新規試験法提案書

眼刺激性試験代替法 再構築ヒト角膜様上皮モデル法 (LabCyte CORNEA-MODEL24 Eye Irritation Test)

平成31年 2 月

国立医薬品食品衛生研究所

新規試験法提案書

平成 31 年 2 月 19 日

No.　2018-04

眼刺激性試験代替法　再構築ヒト角膜様上皮モデル法
（LabCyte CORNEA-MODEL24 Eye Irritation Test）に関する提案

平成 31 年 2 月 19 日に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：LabCyte CORNEA-MODEL24 Eye Irritation Test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。

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などをもとに、眼刺激性試験編纂委員会によりまとめられた文書を用いて、JaCVAM評価会議が評価および検討した結果、その有用性が確認されたことから作成された。

以上の理由により、行政当局の安全性評価方法として LabCyte CORNEA-MODEL24 Eye Irritation Test 法を提案するものである。

大野泰雄

JaCVAM 評価会議　議長

平林容子

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
五十嵐良明 （国立医薬品食品衛生研究所）
石 井 雄 二 （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）
井 上 智 彰 （日本免疫毒性学会）
今 井 教 安 （日本動物実験代替法学会）
岩瀬裕美子 （日本製薬工業協会）
篠 田 和 俊 （独立行政法人　医薬品医療機器総合機構）
杉山真理子 （日本化粧品工業連合会）
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）
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）
沼 澤　　聡 （日本毒性学会）
野 口 真 希 （独立行政法人　医薬品医療機器総合機構）*
森 田　　健 （日本環境変異原学会）
横 関 博 雄 （日本皮膚免疫アレルギー学会）

任期：平成 28 年 4 月 1 日～平成 30 年 3 月 31 日
*：平成 29 年 4 月 1 日～平成 30 年 3 月 31 日

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
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増 村 健 一 （日本環境変異原学会）
横 関 博 雄 （日本皮膚免疫アレルギー学会）

任期：平成 30 年 4 月 1 日～平成 32 年 3 月 31 日

JaCVAM 運営委員会

平 林 容 子（国立医薬品食品衛生研究所　安全性生物試験研究センター）：委員長
石 井 孝 司（国立感染症研究所）
大 原　　拓（厚生労働省　医薬・生活衛生局　医薬品審査管理課）
小川久美子（国立医薬品食品衛生研究所　安全性生物試験研究センター　病理部）
奥 田 晴 宏（国立医薬品食品衛生研究所）
諫 田 泰 成（国立医薬品食品衛生研究所　安全性生物試験研究センター　薬理部）
北 嶋　　聡（国立医薬品食品衛生研究所　安全性生物試験研究センター　毒性部）
小池紘一郎（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）
高 木 篤 也（国立医薬品食品衛生研究所　安全性生物試験研究センター　毒性部　動物管理室）
束 野 正 明（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）
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広 瀬 明 彦（国立医薬品食品衛生研究所　安全性生物試験研究センター　安全性予測評価部）
笛 木　　修（独立行政法人　医薬品医療機器総合機構）
渕 岡　　学（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）
本 間 正 充（国立医薬品食品衛生研究所　安全性生物試験研究センター　変異遺伝部）
小 島　　肇（国立医薬品食品衛生研究所　安全性生物試験研究センター　安全性予測評価部
第二室）：事務局

JaCVAM statement on the LabCyte CORNEA-MODEL24 Eye Irritation Test for assessing ocular irritation

At a meeting held on 19 February 2019 at the National Institute of Health Sciences (NIHS) in Kanagawa, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Proposal: We consider the LabCyte CORNEA-MODEL24 Eye Irritation Test to be a useful means of assessing the ocular irritation potential of chemicals, which when used in a bottom-up approach to identifying chemical substances that do not require classification and labelling under UN GHS* is suitable for use in a regulatory context.

This statement was prepared following a review of the 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” and others 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 LabCyte CORNEA-MODEL24 Eye Irritation Test as a useful means for safety assessment by regulatory agencies.

*UN GHS ： United Nations Globally Harmonized System of Classification and Labelling of Chemicals

Yasuo Ohno
Chairperson
JaCVAM Regulatory Acceptance Board

Yoko Hirabayashi
Chairperson
JaCVAM Steering Committee

February 19, 2019

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. 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)
Mr. Satoshi Numazawa (Japanese Society of Toxicology)
Ms. Maki Noguchi (Pharmaceuticals and Medical Devices Agency) *
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 2017 to 31st March 2018

Mr. Yasuo Ohno (Kihara Memorial Yokohama Foundation for the Advancement of Life Sciences): Chairperson
Ms. Yoko Hirabayashi (BSRC, NIHS)
Mr. Yoshiaki Ikarashi (NIHS)
Mr. Noriyasu Imai (Japanese Society for Alternatives to Animal Experiments)
Mr. Kunifumi Inawaka (Japan Chemical Industry Association)
Mr. Tomoaki Inoue (Japanese Society of Immunotoxicology)
Mr. Yuji Ishii (BSRC, NIHS)
Ms. Yumiko Iwase (Japan Pharmaceutical Manufacturers Association)
Mr. Fumihiro Kubo (Pharmaceuticals and Medical Devices Agency)
Mr. Kenichi Masumura (Japanese Environmental Mutagen Society)
Ms. Ruriko Nakamura (National Institute of Technology and Evaluation)
Mr. Akiyoshi Nishikawa (BSRC, NIHS/ Saiseikai Utsunomiya Hospital)
Mr. Jihei Nishimura (Pharmaceuticals and Medical Devices Agency)
Mr. Satoshi Numazawa (Japanese Society of Toxicology)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Mr. Hiroo Yokozeki (Japanese Society for Cutaneous Immunology and Allergy)

Term: From 1st April 2018 to 31st March 2020

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

Ms. Yoko Hirabayashi (BSRC, NIHS): Chairperson
Mr. Manabu Fuchioka (Ministry of Health, Labour and Welfare)
Mr. Osamu Fueki (Pharmaceuticals and Medical Devices Agency)
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)
Mr. Koichi Hiruta (Pharmaceuticals and Medical Devices Agency)
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)
Mr. Koji Ishii (National Institute of Infectious Diseases)
Mr. Yasunari Kanda (Division of Pharmacology, BSRC, NIHS)
Mr. Satoshi Kitajima (Division of Toxicology, BSRC, NIHS)
Mr. Kouichirou Koike (Ministry of Health, Labour and Welfare)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Mr. Haruhiro Okuda (NIHS)
Mr. Taku Oohara (Ministry of Health, Labour and Welfare)
Mr. Atsuya Takagi (Animal Management Section of the Division of Toxicology, BSRC, NIHS)
Mr. Masaaki Tsukano (Ministry of Health, Labour and Welfare)
Mr. Hajime Kojima (Division of Risk Assessment, BSRC, NIHS): Secretary

眼刺激性試験代替法　再構築ヒト角膜様上皮モデル法

（LabCyte CORNEA-MODEL24 Eye Irritation Test）

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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）
LabCyte CORNEA-MODEL24 眼刺激性試験
（LabCyte CORNEA-MODEL24 EIT）

JaCVAM 評価会議

平成 30 年（2018 年）12 月 25 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
五十嵐良明（国立医薬品食品衛生研究所）
石 井 雄 二（国立医薬品食品衛生研究所　安全性生物試験研究センター）
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横 関 博 雄（日本皮膚免疫アレルギー学会）

任期:平成 30 年 4 月 1 日～平成 32 年 3 月 31 日

再構築ヒト角膜様上皮モデル法（Reconstructed human Cornea-like Epithelium Test Method: RhCE 法）は、ウサギを用いた Draize 眼刺激性試験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 には、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 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™ Human Corneal Epithelium Eye Irritation Test: SkinEthic™ HCE EIT）が ESAC（EURL ECVAM Scientific Advisory Committee）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）として収載されている [1)]。また、OECD は新規 RhCE 組織を用いた試験法に対してその構造および機序が VRM と同等で十分な信頼性を備えていることを評価する性能標準を定めている [2)]。LabCyte CORNEA-MODEL24 を用いた眼刺激性試験（LabCyte CORNEA-MODEL24 Eye Irritation Test: LabCyte CORNEA-MODEL24 EIT）は類似試験法として、OECD の性能標準で指定されている 30 の参照物質を用いた 3 施設での追走的バリデーション研究 [3)] および JaCVAM の第三者評価 [4)]を経て、2018 年に TG492 に収載された [5)]。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「改訂 OECD TG492 再構築角膜様上皮モデル法　LabCyte CORNEA-MODEL24 眼刺激性試験評価報告書」[6)]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）　LabCyte CORNEA-MODEL24 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 法のひとつである LabCyte CORNEA-MODEL24 EIT では、ヒト角膜上皮細胞を重層培養した LabCyte CORNEA-MODEL24 に被験物質が液体の場合は 1 分間、固体の場合は 24 時間被験物質を曝露した後、WST-8（2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)2H-tetrazolium, monosodium salt）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、WST-8 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 WST-8 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。被験物質が液体・固体に関わらず、平均細胞生存率が 40％を超えた場合、被験物質は UN GHS 分類において区分外であると判定する。なお、本試験法で偽陽性を回避するためには、また、UN GHS 分類区分 1 と区分 2 を識別する必要がある場合は、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（LabCyte CORNEA-MODEL24）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。

