新規試験法提案書

眼刺激性試験代替法　Vitrigel®-EIT法

令和 4 年 6 月

国立医薬品食品衛生研究所

新規試験法提案書

令和 4 年 6 月 30 日

No.　2022-01

眼刺激性試験代替法Vitrigel®-EIT法に関する提案

令和 4 年 5 月 10 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： Vitrigel®- EIT 法は適用除外を理解した上で使用し、陰性の結果が得られた場合、UN GHS 区分に該当しない眼刺激性物質を検出する方法として用いることができる。なお、本試験法の利用にあたっては、適用範囲外を十分に配慮した上で使用されるべきである。

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

以上の理由により、行政当局の安全性評価方法として眼刺激性試験代替法 Vitrigel®-EIT 法の使用を提案するものである。

西川秋佳

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

西 川 秋 佳 （国立医薬品食品衛生研究所 病理部/済生会宇都宮病院）：座長
板 垣　 宏 （ITACS コンサルティング）
中村るりこ （独立行政法人 製品評価技術基盤機構）
西 村 次 平 （独立行政法人 医薬品医療機器総合機構）
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）
松 本 一 彦 （名古屋市立大学大学院）

任期：令和 2 年 4 月 1 日～令和 4 年 3 月 31 日

西 川 秋 佳 （国立医薬品食品衛生研究所 病理部/名古屋徳洲会総合病院）：座長
小 島 幸 一 （一般財団法人 食品薬品安全センター）
中村るりこ （独立行政法人 製品評価技術基盤機構）
西 村 次 平 （独立行政法人 医薬品医療機器総合機構）
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任期：令和 4 年 4 月 1 日～令和 6 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）
大久保貴之 （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）
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）
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）
合 田 幸 広 （国立医薬品食品衛生研究所）
杉 山 圭 一 （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）
髙 橋 祐 次 （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）
林　亜紀子 （厚生労働省 医薬・生活衛生局 医薬品審査管理課）
笛 木　修 （独立行政法人 医薬品医療機器総合機構）
増 村 健 一 （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）
横 田 雅 彦 （独立行政法人 医薬品医療機器総合機構）
足利太可雄 （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
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 Vitrigel®-EIT, an alternative method for evaluating ocular irritation

At a meeting held on 10 May, 2022 at National Institute of Health Sciences (NIHS) in Tokyo, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Proposal: Vitrigel®-EIT (Eye Irritancy test) method can be used for eye irritation test to identify chemical substances that do not require classification and labelling under the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) category, if negative results are obtained after understanding exemptions. Furthermore, thorough consideration must be given to the applicability domain when using this test.

This statement was released following a review prepared by the eye irritation test JaCVAM Editorial Committee to acknowledge that the results of the review and study by the JaCVAM Regulatory Acceptance Board have confirmed the usefulness of this assay.

Based on the above, we proposed the Vitrigel®-EIT method as a useful means for assessing eye irritation potential during safety assessments by regulatory agencies.

Akiyoshi Nishikawa
Chairperson,
JaCVAM Regulatory Acceptance Board

Yoko Hirabayashi
Chairperson,
JaCVAM Steering Committee

June 30, 2022

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. Akiyoshi Nishikawa (Division of Pathology, National Institute of Health Sciences: NIHS / Saiseikai Utsunomiya Hospital) : Chairperson
Ms. Yoko Hirabayashi (Center for Biological Safety and Research: CBSR, NIHS)
Mr. Hiroshi Itagaki (ITACS Consulting)
Mr. Kazuhiko Matsumoto (Nagoya City University)
Ms. Ruriko Nakamura (National Institute of Technology and Evaluation)
Mr. Jihei Nishimura (Pharmaceuticals and Medical Devices Agency)

