using the UN GHS (1).
[5]Based on results obtained in the CEFIC CONsortium for *in vitro* Eye Irritation testing strategy (CON4EI) Study.
[6]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.

23. 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 25, 27 and 30). 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

24. The test methods currently covered by this Test Guideline are the scientifically valid EpiOcular™ EIT and SkinEthic™ HCE EIT (9)(12)(13), referred to as the Validated Reference Method (VRM1 and VRM2, respectively). The Standard Operating Procedures (SOP) for the RhCE test methods are available and should be employed when implementing and using the test methods in a laboratory (34)(35). The following paragraphs and Annex II describe the main components and procedures of the RhCE test methods.

RHCE TEST METHOD COMPONENTS

General conditions

25. 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 or sodium dodecyl sulphate (SDS). The barrier function should be demonstrated and may be assessed by determination of either the exposure time required to reduce tissue viability by 50% (ET_{50}) upon application of a benchmark substance at a specified, fixed concentration (e.g., 100 µL of 0.3% (v/v) Triton X-100), or the concentration at which a benchmark substance reduces the viability of the tissues by 50% (IC_{50}) following a fixed exposure time (e.g., 30 minutes treatment with 50 µL SDS) (see paragraph 30). 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 human-derived cells used to establish the RhCE tissue construct should be free of contamination by bacteria, viruses, mycoplasma, and fungi. The sterility of the tissue construct should be checked by the supplier for absence of contamination by fungi and bacteria.

Functional conditions

Viability

26. The assay used for quantifying tissue viability is the MTT assay (16). 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 (36). The OD of the extraction solvent alone should be sufficiently small, i.e., OD < 0.1. Users of the RhCE tissue construct should ensure that each batch of the RhCE tissue construct used meets defined criteria for the negative control. Acceptability ranges for the negative control OD values for the VRMs are given in Table 2. 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 in the test report 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. A similar procedure should be followed by the tissue producer as part of the quality control tissue batch release, but in this case different acceptance criteria than those specified in Table 2 may apply. An acceptability range (upper and lower limit) for the negative control OD values (in the QC test method conditions) should be established by the RhCE tissue construct developer/supplier.

Table 2: Acceptability ranges for negative control OD values (for the test method users)

Test Method	Lower acceptance limit	Upper acceptance limit
EpiOcular™ EIT (OCL-200) – VRM1 (for both the liquids and the solids protocols)	> 0.8[1]	< 2.5
SkinEthic™ HCE EIT (HCE/S) – VRM2 (for both the liquids and the solids protocols)	> 1.0	≤ 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 (37).

Barrier function

27. The RhCE tissue construct should be sufficiently thick and robust to resist the rapid penetration of cytotoxic benchmark substances, as estimated e.g. by ET_{50} (Triton X-100) or by IC_{50} (SDS) (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 30).

Morphology

28. 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). For the VRMs, appropriate morphology has been established by the developer/supplier and therefore does not need to be demonstrated again by a test method user for each tissue batch used.

Reproducibility

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

Quality control (QC)

30. 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 (paragraph 26) and barrier function (see paragraph 27) are the most relevant. An acceptability range (upper and lower limits) for the barrier functions as measured by the ET_{50} or IC_{50} (see paragraphs 25 and 26) should be established by the RhCE tissue construct developer/supplier. The ET_{50} and IC_{50} acceptability range used as QC batch release criterion by the developer/supplier of the RhCE tissue constructs (used in the VRMs) 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.

Table 3: QC batch release criterion

Test Method	Lower acceptance limit	Upper acceptance limit
EpiOcular™ EIT (OCL-200) – VRM1 (100 µL of 0.3% (v/v) Triton X-100)	ET_{50} = 12.2 min	ET_{50} = 37.5 min
SkinEthic™ HCE EIT (HCE/S) – VRM2 (30 minutes treatment with 50 µL SDS)	IC_{50} = 1 mg/mL	IC_{50} = 3.2 mg/mL

Application of the Test Chemical and Control Substances

31. At least two tissue replicates should be used for each test chemical and each control substance in each run. Two different treatment protocols are used, one for liquid test chemicals and one for solid test chemicals (34)(35).For both methods and protocols, the tissue construct surface should be moistened with calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (Ca^{2+}/Mg^{2+}-free DPBS) before application of test chemicals, to mimic the wet conditions of human eye. The treatment of the tissues is initiated with exposure to the test chemical(s) and control substances. For both treatment protocols in both VRMs, 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 32 and 33) (Annex II).