LabCyte CORNEA-MODEL24 EIT については、リードラボとは異なる 3 施設において OECD の性能標準で指定されている 30 の参照物質を用いた追走的バリデーション研究が行われた [3]。その結果、LabCyte CORNEA-MODEL24 EIT は、UN GHS 区分外物質予測性の施設内再現性、施設間再現性および正確性について OECD の性能標準が定めた基準を満たした。さらに JaCVAM の第三者評価 [4]を経て、UN GHS 区分外物質を検出する方法として 2018 年に改訂 OECD 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 LabCyte CORNEA-MODEL24 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 LabCyte CORNEA-MODEL24 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習熟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、UN GHS 分類判定の施設ごとの施設内再現性は 93-100％であり、OECD の性能標準が定めた基準（90％以上）を満たしていた [6]。また、施設間再現性は、87％で、OECD の性能標準が定めた基準（85％以上）を満たしていた [6]。さらに、バリデーション研究で得られたデータによる性能評価では、感度 97.8％、特異度 68.9％および正確度 83.5％となり OECD の性能標準が定めた基準（感度 90%以上、特異度 60%以上、正確度 75%以上）を満たしていた [6]。

但し、細胞生存率の算出に際し、被験物質が WST-8 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（450 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 V のフローチャートを参照する必要がある。

また、本試験法を適用するには、試験法の性能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）およびエアロゾル状物質は適用から除外される。

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、LabCyte CORNEA-MODEL24 は比較的安価であり、その入手は容易で、短時間で実施できる。また、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 LabCyte CORNEA-MODEL24 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習熟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。

参考文献

1) OECD (2017) Guideline for the testing of chemicals. 492, Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage.

2) OECD (2016) Performance standards for the assessment of proposed similar or modified in vitro reconstructed human cornea-like epithelium (RhCE) test methods for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage, based on the validated reference methods Epiocular™ EIT and Skinethic™ HCE EIT described in TG492.

3) LabCyte Validation Management Team (2017) Me-too Validation Report: Validation Study for LabCyte CORNEA-MODEL24 Eye Irritation Test (Version. 3.0).

4) Peer Review Panel (2017) Validation status of the LabCyte CORNEA-MODEL24 EYE IRRITTION TEST, Report of the Peer Review Panel on a JaCVAM co-ordinated study programme addressing the validation status of LabCyte CORNEA-MODEL24 EYE IRRITATION TEST for discriminating eye irritant from non-eye irritant substances.

5) OECD (2018) 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.

6) JaCVAM　眼刺激性試験資料編纂委員会：評価報告書　再構築ヒト角膜様上皮モデル法 (RhCE法) LabCyte CORNEA-MODEL24眼刺激性試験（LabCyte CORNEA-MODEL24 EIT）（2018年10月1日）

評価報告書

再構築ヒト角膜様上皮モデル法（RhCE 法）
LabCyte CORNEA-MODEL24 眼刺激性試験
（LabCyte CORNEA-MODEL24 EIT）

眼刺激性試験資料編纂委員会

平成 30 年（2018 年）12 月 27 日

眼刺激性試験資料編纂委員会

山 本　直 樹　（藤田医科大学）
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略語

CAS: Chemical Abstracts Services

EIT: Eye Irritation Test

ESAC: EURL ECVAM Scientific Advisory Committee

GHS: Globally Harmonized System of Classification and Labeling of Chemicals

JaCVAM: Japanese Center for the Validation of Alternative Methods

OD: Optical Density

OECD: Organization for Economic Co-operation and Development

RhCE: Reconstructed human Cornea-like Epithelium

SDS: Sodium Dodecyl Sulphate

TG: Test Guideline

UN: United Nations

VRM: Validated Reference Method

WST: Water Soluble Tetrazolium Salt

要旨

再構築ヒト角膜様上皮モデル（Reconstructed human Cornea-like Epithelium: 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 TG492 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。LabCyte CORNEA-MODEL24 眼刺激性試験（LabCyte CORNEA-MODEL24 EIT）は RhCE 法の一つで、2018 年に改訂 TG492 に追加された試験法である。本報告では、LabCyte CORNEA-MODEL24 EIT のバリデーション研究報告書、第三者評価報告書、関連論文などをもとに試験法の概要を説明し JaCVAM 眼刺激性試験資料編纂委員会の意見をまとめた。

LabCyte CORNEA-MODEL24 EIT の信頼性・正確性を確認するため、OECD の性能標準（Performance Standard）で指定されている 30 の参照物質（Reference Chemicals）を用いて 3 施設で追走的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 回試験を行った。この追走的バリデーション研究において LabCyte CORNEA-MODL24 EIT の施設内再現性はそれぞれの施設で 93%、97%、100%、また、施設間再現性は 87%であった。正確性は、感度 97.8%、特異度 68.9%、正確度 83.5%であった。これら再現性、正確性の値は、性能標準が定めた基準を満たしていた。

以上より、本委員会は、LabCyte CORNEA-MODEL24 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

再構築ヒト角膜様上皮モデル（Reconstructed human Cornea-like Epithelium: RhCE）法は、RhCE 組織に対する被験物質の細胞毒性を指標に眼刺激性を評価する試験法で、ボトムアップ方式により UN GHS 区分外物質を検出する。

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™ Human Corneal Epithelium Eye Irritation Test: SkinEthic™ HCE EIT）は 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 OECD TG492 に収載されている [1]。また、OECD は新規 RhCE 組織を用いた試験法（me-too 試験法）に対してその構造・機序が VRM と同等であり十分な信頼性と関連性を備えていることを評価する性能標準（Performance Standard）を定めた [2]。LabCyte CORNEA-MODEL24 眼刺激性試験（LabCyte CORNEA-MODEL24 Eye Irritation Test: LabCyte CORNEA-MODEL24 EIT）は me-too 試験法として、OECD の性能標準で指定されている 30 の参照物質（Reference Chemicals）を用いた 3 施設で追走的バリデーション研究 [3]、JaCVAM の第三者評価 [4]を経て 2018 年に TG492 に収載された [1]。

本報告書は LabCyte CORNEA-MODEL24 EIT のバリデーション研究報告書、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損傷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。

LabCyte CORNEA-MODEL24 EIT は、ヒト角膜上皮細胞を重層培養した市販の RhCE 組織（LabCyte CORNEA-MODEL24）を用いる [5]。被験物質が液体の場合は約 1 分間、固体の場合は約 24 時間、被験物質を LabCyte CORNEA-MODEL24 に曝露した後、WST-8 法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、細胞内の脱水素酵素の働きにより電子メディエーターの存在下で WST-8（2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, CAS No.193149-74-5）が還元されて生成される橙色ホルマザン量が生存細胞数に比例することを基本原理としている [6]。細胞生存率が陰性対照と比較して 40%を超えた場合、被験物質は UN GHS 分類において区分外であると判定する。

4．試験手順

LabCyte CORNEA-MODEL24 EIT の手順を以下に示す。詳細は、改訂 TG492[1]およびプロトコル [7]を参照する。使用する RhCE 組織 LabCyte CORNEA-MODEL24 は株式会社ジャパン・ティッシュ・エンジニアリング（J-TEC、日本）より購入できる。

4-1. RhCE 組織の機能的条件

LabCyte CORNEA-MODEL24 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 $0.5 \leq OD \leq 1.6$

（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（25μL）の 60 分間曝露において $1.0 \leq IC_{50}\ (mg/mL) \leq 4.0$

（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は背景データをもとに設定した許容値内にある

これら 4 項目は LabCyte CORNEA-MODEL24 の製造者の出荷基準として採用される。一方、LabCyte CORNEA-MODEL24 の使用者は生存率と再現性の 2 項目を確認する必要がある。