Term: From 1st April 2020 to 31st March 2022

Mr. Akiyoshi Nishikawa (Division of Pathology, NIHS / Nagoya Tokushukai General Hospital) : Chairperson
Ms. Yoko Hirabayashi (CBSR, NIHS)
Mr. Koichi Kojima (Food and Drug Safety Center)
Mr. Kazuhiko Matsumoto (Nagoya City University)
Ms. Ruriko Nakamura (National Institute of Technology and Evaluation)
Mr. Jihei Nishimura (Pharmaceuticals and Medical Devices Agency)

Term: From 1st April 2022 to 31st March 2024

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 (CBSR, NIHS): Chairperson
Mr. Osamu Fueki (Pharmaceuticals and Medical Devices Agency)
Mr. Yukihiro Goda (NIHS)
Ms. Akiko Hayashi (Ministry of Health, Labour and Welfare)
Mr. Koji Ishii (National Institute of Infectious Diseases)
Mr. Yasunari Kanda (Division of Pharmacology, CBSR, NIHS)
Mr. Satoshi Kitajima (Division of Toxicology, CBSR, NIHS)
Mr. Kenichi Masumura (Division of Risk Assessment, CBSR, NIHS)
Ms. Kumiko Ogawa (Division of Pathology, CBSR, NIHS)
Mr. Takayuki Okubo (Ministry of Health, Labour and Welfare)
Mr. Keiichi Sugiyama (Division of Genetics and Mutagenesis, CBSR, NIHS)
Mr. Yuhji Taquahashi (Animal Management Section of the Division of Toxicology, CBSR, NIHS)
Mr. Masaaki Tsukano (Ministry of Health, Labour and Welfare)
Mr. Masahiko Yokota (Pharmaceuticals and Medical Devices Agency)
Mr. Takao Ashikaga (Division of Risk Assessment, CBSR, NIHS): Secretary
Mr. Hajime Kojima (Division of Risk Assessment, CBSR, NIHS): Secretary

眼刺激性試験代替法Vitrigel®-EIT法に関する提案

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評価会議報告書

眼刺激性試験代替法　Vitrigel®-EIT 法

JaCVAM 評価会議

令和 4 年(2022 年)5 月 11 日

JaCVAM 評価会議

西 川 秋 佳 （国立医薬品食品衛生研究所 病理部/済生会宇都宮病院）： 座長
板 垣　 宏 （ITACS コンサルティング）
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任期：令和 2 年 4 月 1 日～令和 4 年 3 月 31 日

西 川 秋 佳 （国立医薬品食品衛生研究所 病理部/名古屋徳洲会総合病院）：座長
小 島 幸 一 （一般財団法人 食品薬品安全センター）
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松 本 一 彦 （名古屋市立大学大学院）

任期：令和 4 年 4 月 1 日～令和 6 年 3 月 31 日

略語

EIT： Eye Irritancy Test（眼刺激性試験）

GHS： Globally Harmonized System of Classification and Labelling of Chemicals
（化学品の分類および表示に関する世界調和システム）

JaCVAM：Japanese Center for the Validation of Alternative Methods
（日本動物実験代替法評価センター）

OECD： Organisation for Economic Co-operation and Development（経済協力開発機構）

TEER： Trans Epithelial Electrical Resistance（経上皮電気抵抗）

TG： Test Guideline（試験法ガイドライン）

UN： United Nations（国際連合）

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「眼刺激性試験代替法 Vitrigel®-EIT 法評価書」[1]をもとに本試験法の科学的妥当性、社会的および行政的な受け入れについて検討した。

1. 試験法の定義

名称：眼刺激性試験代替法 Vitrigel®-EIT（Eye Irritancy Test）法

代替する対象毒性試験：ウサギを用いる眼刺激性試験 [2]

試験法の概略：本試験法では、ウサギ眼の代わりにコラーゲン Vitrigel®[3]膜上で培養したヒト角膜上皮組織シート型培養モデルの経上皮バリア機能を反映する TEER 値の経時変化を用い、眼刺激性を評価する。