32. 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 VRMs, otherwise they should be treated as solids (see paragraph 33). In the VRMs, liquid test chemical are evenly spread over the tissue surface (i.e. a minimum of 60 µL/cm^2 application) (see Annex II, (33)(34)). Capillary effects (surface tension effects) that may occur due to the low volumes applied to the insert (on the tissue surface) should be avoided to the extent possible to guarantee the correct dosing of the tissue. Tissues treated with liquid test chemicals are incubated for 30 min at standard culture conditions (37±2°C, 5±1% CO_2, ≥95% RH). At the end of the

exposure period, the liquid test chemical and the 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 post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) for a pre-defined period of time that varies depending on the VRM used. For VMR1 only, a post-exposure incubation in fresh medium at standard culture conditions is applied prior to performing the MTT assay (see Annex II, (34)(35)).

33. Test chemicals that cannot be pipetted at temperatures up to 37°C are treated as solids in the VRMs. The amount of test chemical applied should be sufficient to cover the entire surface of the tissue, i.e. a minimum of 60 mg/cm^2 application should be used (Annex II). Whenever possible, solids should be tested as a fine powder. Tissues treated with solid test chemicals are incubated for a pre-defined period of time (depending on the VRM used) at standard culture conditions (see Annex II, (34) (35)). At the end of the exposure period, the solid test chemical and the 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 post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) for a pre-defined period of time that varies depending on the VRM used, and a post-exposure incubation in fresh medium at standard culture conditions, prior to performing the MTT assay (see Annex II, (34)(35)).

34. 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 VRMs is neat methyl acetate (CAS No. 79-20-9, commercially available from e.g., Sigma-Aldrich, Cat# 45997; liquid). The recommended negative control substances to be used with the VRM1 and VRM2 are ultrapure H_2O and Ca^{2+}/Mg^{2+}-free DPBS, respectively. These were the control substances used in the validation studies of the VRMs 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. Negative and positive controls should be tested with the same protocol(s) as the one(s) used for the test chemicals included in the run (i.e. for liquids and/or solids). This application should be followed by the treatment exposure, rinsing, a post-exposure immersion, and post-exposure incubation where applicable, as described for controls run concurrently to liquid test chemicals (see paragraph 32) or for controls run concurrently to solid test chemicals (see paragraph 33), prior to performing the MTT assay (see paragraph 35) (34)(35). 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

35. The MTT assay is a standardised quantitative method (16) 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 VRMs, the RhCE tissue construct sample is placed in 0.3 mL of MTT solution at 1 mg/mL for 180±15 min 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 an appropriate volume of isopropanol (or a similar solvent) (34)(35). 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 42) (11)(36).

36. 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 37-41). 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 570±30 nm, an HPLC/UPLC-spectrophotometry procedure to measure MTT formazan may be employed (see paragraphs 41 and 42) (11)(36). A detailed description of how to detect and correct for direct MTT reduction and interferences by colouring agents is available in the VRMs SOPs (34)(35). Illustrative flowcharts providing guidance on how to identify and handle direct MTT-reducers and/or colour interfering chemicals for VRM1 and VRM2 are also provided in Annexes III and IV, respectively.

37. 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, the test chemical is added to water and/or isopropanol and incubated for an appropriate time at room temperature (see Annex II, (34)(35)). If the test chemical in water and/or isopropanol absorbs sufficient light in the range of 570±20 nm for VRM1 (see Annex III), or if a coloured solution is obtained when mixing the test chemical with water for VRM2 (see Annex IV), the test chemical is presumed to interfere with the standard absorbance (OD) measurement of MTT formazan and further colourant controls should be performed or, alternatively, an HPLC/UPLC-spectrophotometry procedure should be used in which case these controls are not required (see paragraphs 41 and 42 and Annexes III and IV)(34)(35). 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 (34)(35). 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 = $[\%Viability_{test}] - [\%NSC_{living}]$.