4-2. 被験物質の適用

物質あたり 3 RhCE 組織を用いる。被験物質は調製せず、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、LabCyte CORNEA-MODEL24 に 50 μL を適用し、室温で 1 分間（±5 秒）培養する。その後、Ca^{2+}/Mg^{2+}不含 Dulbecco リン酸緩衝生理食塩水（DPBS）の水流で 10 回あるいはそれ以上洗浄して被験物質を除去し、24±1 時間標準培養条件*で培養する。固体被験物質の場合、10 mg を均一に適用し、標準培養条件で 24±1 時間培養する。その後、Ca^{2+}/Mg^{2+}不含 DPBS の水流で 10 回あるいはそれ以上洗浄して被験物質を除去する。洗浄後の培養は行わない。

同時陽性対照には液体被験物質の場合はエタノール（CAS No. 64-17-5）、固体被験物質の場合はラウリン酸（CAS No. 143-07-7）が推奨される。同時陰性対照には液体被験物質の場合は Ca^{2+}/Mg^{2+}不含 DPBS が推奨され、固体被験物質の場合は無処理を用いる。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 WST-8 法を用いる。洗浄・後培養（液体のみ）後、RhCE 組織を WST-8 溶液（Cell Count Kit-8 溶液:アール平衡塩溶液= 1:10）0.3 mL 中で 240±15 分間標準培養条件下で反応させる。WST-8 溶液中のホルマザンの定量は OD 測定（OD_{450nm}－OD_{650nm}）または HPLC/UPLC で行う。

被験物質が WST-8 還元物質の場合、あるいはホルマザンと同じような波長（450 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。被験物質が WST-8 法を干渉する物質であるかどうかの事前検討とその場合の細胞生存率の補正の手順については TG 本文の説明および ANNEX V 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 $0.5 \leq OD \leq 1.6$ であること。

2）陽性対照の平均細胞生存率が 40%以下であること。

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* 37±2˚C、5±1% CO_2、≥95% 湿度

3）被験物質および陰性・陽性対照のそれぞれにおいて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. 判定

被験物質の平均細胞生存率が 40%を超えた場合、被験物質は UN GHS 区分外と判断される。平均細胞生存率が 40%以下の場合は、その結果のみでは被験物質の眼刺激性の予測はできない。本試験法では偽陽性の可能性があり、また UN GHS 区分 1 と区分 2 を区別できない。その場合は、被験物質の眼刺激性を分類するには追加の情報が必要となる。

各組織の測定値での判定が一致しない、平均細胞生存率が 40±5%の範囲にある等、得られた結果がボーダーラインの場合は 2 回目の試験を検討する。1 回目と 2 回目の試験で結果が一致しない場合は 3 回目の試験を検討する。

表 1. VRM と LabCyte CORNEA-MODEL24 EIT の主な項目の比較

	EpiOcular™ IET (VRM1)	SkinEthic™ HCE EIT (VRM2)	LabCyte CORNEA-MODEL24 EIT
RhCE 組織の由来	ヒト表皮角化細胞	ヒト不死化角膜上皮細胞	ヒト角膜上皮細胞
曝露量	50 µL または50 mg (0.6 cm^2)	30 µL または 30 mg (0.5 cm^2)	50 µL または 10 mg (0.3 cm^2)
曝露時間	30 分（液体）、6 時間（固体）	30 分（液体）、4 時間（固体）	1 分（液体）、24 時間（固体）
陽性・陰性対照	陽性：酢酸メチル 陰性：超純水	陽性：酢酸メチル 陰性：Ca^{2+}/Mg^{2+}不含 DPBS	陽性：エタノール（液体）、ラウリン酸（固体） 陰性：Ca^{2+}/Mg^{2+}不含 DPBS（液体）、無処理（固体）
生存率測定	MTT 法	MTT 法	WST-8 法
UN GHS 区分外判定基準（平均細胞生存率）	>60%	>60%（液体）、>50%（固体）	>40%

5．追走的バリデーション研究

開発者の J-TEC（リードラボ）とは異なる 3 施設（株式会社薬物安全性試験センター、日本コルマー株式会社、富士フィルム株式会社）で LabCyte CORNEA-MODEL24 EIT の追走的バリデーション研究が行われた [3)]。これら 3 施設は追走的バリデーション研究の前に技術移転性の確認を行った総当り試験に参加した施設である [8)]。被験物質には OECD の性能標準で指定されている 30 の参照物質を用いた（Appendix 1）。試験はすべての被験物質について 3 施設でそれぞれ 3 回実施された。

5-1．試験法の信頼性

5.1.1. 技術移転性

技術移転性については、追走的バリデーションの実施前に行われた 24 施設が参加した総当り試験

で検討され、良好な結果が得られている[8)]。

5-1-2. 施設内再現性

UN GHS 分類判定の施設ごとの施設内再現性は 93%、97%、100%であった。これらの値は OECD の性能標準が定めた基準（90%以上）を満たした[3)]。

なお、リードラボである J-TEC の社内データ（139 物質）では、施設内再現性は 97%であった[8)]。

5-1-3. 施設間再現性

UN GHS 分類判定の施設間再現性は 87%であった。この値は OECD の性能標準が定めた基準（85%以上）を満たした[3)]。

5-2. 試験法の正確性

正確性の評価には、3 施設で行われた全試験のデータを用いた。その結果、感度 97. 8%、特異度 68.9%、正確度 83.5%であった。これらの値は OECD の性能標準が定めた基準（感度 90%以上、特異度 60%以上、正確度 75%以上）を満たした[3)]。

なお、リードラボである J-TEC の社内データ（139 物質）では、感度 100.0%、特異度 73.0%、正確度 87.8%であった[8)]。

6. 試験法の適用範囲

TG492 は、試験法の適正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) UN GHS 区分 1、区分 2（2A・2B）物質の検出には用いることはできない。

7. 本委員会の結論

LabCyte CORNEA-MODEL24 EIT の追走的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 OECD の性能標準が定めた基準を満たしていた。

LabCyte CORNEA-MODEL24 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技術習得がガイドラインの習熟度確認物質（Appendix2）で確かめられていれば、LabCyte CORNEA-MODEL24 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。

8. 文献

1) OECD (2018) 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.

2) OECD (2015) Performance Standards for the Assessment of Proposed Similar or Modified In Vitro Reconstructed Human Cornea-like Epithelium (RhCE) Test Methods for Eye Hazards, Series on Testing & Assessment No. 216

3) LabCyte Validation Management Team (2017) Me-too Validation Report: Validation Study for LabCyte CORNEA-MODEL24 Eye Irritation Test (Version. 3.0), JaCVAM

4) Peer Review Panel (2017) Validation status of the LabCyte CORNEA-MODEL24 EYE IRRITTION TEST, Report of the Peer Review Panel on a JaCVAM co-ordinated study programme addressing the validation status of LabCyte CORNEA-MODEL24 EYE IRRITION TEST for discriminating eye irritant from non eye irritant substances

5) Katoh, M et al (2013) Establishment of a new *in vitro* test method for evaluation of eye irritation using a reconstructed human corneal epithelial model, LabCyte CORNEA-MODEL, Toxicol. in Vitro 27, 2184

6) Ishiyama, M. et al (1997) A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability, Talana 44, 1299

7) Japan Tissue Engineering Co., Ltd. (2017) *In Vitro* Eye Irritation Test Using Human Corneal Tissue Model: LabCyte CORNEA-MODEL24 (Version. 2.5.6), JaCVAM

8) Katoh, M et al (2017) Background Review Document: New Established *In Vitro* Eye Irritation Test Method using Reconstructed Human Corneal Epithelium, LabCyte CORNEA-MODEL24 (Version 2.2), JaCVAM

Appendix 1

追走的バリデーションに用いられた参照物質

物質名	CAS 番号	性状	UN GHS 分類
(Ethylenediaminepropyl)-trimethoxysilane	1760-24-3	液体	区分 1
Methylthioglycolate	2365-48-2	液体	区分 1
Tetraethylene glycol diacrylate	17831-71-9	液体	区分 1
1,2-Benzisothiazol-3(2H)-one	2634-33-5	固体	区分 1
2,5-Dimethyl-2,5-hexanediol	110-03-2	固体	区分 1
Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis- (benzenesulphonate)	27344-41-8	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
gamma-Butyrolactone	96-48-0	液体	区分 2A
1,5-Naphthalenediol	83-56-7	固体	区分 2A
Sodium benzoate	532-32-1	固体	区分 2A
2-Methyl-1-pentanol	105-30-6	液体	区分 2B
Diethyl toluamide	134-62-3	液体	区分 2B
1,4-Dibutoxy benzene	104-36-9	固体	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	342573-75-5	液体	区分外
2-Ethoxyethyl methacrylate	2370-63-0	液体	区分外
3-Phenoxybenzyl alcohol	13826-35-2	液体	区分外
4-(Methylthio)-benzaldehyde	3446-89-7	液体	区分外
Dipropyl disulphide	629-19-6	液体	区分外
Ethyl thioglycolate	623-51-8	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	区分外
1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	固体	区分外
2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol	3179-89-3	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
4,4'-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	固体	区分外
Cellulose, 2-(2-hydroxy -3-(trimethylammonium) propoxy) ethyl ether chloride (91%)	68610-92-4	固体	区分外
Potassium tetrafluoroborate	14075-53-7	固体	区分外
Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	66170-10-3	固体	区分外

Appendix 2

RhCE 法の習熟度確認物質

物質名	CAS 番号	性状	UN 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:
25 June 2018

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 (IATA) for Serious Eye Damage and Eye irritation (3).