試験法の科学的妥当性：

本試験法である Vitrigel®-EIT法 は、ヒト角膜上皮組織シート型培養モデルに被験物質を曝露した際の経上皮バリア機能の経時変化を指標として、被験物質の眼刺激性の有無を判定する試験法である[4]。

経上皮バリア機能を反映する TEER 値の経時変化から、被験物質の UN GHS 区分における区分に該当しないと判定する試験法であり、科学的にも妥当である。バリデーション研究の結果、本試験法はバリデーション実行委員会の定めた再現性の基準を満たしていた [5]。また、追加検証の結果、適用除外を理解した上で使用すれば、感度も十分であると確認された [6]。

2. 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、通常の培養技術を習熟した施設であれば実施できる試験法である。OECD TG494[6]に承認された HCE-T 細胞は理研バイオリソース研究センターから提供を受けることができ、Vitrigel®は市販されており、簡便に短時間で UN GHS 区分に該当しない眼刺激性物質を評価できる。また、生きた動物を用いないという点で、3Rs の精神に合致しており、社会的受け入れ性は高い。

行政上の利用性：

Vitrigel®- EIT 法の適用除外を理解した上で使用し、陰性の結果が得られた場合、UN GHS 区分に該当しない眼刺激性物質を検出する方法として用いることができる。なお、本試験法の利用にあたっては、適用範囲を十分に配慮した上で使用されるべきである。

参考文献

1) JaCVAM　眼刺激性試験資料編纂委員会：眼刺激性試験代替法 Vitrigel®-EIT 法評価書（2022 年 4 月 8 日）

2) OECD (2021) Test Guideline 405, Acute Eye Irritation/Corrosion

3) Takezawa,T., Ozaki, K., Nitani, A., Takabayashi, C., Shimo-Oka, T., Collagen vitrigel: A novel scaffold that can facilitate a three-dimensional culture for reconstructing organoids. *Cell Transplant,* 13 (2004):463–473.

4) Takezawa, T., Nishikawa, K., and Wang, P. C., "Development of a human corneal epithelium model utilizing a collagen vitrigel membrane and the changes of its barrier function induced by exposing eye irritant chemicals," *Toxicology In Vitro* 25 (2011a): 1237–1241.

5) VITRIGEL-EIT Validation Management Team (2017) Validation Study of the Vitrigel-EIT method as an alternative to in vivo eye irritation testing Study Report, Version 2.0.

6) OECD (2021) Test Guideline 494, Vitrigel-Eye Irritancy Test Method for Identifying Chemicals not requiring Classification and Labelling for Eye Irritation or Serious Eye Damage

評価報告書

眼刺激性試験代替法 Vitrigel®-EIT 法

眼刺激性試験資料編纂委員会

令和 4 年(2022 年)4 月 8 日

眼刺激性試験資料編纂委員会

山本　直樹　（委員長：藤田医科大学/金沢医科大学）
佐々木 正治　（アレクシオンファーマ合同会社）
竹内　小苗　（P&G イノベーション合同会社）
波多野 浩太　（ホーユー株式会社）
原　　範 子　（藤田医科大学）
山下　晴洋　（大正製薬株式会社）

略語

CAS：	Chemical Abstracts Services
EIT：	Eye Irritancy Test
IATA：	Integrated Approaches to Testing and Assessment
GHS：	Globally Harmonized System of Classification and Labelling of Chemicals
JaCVAM：	Japanese Center for the Validation of Alternative Methods
OECD：	Organization for Economic Co-operation and Development
RhCE：	Reconstructed human Cornea-like Epithelium
TEER：	Trans Epithelial Electrical Resistance
TG：	Test Guideline
UN：	United Nations