38. To identify direct MTT reducers, each test chemical should be added to freshly prepared MTT solution. An appropriate amount of test chemical is added to a MTT solution and the mixture is incubated for approximately 3 hours at standard culture conditions (see Annexes III and IV)(34)(35). 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. 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. Killed tissues of VRM1 are prepared by exposure to low temperature ("freeze-killed"). Killed tissues of VRM2 are prepared by prolonged incubation (e.g., at least 24±1 hours) in water followed by storage to low temperature ("water-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 (34)(35). 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].

39. Test chemicals that are identified as producing both colour interference (see paragraph 37) and direct MTT reduction (see paragraph 38) 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 capacity to directly reduce MTT as described in paragraph 38. 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 Annexes III and IV)(34)(35). 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}].

40. 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.

41. 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 $\%NSC_{living} \geq 60\%$ (VRM1, and VRM2 for liquids' protocol) or 50% (VRM2 for solids' protocol) of the negative control should be taken with caution as this is the established cut-off used in the VRMs to distinguish classified from not classified chemicals (see paragraph 44). 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 Annexes III and IV). This is because the HPLC/UPLC system allows for the separation of the MTT formazan from the chemical before its quantification (36). 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 38). 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 38. 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 38. Finally, it should be noted that direct MTT-reducers or direct MTT-reducers that are also colour interfering, which are retained 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 RhCE test methods, although these are expected to occur in only very rare situations.

42. 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 (11)(36). 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 (36)(38). These key parameters and their acceptance criteria are shown in Annex V. Once the acceptance criteria defined in Annex V 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

43. For each run using RhCE tissue batches that met the quality control (see paragraph 30), 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 26). Similarly, tissues treated with the positive control substance, i.e., methyl acetate, should show a mean tissue viability < 50% relative to the negative control in the VRM1 with either the liquids' or the solids' protocols, and ≤ 30% (liquids' protocol) or ≤ 20% (solids' protocol) relative to the negative control in the VRM2, thus reflecting the ability of the tissues to respond to an irritant test chemical under the conditions of the test method (34)(35). 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 standard deviation (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

44. 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 for identifying test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) is given in Table 4. 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 (>) the established percentage tissue viability cut-off value, as shown in Table 4. In this case no further testing in other test methods is required.

- If the mean percent tissue viability after exposure and post-exposure incubation is less than or equal (≤) to the established percentage tissue viability cut-off value, no prediction can be made, as shown in Table 4. In this case, further testing with other test methods will be required because RhCE test methods show a certain number of false positive results (see paragraphs 14-15) and cannot resolve between UN GHS Categories 1 and 2 (see paragraph 17).

Table 4: Prediction Models according to UN GHS classification

VRM	No Category	No prediction can be made
EpiOcular™ EIT (for both protocols)	Mean tissue viability > 60%	Mean tissue viability ≤ 60%
SkinEthic™ HCE EIT (for the liquids' protocol)	Mean tissue viability > 60%	Mean tissue viability ≤ 60%
SkinEthic™ HCE EIT (for the solids' protocol)	Mean tissue viability > 50%	Mean tissue viability ≤ 50%

45. 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 measurements and/or mean percent tissue viability equal to 60±5% (VRM1, and VRM2 for liquids'

protocol) or 50±5% (VRM2 for solids' protocol), a second test should be considered, as well as a third one in case of discordant results between the first two tests.

46. 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 test chemicals 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

47. 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 RhCE test method 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 difference of viability between two tissue replicates (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

48. 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.

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 or Ca^{2+}/Mg^{2+}-free DPBS, 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 Tissue Construct 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 HPLC/UPLC-spectrophotometry system used, 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 quality control (supplier)
  ii) Viability under test method conditions (user);
  iii) Barrier function quality control;
  iv) Morphology, if available;
  v) Reproducibility and predictive capacity;
  vi) Other quality controls (QC) of the RhCE tissue construct, if available;

- Reference to historical data of the RhCE tissue construct. This should include, but is not limited to: Acceptability of the QC data with reference to historical batch data;

- Statement that the testing facility has demonstrated proficiency in the use of 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;
- 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);

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 value 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 value 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

LITERATURE

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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) for its identification and characterisation; (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 chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Interchangeable with "Reversible effects on the eye" and with "UN GHS Category 2" (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.

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.