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

In accordance with the Decision of the Council on a Delegation of Authority to amend Annex I of the Decision of the Council on the Mutual Acceptance of Data in the Assessment of Chemicals [C(2018)49], this Guideline was amended by the OECD's Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology by written procedure on 25 June 2018.guideline was adopted by the OECD Council by written procedure on 25 June 2018.

adopted as OECD Test Guidelines (TGs) 437 (4), 438 (5), 460 (6) and 491 (7) to address the human health endpoint serious eye damage or no classification.

4. Three 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 (8)(9)(10)(11)(12)(13)(14)(15) (16) using the EpiOcular™ Eye Irritation Test (EIT), the SkinEthic™ Human Corneal Epithelium (HCE) EIT and the LabCyte CORNEA-MODEL24 EIT. Each of these methods makes use of commercially available RhCE tissue constructs as test system, two of them are referred to in the following text as the Validated Reference Methods – EpiOcular™ EIT (VRM1) and SkinEthic™ HCE EIT (VRM2), respectively. From these validation studies and their independent peer review (10)(13) it was concluded that EpiOcular™ EIT, SkinEthic™ HCE EIT and LabCyte CORNEA-MODEL24 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 (14). Annexes II-V provide a synopsis of the important elements of the test methods, as well as flowcharts providing guidance for specific situations.

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)(16) 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 (17). The Bottom-Up approach 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 is designed to be used when, based on existing information, a chemical is expected to cause serious eye damage. The EpiOcular™ EIT, SkinEthic™ HCE EIT and LabCyte CORNEA-MODEL24 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. However, the EpiOcular™ EIT, SkinEthic™ HCE EIT and LabCyte CORNEA-MODEL24 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 (3). A test chemical that is identified as requiring classification for eye irritation/serious eye damage with EpiOcular™ EIT, SkinEthic™ HCE EIT or LabCyte CORNEA-MODEL24 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, 491, or if necessary TG 405.

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 tetrazolium dye {TD; e.g., MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide; CAS RN 298-93-1] for VRM1 and VRM2, or WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; CAS RN 193149-74-5] for LabCyte CORNEA-MODEL24 EIT } assay (18)(19) (see paragraph 22). 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 (20) are available to facilitate the validation of new or modified *in vitro* RhCE-based test methods similar to EpiOcular™ EIT, SkinEthic™ HCE EIT and LabCyte CORNEA-MODEL24 EIT, in accordance with the principles of Guidance Document No. 34 (21), 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.

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), human immortalized corneal epithelial cells (i.e., SkinEthic™ HCE/S), or primary human corneal epithelial cells (i.e., LabCyte CORNEA-MODEL24). The EpiOcular™ OCL-200, SkinEthic™ HCE/S, and LabCyte CORNEA-MODEL24 RhCE tissue constructs are similar to the *in vivo* corneal epithelium three-dimensional structure and are produced using cells from the species of interest (22)(23)(24). Moreover, the test methods directly measure cytotoxicity resulting from penetration of the chemical through the cornea and production of cell and tissue damage following chemical exposure, which determines the overall *in vivo* serious eye damage/eye irritation response. Cell damage can occur by several modes of action (see paragraph 21), 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 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 (9). 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 (9)(11)(12). 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 (11)(12). The LabCyte CORNEA-MODEL24 EIT catch-up validation database

contained 30 reference chemicals listed in the performance standards (20) of this Test Guideline 492.

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 formazan dye (FD, naturally or after treatment) and test chemicals able to directly reduce the vital dye TD (to FD) 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 each FD (see paragraphs 37-43).

13. Results generated in pre-validation (25)(26)(27) and validation (9)(11)(12)(15) studies have demonstrated that EpiOcular™ EIT, SkinEthic™ HCE EIT and LabCyte CORNEA-MODEL24 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. The level of reproducibility in terms of concordance of predictions that can be expected from LabCyte CORNEA-MODEL24 EIT from data on 30 reference chemicals listed in the performance standards (21) is in the order of 96% within laboratories and 87% 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 (9), 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)(16) 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 (28). 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)(16). 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 (28).

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 (11)(12), 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)(16) classified according to the UN GHS classification system (1).

16. The LabCyte CORNEA-MODEL 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 catch up validation study (15), the LabCyte CORNEA-MODEL EIT meets the criteria for reproducibility and reproductive capacity, as required by the Performance Standards of this Gguideline (20). Additionally, a database consisting of 139 chemicals were tested by the tissue construct developer (unpublished data).

17. The false negative rates obtained with these RhCE test methods with either substances or mixtures are within the *in vivo* Draize eye test within-test variability of 12 % (29). The false positive rates obtained with any 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 45) will require further testing with (an)other *in vitro* test method(s), or as a last option in rabbits, depending on regulatory requirements, according to the OECD Guidance Document on an Integrated Approaches on Testing and Assessment for Serious Eye Damage and Eye irritation (3). 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, SkinEthic™ HCE EIT and LabCyte CORNEA-MODEL24 EIT under classification schemes other than UN GHS.

18. 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 guidelines is required (3).

19. 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

20. 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, human immortalized corneal epithelial cells, or primary culture human corneal epithelial cells, which have been cultured for several days to form a stratified, highly differentiated squamous epithelium morphologically similar to that found

[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.

in the human cornea. The EpiOcular™ and LabCyte CORNEA-MODEL24 RhCE tissue construct consists of at least 3 viable layers of cells and a non-keratinized surface, showing a corneal-like structure analogous to that found *in vivo* (24). 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 (23)(30).

21. 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) (17)(31)(32). 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 (33)(34). 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 (33) and with the extent of the subsequent responses and eventual outcomes (36). 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 (34)(37). 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.

22. RhCE tissue viability is classically measured by enzymatic conversion of TD (MTT for VRM1 and VRM2, or WST-8 for LabCyte CORNEA-MODEL24 EIT) by the viable cells of the tissue into coloured FD (blue MTT formazan or yellow WST-8 formazan). Blue MTT FD is quantitatively measured after extraction from tissues (18), whereas yellow WST-8 FD does not require extraction because of its water solubility and is quantitatively measured directly from the WST-8 solution on which the tissues are incubated during the WST-8 assay (19). 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% for EpiOcular™ EIT and SkinEthic™ HCE EITL[2]; > 50% for SkinEthic™ HCE EITS[3]; or > 40% for LabCyte CORNEA-MODEL24 EIT) (see paragraph 45).

[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

23. 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 VRM1 and VRM2 (9)(11)(12). 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)(16) 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 (29)(38); (iv) are representative of the chemical classes used in the validation study (9)(11)(12); (v) cover a good and wide representation of organic functional groups (9)(11)(12); (vi) have chemical structures that are well-defined (9)(11)(12); (vii) are coloured and/or direct TD 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 VRMs, could be used. Such deviations should however be justified.