要旨

Vitrigel®-EIT（Eye Irritancy Test）法は、コラーゲンVitrigel®膜上で培養したヒト角膜上皮組織シート型培養モデルの経上皮バリア機能を指標として、TEER（Trans Epithelial Electrical Resistance）を測定し、その物質の眼刺激性を評価する試験法である。経済協力開発機構（OECD：Organization for Economic Co-operation and Development）の試験法ガイドライン（TG：Test Guideline）494としてボトムアップ方式で国際連合化学品の分類および表示に関する世界調和システム（UN GHS: United Nations Globally Harmonized System of Classification and Labelling of Chemicals）区分に該当しない物質を検出する方法として採択されている。本報告書では、Vitrigel®-EIT法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。

Vitrigel®-EIT 法の信頼性・正確性を確認するため、36 物質を用いて 3 施設でバリデーション研究が行われた。この研究において Vitrigel®- EIT 法の施設内再現性はそれぞれの施設で 80%、90%、100%、また、施設間再現性は 92%であり、再現性はバリデーション実行委員会の定めた基準を満たした。一方、正確性は、感度 75~83%、特異度 42%、正確度 64~69%であった。この値は行政的利用を目指す試験法にとって十分なものではなく、開発者は予測性を改良するため、適用除外を「培養液に 2.5%になるよう混合した調製物において、pH が5 以下となる酸性の物質および調製後 3 分以内の短時間に相分離を示す物質」と定めた。これにより、開発者が改良プロトコルを用いて実施した 158 物質のデータ（バリデーション結果を含む）から、適用除外を考慮した 107 物質のボトムアップ方式による感度は 96%、特異度は 67%、正確度は 81%となった。

以上より、本委員会は、Vitrigel®- EIT 法は適用除外を理解した上で使用すれば、ボトムアップ方式で UN GHS 区分に該当しない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

Vitrigel®-EIT 法は、竹澤らにより開発されたコラーゲン Vitrigel®膜 [1]上で培養したヒト角膜上皮組織シート型培養モデルの経上皮バリア機能の経時変化を指標に用い、眼刺激性を評価する試験法である。本試験法は、ボトムアップ方式により UN GHS 区分に該当しない物質を検出することができる [2-5]。

3 次元角膜様モデルを用いた眼刺激性試験として、EpiOcular™ 眼刺激性試験、SkinEthic™ ヒト角膜上皮モデル眼刺激性試験および LabCyte CORNEA-MODEL24 眼刺激性試験が TG492 に収載されているが [6]、いずれも細胞毒性を指標としている。本試験法は、これらと異なり経上皮バリア機能を反映する TEER 値の経時変化を指標として、簡便に短時間で眼刺激性を評価できる方法として国立研究開発法人農業生物資源研究所（現　国立研究開発法人農業・食品産業技術総合研究機構）および関東化学株式会社の共同研究において開発され [2,4,5]、JaCVAM によるバリデーション研究[3] および第三者評価[7] を経て 2019 年に TG 494 に収載された[8]。その後、開発者より TG494 の適応範囲に関する改定の提案があり（Appendix 1）、JaCVAM および OECD における検討を経て、2021 年に OECD TG494 改定案が作成された [9]。本試験法は、作用機構に基づいたものであり、OECD ガイダンス文書 263 に示す IATA（Integrated Approaches to Testing and Assessment）の構成要件として [10]、有用であると考えられる。

本報告書では、Vitrigel®-EIT 法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ものである。

2. 試験法の位置づけ

Vitrigel®-EIT 法は、UN GHS 区分に該当しない物質（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

Vitrigel®-EIT法 は、ヒト角膜上皮組織シート型培養モデルに被験物質を曝露した際の経上皮バリア機能の経時変化を指標として、被験物質の眼刺激性の有無を判定する試験法である。

経上皮バリア機能を反映する TEER 値の経時変化から、被験物質の UN GHS 区分における区分に該当しないと判定する。

4. 試験手順

Vitrigel® -EIT 法の手順を以下に示す。詳細は、TG 494[9] およびプロトコル[11] を参照する。使用する細胞は HCE-T 細胞（理研バイオリソース研究センター RCB2280）を購入して用いる。 コラーゲン Vitirigel® 膜チャンバーおよび TEER 測定装置は関東化学株式会社より購入できる。