HCE: SkinEthic™ Human Corneal Epithelium.

HPLC: High Performance Liquid Chromatography.

IC_{50}: Concentration at which a benchmark chemical reduces the viability of the tissues by 50% following a fixed exposure time (e.g., 30 minutes treatment with SDS).

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

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 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).

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.

VRM1: EpiOcular™ EIT is referred as the Validated Reference Method 1.

VRM2: SkinEthic™ HCE EIT is referred to as the Validated Reference Method 2.

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.

ANNEX II

MAIN TEST METHOD COMPONENTS OF THE RhCE TEST METHODS VALIDATED FOR IDENTIFYING CHEMICALS NOT REQUIRING CLASSIFICATION AND LABELLING FOR EYE IRRITATION OR SEERIOUS EYE DAMAGE

Test Method Components	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)	
Protocols	Liquids (pipetteable at 37±1°C or lower temperatures for 15 min)	Solids (not pipetteable)	Liquids and viscous (pipetteable)	Solids (not pipetteable)
Model surface	0.6 cm^2	0.6 cm^2	0.5 cm^2	0.5 cm^2
Number of tissue replicates	At least 2	At least 2	At least 2	At least 2

Test Method Components	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)	
Pre-check for colour interference	50 µL + 1 mL H_2O for 60 min at 37±2ºC, 5±1% CO_2, ≥95% RH (non-coloured test chemicals), or 50 µL + 2 mL isopropanol mixed for 2-3h at RT (coloured test chemicals) → if the OD of the test chemical at 570±20 nm, after subtraction of the OD for isopropanol or water is > 0.08 (which corresponds to approximately 5% of the mean OD of the negative control), living adapted controls should be performed.	50 mg + 1 mL H_2O for 60 min at 37±2ºC, 5±1% CO_2, ≥95% RH (non-coloured test chemicals) and/or 50 mg + 2 mL isopropanol mixed for 2-3h at RT (colored and non-colored test chemicals) → if the OD of the test chemical at 570±20 nm after subtraction of the OD for isopropanol or water is > 0.08 (which corresponds to approximately 5% of the mean OD of the negative control), living adapted controls should be performed.	10 µL + 90 µL H_2O mixed for 30±2 min at Room Temperature (RT, 18-28°C) → if test chemical is coloured, living adapted controls should be performed	10 mg + 90 µL H_2O mixed for 30±2 min at RT → if test chemical is coloured, living adapted controls should be performed
Pre-check for direct MTT reduction	50 µL + 1 mL MTT 1 mg/mL solution for 180±15 min at 37±2ºC, 5±1% CO_2, ≥95% RH → if solution turns blue/purple,freeze-killed adapted controls should be performed (50 µL of sterile deionized water in MTT solution is used as negative control)	50 mg + 1 mL MTT 1 mg/mL solution for 180±15 min at 37±2ºC, 5±1% CO_2, ≥95% RH → if solution turns blue/purple, freeze-killed adapted controls should be performed (50 µL of sterile deionized water in MTT solution is used as negative control)	30 µL + 300 µL MTT 1 mg/mL solution for 180± 15 min at 37±2ºC, 5±1% CO_2, ≥95% RH → if solution turns blue/purple, water-killed adapted controls should be performed (30 µL of sterile deionized water in MTT solution is used as negative control)	30 mg + 300 µL MTT 1 mg/mL solution for 180± 15 min at 37±2ºC, 5±1% CO_2, ≥95% RH → if solution turns blue/purple, water-killed adapted controls should be performed (30 µL of sterile deionized water in MTT solution is used as negative control)