Table 1: List of proficiency chemicals

Chemical Name	CAS RN	Organic Functional Group[1]	Physical state	VRM1 viability (%)[2]	VRM2 viability (%)[3]	LabCyte viability (%)[3-1]	VRM Prediction	TS 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	1.7±1.2	No prediction can be made	Y (strong)	N
Hydroxyethyl acrylate	818-61-1	Acrylate; Alcohol	L	7.5±4.75[5]	1.6±1.0	7.5±4.75	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	2.8±2.6	No prediction can be made	N	N
Sodium oxalate	62-76-0	Oxocarboxylic acid	S	29.0±1.2	5.3±4.1	3.7±1.5	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	0.4±0.4	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	2.9±2.6	No prediction can be made	N	N
In Vivo Category 2B[4]									
Diethyl toluamide	134-62-3	Benzamide	L	15.6±6.3	2.8±0.9	32.4±9.3	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	S	4.7±1.5	15.8±1.1	2.2±2.6	No prediction can be made	N	N

		carbocycles; Cycloalkane							
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	48.0±8.9	No Cat	N	N
Dicaprylyl ether	629-82-3	Alkoxy; Ether	L	97.8±4.3	95.2±3.0	92.7±5.0	No Cat	N	N
Piperonyl butoxide	51-03-6	Alkoxy; Benzodioxole; Benzyl; Ether	L	104.2±4.2	96.5±3.5	95.6±14.0	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	62.6±11.5	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	77.8±9.0	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	90.2±5.8	No Cat	N	N
Potassium tetrafluoroborate	14075-53-7	Inorganic Salt	S	88.6±3.3	92.9±5.1	66.6±0.2	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 FD.

[1]Organic functional group assigned according to an OECD Toolbox 3.1 nested analysis (9).

[2]Based on results obtained with EpiOcular™ EIT in the EURL ECVAM/Cosmetics Europe Eye Irritation Validation Study (EIVS) (9).

[3] Based on results obtained with SkinEthic™ HCE EIT in the validation study (11)(12).

[3-1] Based on results obtained with LabCyte CORNEA-MODEL24 EIT in the validation study (15).

[4]Based on results from the *in vivo* rabbit eye test (OECD TG 405) (2)(15) 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.

24. 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 26, 28 and 31). 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

25. The test methods currently covered by this Test Guideline are the scientifically valid EpiOcular™ EIT, SkinEthic™ HCE EIT and LabCyte CORNEA-MODEL24 EIT(10)(13)(14), the first two being referred as the Validated Reference Methods (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 (39)(40)(41). The following paragraphs and Annex II describe the main components and procedures of the RhCE test methods.

RhCE TEST METHOD Components

GENERAL CONDITIONS

26. 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 or 60 minutes treatment with 25 µL SDS) (see paragraph 31). 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

27. The assay used for quantifying tissue viability is the TD (MTT for VRM1 and VRM2, or WST-8 for LabCyte CORNEA-MODEL24 EIT) assay (18)(20). 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). Alternatively, viable cells of the RhCE tissue construct reduce the vital dye WST-8 into a water soluble yellow formazan. The extracted FD may be quantified using either a standard absorbance (Optical Density (OD)) measurement or an HPLC/UPLC-spectrophotometry procedure (42). The OD of the blank solution alone (which is the extraction solvent for MTT assay, or the diluted WST-8 medium for the WST-8 assay) 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**
LabCyte CORNEA-MODEL24 EIT (for both the liquids and the solids protocols)	**≥ 0.5**	**≤ 1.6**

[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 (43).

Barrier function

28. 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 31).

Morphology

29. 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 three test methods, 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

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

Quality control (QC)

31. 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 27) and barrier function (see

paragraph 28) 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 26 and 28) 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 test methodsis 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 criteria

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 minutes	**ET_{50} = 37.5 minutes**
SkinEthic™ HCE EIT (HCE/S) – VRM2 (30 minutes treatment with 50 µL SDS)	IC_{50} = 1.0 mg/mL	**IC_{50} = 3.2 mg/mL**
LabCyte CORNEA-MODEL24 EIT – (60 minutes treatment with 25 µL SDS)	**IC_{50} = 1.0 mg/mL**	**IC_{50} = 4.0 mg/mL**

Application of the Test Chemical and Control Substances

32. 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 (39)(40)(41). For the two VRMs, 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 the human eye. The treatment of the tissues is initiated with exposure to the test chemical(s) and control substances. For any treatment protocols in any of the two 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 33 and 34) (Annex II).

33. 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 three test methdos, otherwise they should be treated as solids (see paragraph 34). In the test methods, liquid test chemical are evenly spread over the tissue surface (i.e. a minimum of 60 µL/cm^2 application) (see Annex II, (39)(40)(41). 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 1 minute (LabCyte CORNEA-MODEL24 EIT) or 30 minutes (VRM1 and VRM2) at the standard conditions of each method. 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. For the two VRMs, this rinsing step should be 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 and for LabCyte CORNEA-MODEL24 EIT, a post-exposure incubation in fresh medium at standard culture conditions is applied prior to performing the TD assay (see Annex II, (39)(40)(41)).

34. Test chemicals that cannot be pipetted at temperatures up to 37°C are treated as solids in the three test methods. The amount of test chemical applied should be sufficient to cover the entire surface of the tissue, i.e. a minimum of 33 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 method used) at standard culture conditions (see Annex II, (39)(40)(41)). 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. For the two VRMs, this rinsing step should be 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, followed by a post-exposure incubation in fresh medium at standard culture conditions, prior to performing the TD assay (see Annex II, (39)(40)(41)(42)).

35. 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 RN. 79-20-9, commercially available from e.g., Sigma-Aldrich, Cat# 45997; liquid) for both liquids and solids protocols. The recommended positive control substances to be used with the LabCyte CORNEA-MODEL24 EIT are ethanol (CAS RN. 64-17-5) for liquids protocol and lauric acid (CAS RN 143-07-7) for solids protocol. The recommended negative control substances to be used with the VRM1 and VRM2 are ultrapure H_2O and Ca^{2+}/$Mg2^{+}$-free DPBS for both liquids and solids protocols, respectively. The recommended negative control to be used with the LabCyte CORNEA-MODEL24 EIT is Ca^{2+}/Mg^{2+}-free DPBS (CAS RN. 64-17-5) for liquids protocol and no treatment for solids protocol. 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 33) or for controls run concurrently to solid test chemicals (see paragraph 34), prior to performing the TD assay (see paragraph 36) (39)(40)(41). 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

36. The TD assay is a standardised quantitative method (18)(19) that should be used to measure tissue viability under this Test Guideline. It is compatible with use in a three-

dimensional tissue construct. The TD assay is performed immediately following the post-exposure procedures. The VRMs use the MTT assay. In the VRMs, the RhCE tissue construct sample is placed in 0.3 mL of MTT solution at 1 mg/mL for 180±15 minutes at standard culture conditions. The vital dye MTT is reduced into a blue MTT formazan precipitate by the viable cells of the RhCE tissue construct. The precipitated blue MTT formazan product is then extracted from the tissue using an appropriate volume of isopropanol (or a similar solvent) (39)(40). 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. LabCyte CORNEA-MODEL24 EIT uses the WST-8 assay. In the LabCyte CORNEA-MODEL24 EIT, the RhCE tissue construct sample is placed in 0.3 mL of diluted WST-8 solution, prepared according to the standard operating procedures (41), for 240 minutes at standard culture conditions and the vital dye WST-8 is reduced into a yellow WST-8 formazan by the viable cells of the RhCE tissue construct, which is dissolved into the diluted WST-8 solution (41). The concurrently tested negative and positive control substances should be treated similarly to the tested chemical. In the VRM1 and VRM2, 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 43) (12)(42). In the LabCyte CORNEA-MODEL24 EIT, the WST-8 formazan may be directly quantified (i.e. without the need of an extraction procedure) either by a standard absorbance (OD) measurement at 450 nm using a filter band pass of maximum ±30 nm or by using an HPLC/UPLC-spectrophotometry procedure (see paragraph 43) .

37. Optical properties of the test chemical or its chemical action on TD (MTT for VRM1 and VRM2, or WST-8 for LabCyte CORNEA-MODEL24 EIT) may interfere with the measurement of FD leading to a false estimate of tissue viability. Test chemicals may interfere with the measurement of FD by direct reduction of the TD into coloured FD (blue MTT formazan or yellow WST-8 formazan) and/or by colour interference if the test chemical absorbs, naturally or due to treatment procedures, in the same OD range as FD (i.e., MTT formazan: around 570 nm; WST-8 formazan: around 450 nm). Pre-checks should be performed before testing to allow identification of potential direct TD 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 38-42). 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 TD assay is performed. For test chemicals absorbing light in the same range as FD (naturally or after treatment), which are not compatible with the standard absorbance (OD) measurement of FD due to strong interference, i.e., strong absorption at 570±30 nm (with MTT formazan) or 450±30 nm (with WST-8 formazan), an HPLC/UPLC-spectrophotometry procedure to measure FD may be employed (see paragraphs 42 and 43) (11)(40). A detailed description of how to detect and correct for direct TD reduction and interferences by colouring agents is available in the test methods' respective SOPs (39)(40)(41). Illustrative flowcharts providing guidance on how to identify and handle direct TD-reducers and/or colour interfering chemicals for VRM1, VRM2 and LabCyte CORNEA-MODEL24 EIT are also provided in Annexes III, IV and V, respectively.