4-1. モデルの構築

コラーゲンVitrigel® 膜チャンバーを 12wellプレートにセットし、ウェル内に培養液を 1.5mL を加え、チャンバー内に1.2×10^5cells/mLに調製した細胞懸濁液を 0.5mLずつ分注しCO_2インキュベーター(37℃, 5%CO_2-Air)内に静置する。播種後 2日目に、チャンバー内の培養液を除き気相‐液相界面培養を開始する。播種後 6日目のモデルのチャンバー内に培養液を 0.5mL入れ、温度を 28℃±2℃に調節する。TEER 測定用電極をチャンバーにセットし TEER 値を測定する。TEER値がモデルの成立基準を満たしているかを確認する。

4-2. 被験物質の調製

培養液で 2.5%(w/v) になるように被験物質を溶解または懸濁し、調製物を作製する。

調製物を pH 試験紙(測定範囲に pH 1～11 が含まれるもの)または pH メーターにて測定し、pH が 5 以下となる酸性の物質は試験に適応できない。また、調製物の作製後 0 および 3 分に UV/VIS 分光光度計にて 660 nm の吸光度を測定し、絶対値で 0.1 を超える差がみられた物質は短時間に相分離を示す物質として試験に適応できない。被験物質が吸光度の測定を阻害する場合には、異なった波長を用いることもできる。

4-3. 被験物質の適用および測定

成立基準を満たすモデルに TEER 測定用電極を設置した後、被験物質 2.5%(w/v)水溶液または懸濁液をモデルに曝露する。被験物質の適用時のモデル温度は、28℃±2℃とする。

1 種類の被験物質について 3 個のモデルを用いて試験を行い、TEER値を 10 秒毎に 3 分間記録し、それぞれのTEERの初期値に対する変化率の平均値から、3 つの指標（Time lag、Intensity、Plateau level）を求める。これら指標の経時変化を自動解析し、眼刺激性の有無を判定する。

4-4. 判定

<u>計算手順</u>

3 つの指標は以下の式から算出される（図 1 参照）

Time lag (sec) ：t_1

Intensity (%/sec)：$-[P_2-P_1]/[t_2-t_1]$

Plateau level (%)：$100-P_2$

t_1 (sec)：TEER 値の変化速度が $0 \geq dP/dT > -0.03$%/sec の範囲内にある最大の曝露時間。

t_2 (sec)：t_1 後、TEER 値の変化速度が $dP/dT \leq -0.03$%/sec の条件を満たした後、$0 \geq dP\,(P_3-P_2)/dT\,(t_3-t_2) > -0.03$%/sec の条件を満たした最初の曝露時間。