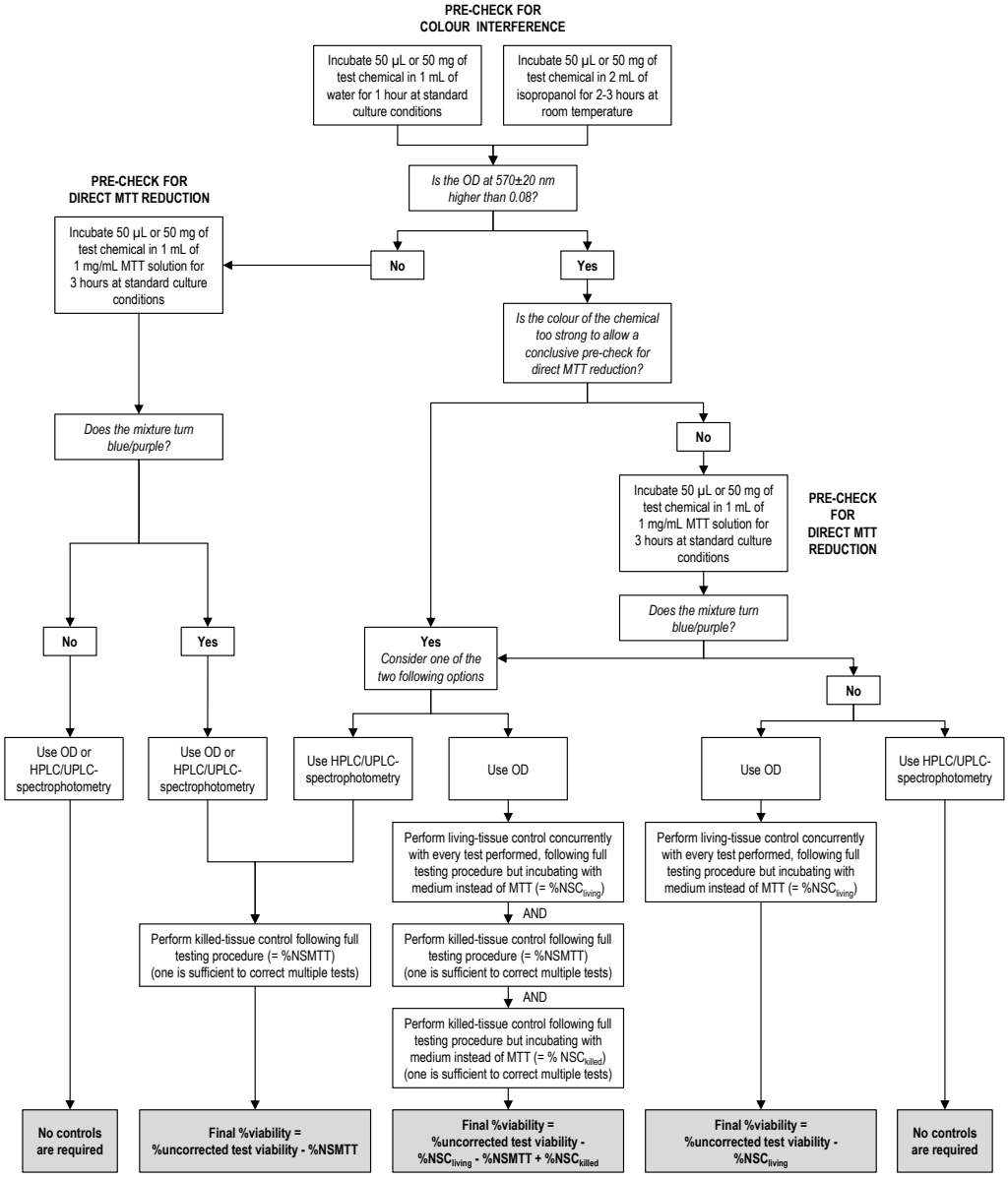
Test Method Components	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)	
Pre-treatment	20 µL Ca^{2+}/Mg^{2+}-free DPBS for 30 ± 2 min at 37±2ºC, 5±1% CO_2, ≥95% RH, protected from light.	20 µL Ca^{2+}/Mg^{2+} -free DPBS for 30±2 min at 37±2ºC, 5±1% CO_2, ≥95% RH, protected from light.	-	-
Treatment doses and application	50 µL (83.3 µL/cm^2)	50 mg (83.3 mg/cm^2) using a calibrated tool (e.g., a levelled spoonful calibrated to hold 50 mg of sodium chloride).	10 µL Ca^{2+}/Mg^{2+}-free DPBS + 30 ± 2 µL (60 µL/cm^2) For viscous, use a nylon mesh	30 µL Ca^{2+}/Mg^{2+}-free DPBS + 30 ± 2 mg (60 mg/cm^2)
Exposure time and temperature	30 min (± 2 min) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	6 hours (± 0.25 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	30 min (± 2 min) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	4 hours (± 0.1 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH
Rinsing at room temperature	3 times in 100 mL of Ca^{2+}/Mg^{2+}-free DPBS	3 times in 100 mL of Ca^{2+}/Mg^{2+}-free DPBS	20 mL Ca^{2+}/Mg^{2+}-free DPBS	25 mL Ca^{2+}/Mg^{2+}-free DPBS
Post-exposure immersion	12 min (± 2 min) at RT in culture medium	25 min (± 2 min) at RT in culture medium	30 min (± 2 min) at 37ºC, 5% CO_2, 95% RH in culture medium	30 min (± 2 min) at RT in culture medium
Post-exposure incubation	120 min (± 15 min) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	18 h (± 0.25 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	none	18 h (± 0.5 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH
Negative control	50 µL H_2O Tested concurrently	50 µL H_2O Tested concurrently	30 ± 2µL Ca^{2+}/Mg^{2+}-free DPBS Tested concurrently	30 ± 2µL Ca^{2+}/Mg^{2+}-free DPBS Tested concurrently