38. To identify potential interference by test chemicals absorbing light in the same range as FD (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, (39)(40)(41). 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) and LabCyte CORNEA-MODEL24 EIT(see Annex V), the test chemical is presumed to interfere with the standard absorbance (OD) measurement of FD and further colurant controls should be performed or, alternatively, an HPLC/UPLC-spectrophotometry procedure should be used in which case these controls are not required (see paragraphs 42 and 43 and Annexes III, IV and V)(39)(40)(41). 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 TD solution during the TD incubation step, to generate a non-specific colour in living tissues (NSC_{living}) control (39)(40)(41). 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 the MTT or WST-8 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 or WST-8, run concurrently to the test being corrected ($\%NSC_{living}$), i.e., True tissue viability = [$\%Viability_{test}$] - [$\%NSC_{living}$].

39. To identify direct MTT or WST-8 reducers, each test chemical should be added to freshly prepared TD. An appropriate amount of test chemical is added to a TD solution and the mixture is incubated for approximately 3 or 4 hours at standard culture conditions (see Annexes III, IV and V) (39)(40)(41). If the TD mixture containing the test chemical (or suspension for insoluble test chemicals) turns blue/purple (for MTT solution) or yellow/orange (for WST solution), the test chemical is presumed to directly reduce the TD 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"). Killed tissues of LabCyte CORNEA-MODEL24 EIT are prepared by freezing tissues at -80°C or below, for 30 minutes twice (“freeze-killed”). Each TD reducing test chemical is applied on at least two killed tissue replicates, which undergo the entire testing procedure, to generate a non-specific TD reduction (NSMTT or NSWST) control (39)(40)(41). A single NSMTT or NSWST 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 TD reducer ($\%Viability_{test}$) minus the percent non-specific TD reduction obtained with the killed tissues exposed to the same reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT or %NSWST), i.e. true tissue viability = [$\%Viability_{test}$] - [%NSMTT or %NSWST].

40. Test chemicals that are identified as producing both colour interference (see paragraph 38) and direct TD reduction (see paragraph 39) will also require a third set of controls when performing the standard absorbance (OD) measurement, apart from the NSMTT or NSWST, 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 for MTT formazan (e.g., blue, purple, black) or with lightly colored test chemicals absorbing light in the range of 450±30 nm for WST-8 formazan (e.g. yellow, orange) because their intrinsic colour impedes the assessment of their capacity to directly reduce MTT or WST-8 as described in paragraph 39. This forces the use of NSMTT or NSWST controls, by default, together with the NSC_{living} controls. Test chemicals for which both NSMTT or NSWST, and NSC_{living} controls are performed may be absorbed and retained by both living and killed tissues. Therefore, in this case, the NSMTT or NSWST control may not only correct for potential direct TD 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)(39)(40)(41). 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 TD solution during the TD 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 or NSWST 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 or %NSWST minus $\%NSC_{living}$ plus the percent non-specific colour obtained with killed tissues exposed to the interfering test chemical and incubated with medium without TD, calculated relative to the negative control ran concurrently to the test being corrected ($\%NSC_{killed}$), i.e., True tissue viability = [$\%Viability_{test}$] - [%NSMTT or %NSWST] - [$\%NSC_{living}$] + [$\%NSC_{killed}$].

41. It is important to note that non-specific TD reduction and non-specific colour interferences may increase the OD (when performing standard absorbance measurements) of the sample above the linearity range of the spectrophotometer and that non-specific TD reduction can also increase the FD peak area (when performing HPLC/UPLC-spectrophotometry measurements) of the sample above the linearity range of the spectrophotometer. On this basis, when using RhCEs, it is important for each laboratory to determine the OD/peak area linearity range of their spectrophotometer with MTT formazan (CAS RN# 57360-69-7) which is commercially available from e.g., Sigma-Aldrich (Cat# M2003), or WST-8 formazan (CAS RN193149-76-7), commercially available from Dojindo Molecular Technologies.

42. The standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess direct TD-reducers and colour interfering test chemicals, when the observed interference with the measurement of FD is not strong (i.e., the ODs of the samples obtained with the test chemical without any correction for direct TD reduction and/or colour interference are within the linear range of the spectrophotometer). Nevertheless, results for test chemicals producing %NSMTT or %NSWST and/or $\%NSC_{living} \geq 60\%$ (VRM1, and VRM2 for liquids protocol) or 50% (VRM2 for solids protocol) or 40% (LabCyte CORNEA-MODEL24 EIT) 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 45). Standard absorbance (OD) can however not be measured when the interference with the measurement of FD is strong (*i.e.*, leading to uncorrected ODs of the test samples 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 strongly with the standard absorbance (OD) measurement of FD may still be assessed using HPLC/UPLC-spectrophotometry (see Annexes III, IV and V). This is because the HPLC/UPLC system allows for the separation of the FD from the chemical before its quantification (40). For this reason, NSC_{living} or NSC_{killed} controls are never required when using HPLC/UPLC-spectrophotometry, independently of the chemical being tested. NSMTT or NSWST controls should nevertheless be used if the test chemical is suspected to directly reduce TD (following the procedure described in paragraph 39). NSMTT or NSWST 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 TD as described in paragraph 39. When using HPLC/UPLC-spectrophotometry to measure FD, the percent tissue viability is calculated as percent FD peak area obtained with living tissues exposed to the test chemical relative to the FD peak obtained with the concurrent negative control. For test chemicals able to directly reduce TD, true tissue viability is calculated as: $\%Viability_{test}$ minus %NSMTT or %NSWST, as described in the last sentence of paragraph 39. Finally, it should be noted that direct TD-reducers or direct TD-reducers that are also colour interfering, which are retained in the tissues after treatment and reduce TD so strongly that they lead to ODs (using standard OD measurement) or peak areas (using UPLC/HPLC-spectrophotometry) of the tested samples 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.

43. HPLC/UPLC-spectrophotometry may be used with all types of test chemicals (coloured, non-coloured, TD-reducers and non-TD reducers) for measurement of FD (12)(42). 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 TD from samples 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 (42)(44). These key parameters and their acceptance criteria are shown in Annex IV. Once the acceptance criteria defined in Annex VI have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure FD under the experimental conditions described in this Test Guideline.

Acceptance Criteria

44. For each run using RhCE tissue batches that met the quality control (see paragraph 31), 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 27). Similarly, tissues treated with the positive control substance, i.e., methyl acetate (for VRM1 and VRM2), ethanol (for LabCyte CORNEA-MODEL24 EIT with liquid protocol) or lauric acid (LabCyte CORNEA-MODEL24 EIT with solid protocol), should show a mean tissue viability < 50% relative to the negative control in the VRM1 with either the liquids or the solids protocols, ≤ 30% (liquids protocol) or ≤ 20% (solids

protocol) relative to the negative control in the VRM2, and ≤ 40% relative to the negative control in the LabCyte CORNEA-MODEL24 EIT, thus reflecting the ability of the tissues to respond to an irritant test chemical under the conditions of the test method (39)(40)(41). 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

45. The OD values/peak areas obtained with the replicate samples 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 from this result in isolation, 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 14-16) and cannot resolve between UN GHS Categories 1 and 2 (see paragraph 18). The “No prediction can be made” result of the RhCE test in these cases, would require additional information for classification purposes [see (3) for guidance].

Table 4: Prediction Models according to UN GHS classification

Test Method	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%**
LabCyte CORNEA-MODEL24 EIT (for both protocols)	**Mean tissue viability > 40%**	**Mean tissue viability ≤ 40%**

46. 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), 50±5% (VRM2 for solids' protocol) or 40±5% (LabCyte CORNEA-MODEL24 EIT), a second test should be considered, as well as a third one in case of discordant results between the first two tests.

47. 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

48. Data from individual replicate tissues in a run (*e.g.*, OD values/FD 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 FD through direct TD reduction and/or coloured interference should be reported for each tested chemical.

TEST REPORT

49. 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 those referenced in Annex II , if applicable;
- Justification for the use of a different positive control than those referenced in Annex II , 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 FD, and linearity range of measuring device (e.g., spectrophotometer);
- Description of the method used to quantify FD;

- 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 TD-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (positive control, negative control, NSMTT or NSWST, 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 FD 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 TD-reducers and/or coloured test chemicals, including OD value or FD peak area, %NSMTT or %NSWST, $\%NSC_{living}$,

$\%NSC_{killed}$, 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 (21).