Test Method Components	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)	
Positive control	50 µL Methyl acetate Tested concurrently	50 µL Methyl acetate Tested concurrently	30 ± 2µL Methyl acetate Tested concurrently	30 ± 2µL Methyl acetate Tested concurrently
MTT solution	300 µL 1 mg/mL	300 µL 1 mg/mL	300 µL 1 mg/mL	300 µL 1 mg/mL
MTT incubation time and temperature	180 min (± 15 min) at 37±2ºC, 5±1% CO_2, ≥95% RH	180 min (± 15 min) at 37±2ºC, 5±1% CO_2, ≥95% RH	180 min (± 15 min) at 37±2ºC, 5±1% CO_2, ≥95% RH	180 min (± 15 min) at 37±2ºC, 5±1% CO_2, ≥95% RH
Extraction solvent	2 mL isopropanol (extraction from top and bottom of insert by piercing the tissue)	2 mL isopropanol (extraction from bottom of insert by piercing the tissue)	1.5 mL isopropanol (extraction from top and bottom of insert)	1.5 mL isopropanol (extraction from bottom of insert)
Extraction time and temperature	2-3 h with shaking (~120 rpm) at RT or overnight at 4-10°C	2-3 h with shaking (~120 rpm) at RT or overnight at 4-10°C	4 h with shaking (~120 rpm) at RT or at least overnight without shaking at 4-10°C	At least 2 h with shaking (~120 rpm) at RT
OD reading	570 nm (550 - 590 nm) without reference filter	570 nm (550-590 nm) without reference filter	570 nm (540 - 600 nm) without reference filter	570 nm (540 - 600 nm) without reference filter
Tissue Quality Control	Treatment with 100 µL of 0.3% (v/v) Triton X-100 12.2 min ≤ ET_{50} ≤ 37.5 min	Treatment with 100 µL of 0.3% (v/v) Triton X-100 12.2 min ≤ ET_{50} ≤ 37.5 min	30 min treatment with SDS (50 µL) 1.0 mg/mL ≤ IC_{50} ≤ 3.5 mg/mL	30 min treatment with SDS (50 µL) 1.0 mg/mL ≤ IC_{50} ≤ 3.2 mg/mL

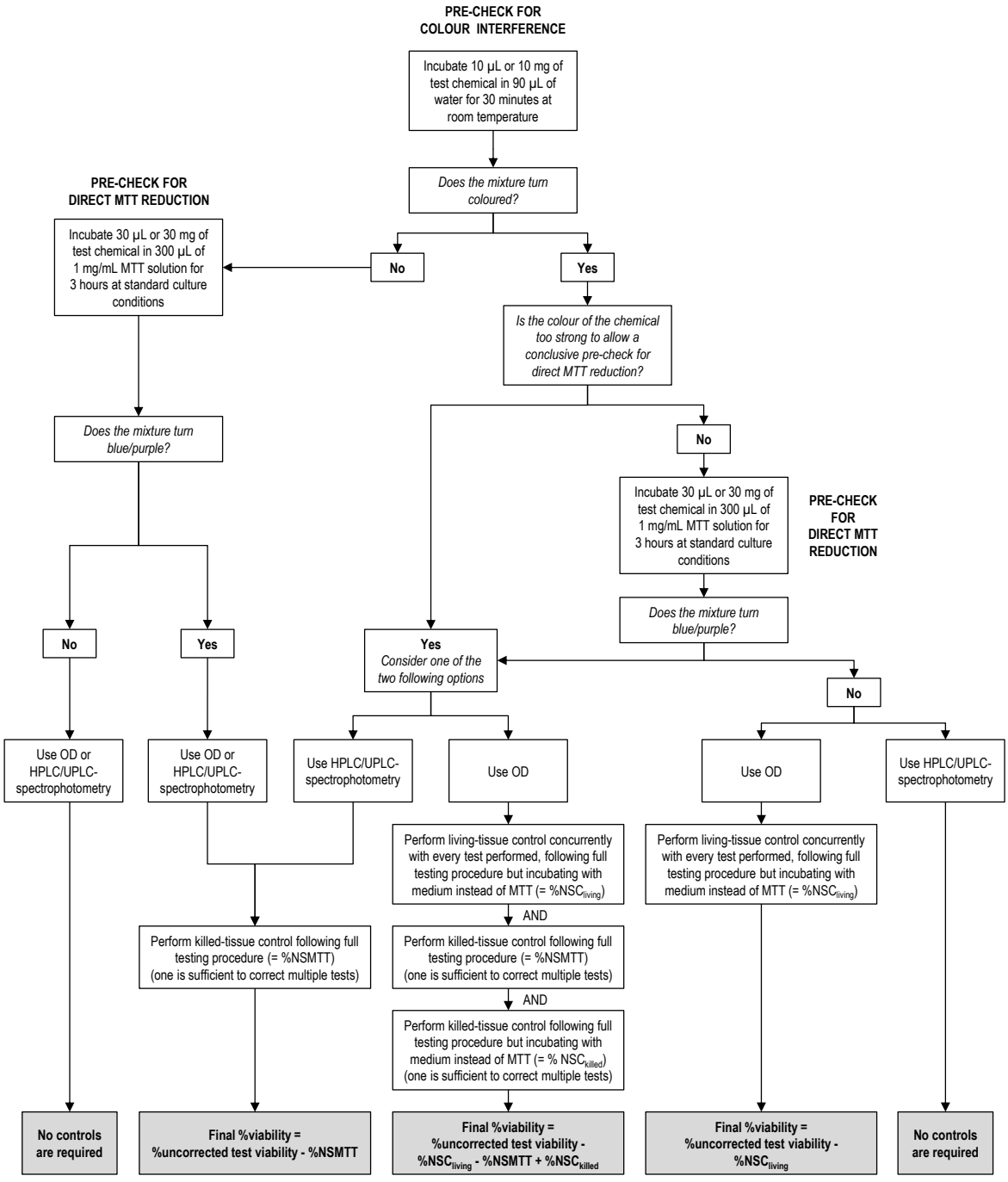
Test Method Components	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)	
Acceptance Criteria	1. Mean OD of the tissue replicates treated with the negative control should be > 0.8 and < 2.5 2. Mean viability of the tissue replicates exposed for 30 min with the positive control, expressed as % of the negative control, should be < 50% 3. The difference of viability between two tissue replicates should be less than 20%.	1. Mean OD of the tissue replicates treated with the negative control should be > 0.8 and < 2.5 2. Mean viability of the tissue replicates exposed for 6 hours with the positive control, expressed as % of the negative control, should be < 50% 3. The difference of viability between two tissue replicates should be less than 20%.	1. Mean OD of the tissue replicates treated with the negative control should be > 1.0 and ≤ 2.5 2. Mean viability of the tissue replicates exposed for 30 min with the positive control, expressed as % of the negative control, should be ≤ 30% 3. The difference of viability between two tissue replicates should be less than 20%.	1. Mean OD of the tissue replicates treated with the negative control should be > 1.0 and ≤ 2.5 2. Mean viability of the tissue replicates exposed for 4 hours with the positive control, expressed as % of the negative control, should be ≤ 20% 3. The difference of viability between two tissue replicates should be less than 20%.

ANNEX III

ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT MTT-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE VRM1 SOP



ANNEX IV
ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT MTT-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE VRM2 SOP



ANNEX V

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 (36)(38)	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.