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) (3).

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.

Formazan dye (FD): Chromogenic product of the reduction of MTT and WST-8.

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 or 60 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. (CAS RN 298-93-1)

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.

NSWST: Non-Specific WST-8 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 (21).

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 (19).

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 (21).

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 (21).

Reproducibility: The agreement among results obtained from repeated testing of the same test chemical using the same test protocol (See "Reliability") (21).

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 (21).

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

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.

Tetrazolium dye (TD) : Tetrazolium salts MTT and WST-8.

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) (3).

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 (21).

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 (21).

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 (21).

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 chemical.

WST: Water soluble tetrazolium salt.

WST-8: Water soluble tetrazolium salt-8. [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt. (CAS RN 193149-74-5).

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 Component	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)		LabCyte CORNEA-MODEL24 EIT	
Protocol	Liquids (pipetteable at 37±1°C or lower temperatures for 15 min)	Solids (not pipetteable)	Liquids and viscous (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solid (not pipetteable)
Model surface	0.6 cm^2	0.6 cm^2	0.5 cm^2	0.5 cm^2	0.3 cm^2	0.3 cm^2
Number of tissue replicates	At least 2	At least 2	At least 2	At least 2	3 tissues	3 tissues
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	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, 18-28ºC) → 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	50 µL + 0.5 mL distilled water mixed for 15 minutes at 37±2ºC, 5±1% CO_2, ≥95% RH. If test chemical is coloured, living adapted controls should be performed	10 mg + 0.5 mL distilled water mixed for 15 minutes at 37±2ºC, 5±1% CO_2, ≥95% RH. If test chemical is coloured, living adapted controls should be performed

	performed.					

Test Method Component	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)		LabCyte CORNEA-MODEL24 EIT	
Protocol	Liquid	Solid	Liquid	Solid	Liquid	Solid
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)	50 µL + 300 µL diluted WST-8 medium for 240±20 minutes at 37±2ºC, 5±1% CO_2, ≥95% RH If solution turns yellow, freeze-killed adapted controls should be performed.	10 mg + 300 µL diluted WST-8 medium for 240±20 minutes at 37±2ºC, 5±1% CO_2, ≥95% RH If solution turns yellow, freeze-killed adapted controls should be performed.
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)	50 µL (167 µL/cm^2)	10 mg (33 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	1 minutes (± 5 second) in culture medium at RT	24 hours (± 1 hr) 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	10 times or over by stream of Ca^{2+}/Mg^{2+} -free DPBS	10 times or over by stream of 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	-	-

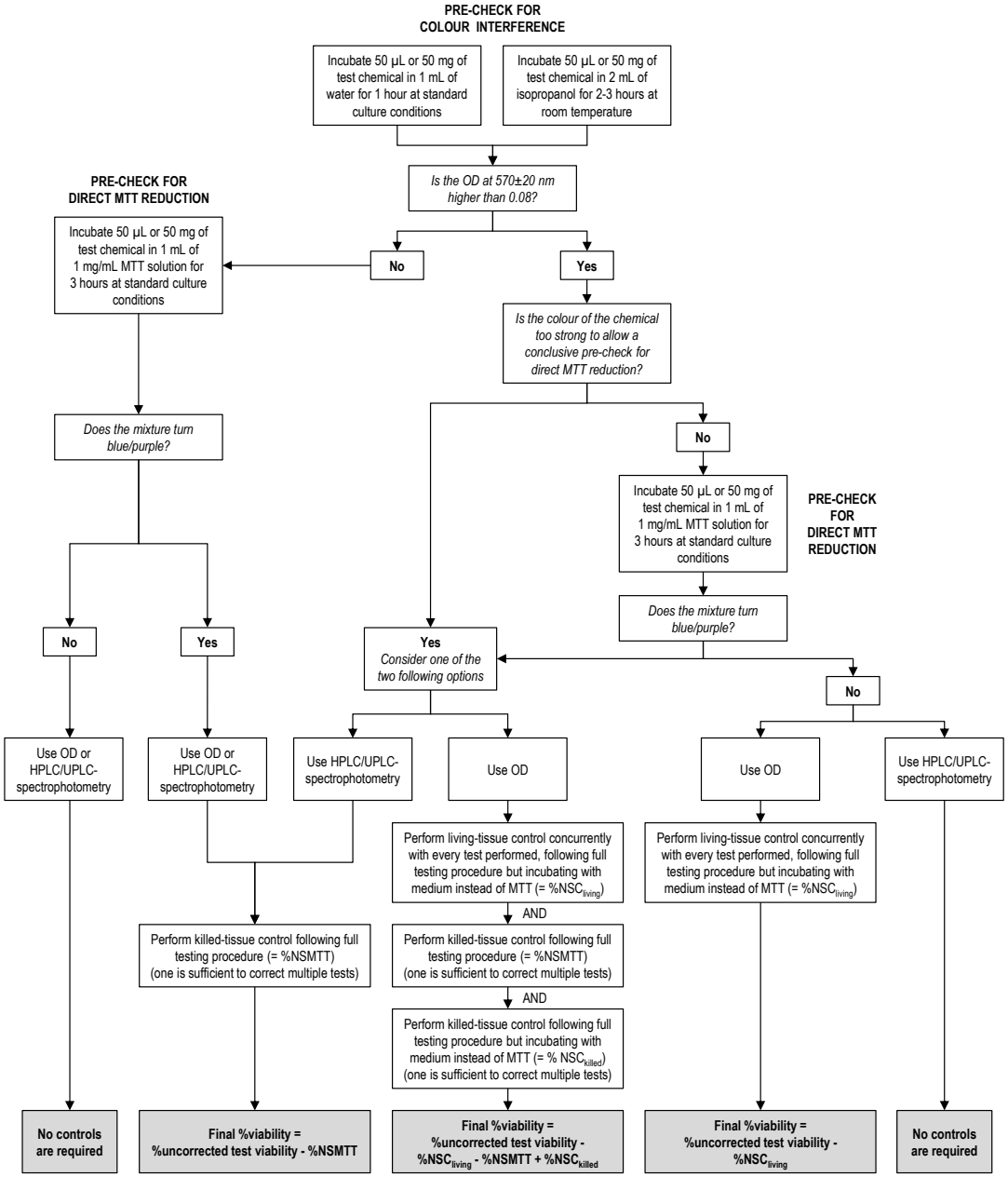
Test Method Component	**EpiOcular™ EIT (VRM 1)**		**SkinEthic™ HCE EIT (VRM 2)**		**LabCyte CORNEA-MODEL24 EIT**	
Protocol	Liquid	Solid	Liquid	Solid	Liquid	Solid
Post-exposure incubation	120 min (± 15 min) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	18 h (± 0.25 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	none	18 h (± 0.5 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	24 h (± 1 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	none
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	50 µL Ca^{2+}/Mg^{2+} -free DPBS Tested concurrently	No treatment Tested concurrently
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	50 µL Ethanol Tested concurrently	10 mg Lauric acid Tested concurrently
Tetrasolium salt solution	300 µL 1 mg/mL	300 µL 1 mg/mL	300 µL 1 mg/mL	300 µL 1 mg/mL	300 µL of diluted WST-8 solution (10 fold dilution of CCK-8)	300 µL of diluted WST-8 solution (10 fold dilution of CCK-8)
Tetrasolium salt incubation 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	240 minutes (± 15 minutes) at 37±2ºC, 5±1% CO_2, ≥95% RH	240 minutes (± 15 minutes) 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)	Not required	Not required
Extraction time	2-3 h with shaking (~120	2-3 h with shaking	4 h with shaking (~120	At least 2 h with	Not required	Not required

and temperature	rpm) at RT or overnight at 4-10°C	(~120 rpm) at RT or overnight at 4-10°C	rpm) at RT or at least overnight without shaking at 4-10°C	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	450 nm with reference filter (650 nm)	450 nm with reference filter (650 nm)
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	60 minutes treatment with SDS (25 µL) 1.0 mg/mL ≤ IC_{50} ≤ 4.0 mg/mL	60 minutes treatment with SDS (25 µL) 1.0 mg/mL ≤ IC_{50} ≤ 4.0 mg/mL

Test Method Component	**EpiOcular™ EIT (VRM 1)**		**SkinEthic™ HCE EIT (VRM 2)**		**LabCyte CORNEA-MODEL24 EIT**	
Protocol	Liquid	Solid	Liquid	Solid	Liquid	Solid
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 minutes 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 minutes 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%.	1. Mean OD of the tissue replicates treated with the negative control should be ≥ 0.5 and ≤ 1.3. 2. Mean viability of the tissue replicates treated with the positive control should be ≤ 40% 3. The standard deviation (SD) between three tissue replicates should not exceed 18%	1. Mean OD of the tissue replicates treated with the negative control should be ≥ 0.5 and ≤ 1.3. 2. Mean viability of the tissue replicates treated with the positive control should be ≤ 40% 3. The standard deviation (SD) between three tissue replicates should not exceed 18%

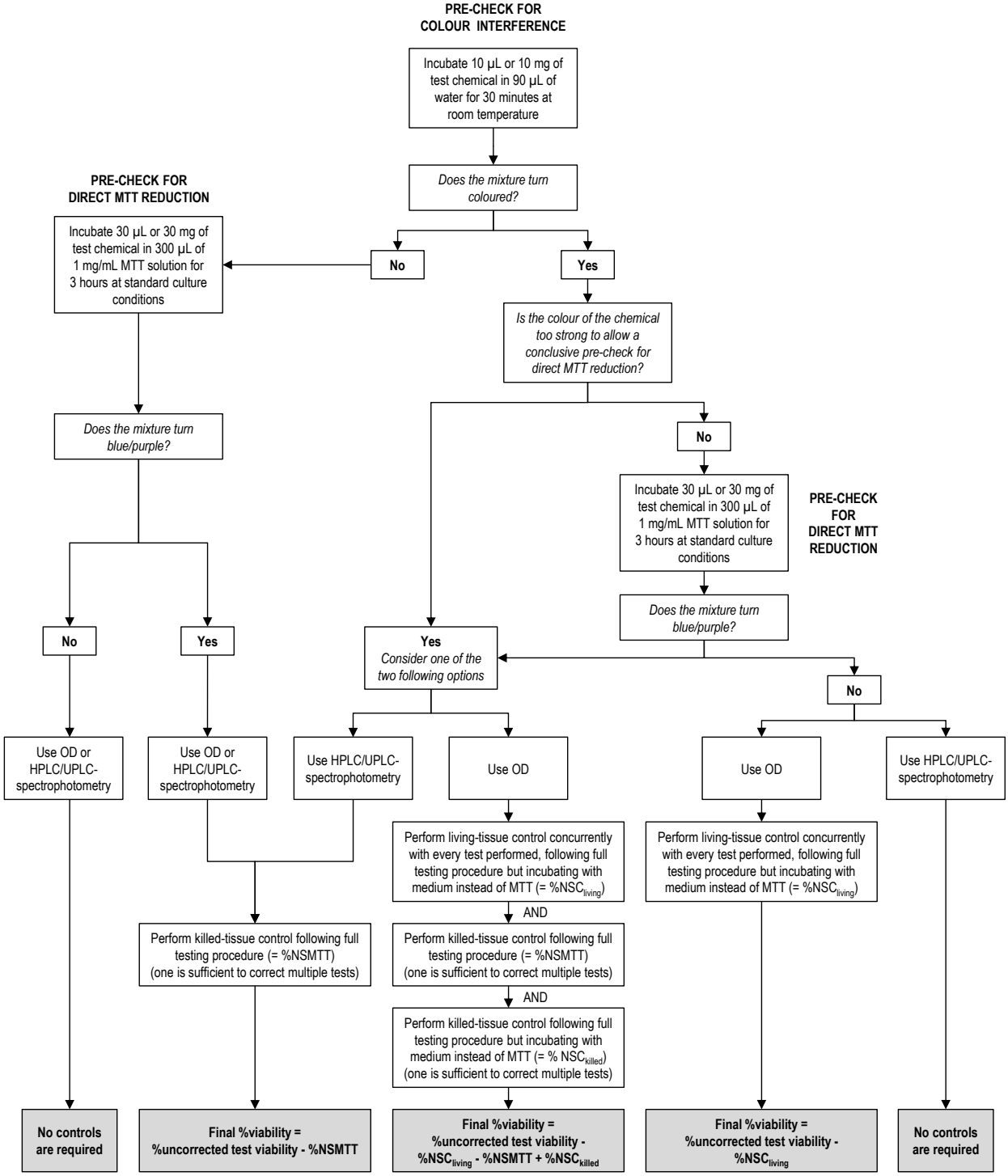
ANNEX III

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



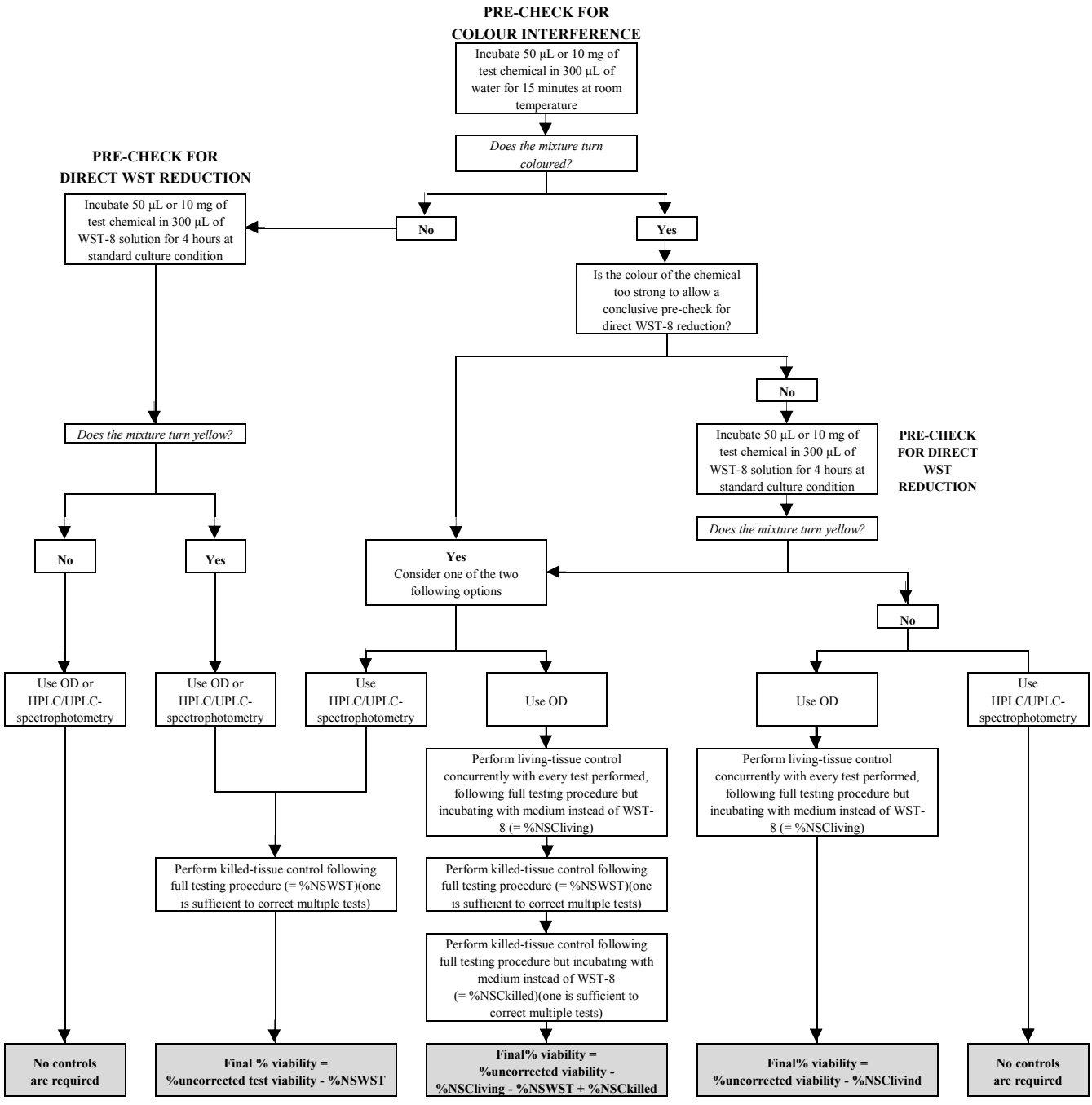
ANNEX IV

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

ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT WST-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE LABCYTE CORNEA-MODEL24 EIT SOP



ANNEX VI

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 (43)(45)	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 AreaLLOQ[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.