t_3 (sec)：$t_2 + 30$ sec

P_1, P_2, P_3 (%)：曝露時間 t_1, t_2, t_3 の時 TEER 値の t_0 時の TEER 値に対する百分率。

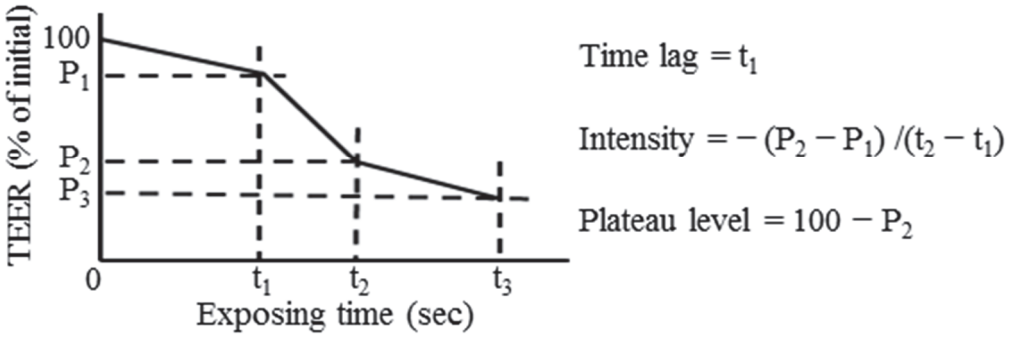

図 1．TEER 測定値の推移

上記の指標をもとにした予測モデルを表 1 に示す。

表 1．予測モデル

基準	予測
Time lag ≤ 180 (sec) or Intensity ≥ 0.05 (%/sec) or Plateau level > 5.0 (%)	不可（I）(1)
Time lag > 180 (sec) and Intensity < 0.05 (%/sec) and Plateau level ≤ 5.0 (%)	区分外（NI）(2)

注釈：

(1) 不可の場合には、IATA[9] の既存データや情報をもとに、被験物質におけるすべての眼刺激性作用機構の妥当性を考慮する必要がある。

(2) 区分外とは、UN GHS 区分に該当しない化学物質を指す。

4-5. 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) 初期の TEER 値が 140～220Ω・cm^2 の範囲にあるモデルである。
2) 陰性対照（生理食塩水：Saline）のPlateau level（PL）が 5 %以下である。
3) 陽性対照（塩化ベンザルコニウム：BAC）のPL が40%以上である。
4) 参照試料（エタノール：EtOH）のPL が10%以上である。
5) 被験物質の 3 つの指標の平均標準偏差が 15%以下である。

5. バリデーション研究

開発者とは異なる 3 施設（株式会社ダイセル：Daicel、株式会社ボゾリサーチセンター：BRC、一般財団法人食品薬品安全センター：FDSC）の協力を得て、Vitrigel®- EIT 法のバリデーション研究が行われた[3]。これら 3 施設はバリデーション研究の前に開発者から技術実習を受けた後、技術移転性を確認した。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。バリデーション結果は Appendix2 に示した。

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、プレバリデーション研究(phase 0)にて、3 施設による 5 物質(BAC、2-Propanol、Glycerol、n-Hexanol、Silicon dioxide n-hydrate)および陽性対照物質(EtOH)を用いた試験が行われ、プロトコルの確認がなされた[3]。その結果、EtOH の受け入れ PL 基準(20~30%)が厳しく、1 施設で基準を満たせなかった。バリデーション実行委員会で議論した結果、この基準値を 10~30%に広げ、この物質を自家製モデルの適正を評価する参照試料とし、陽性対照を BAC とすることになった。

5-1-2. 施設内再現性

Phase I バリデーション研究において、10 物質を用いて施設内再現性が評価され、UN GHS 区分判定の施設内再現性は 80%、90%、100%とバリデーション実行委員会の基準(80%以上)を満たした。バリデーション実行委員会で議論した結果、EtOH の受け入れ PL 基準値を 10~40%に広げ、適用温度を 18℃~30℃から 22℃~30℃に上げることが合意された。

5-1-3. 施設間再現性

Phase II バリデーション研究において、10 物質を用いて施設間再現性が評価された。その結果、施設間再現性は 80%以上であるバリデーション実行委員会の基準を満たした。ただし、UN GHS 区分 1 にあたるイミダゾール(No.2-1)を 2 施設が陰性と判断した。バリデーション実行委員会はこの結果を重く受け止め、Phase II の結果を不採用とし、プロトコルの改善を開発者に依頼した。その結果、開発者より、被験物質適用温度を 28℃±2℃とする改訂が提案された。このプロトコルを用いて、3 施設がイミダゾールで陽性結果を得たことを確認した上で 36 物質を用いて Phase III バリデーション研究を実施した。その結果、施設間再現性は 92%であり、バリデーション実行委員会の定めた基準(80%以上)を満たした。施設内・施設間再現性を高めるためのプロトコルの変遷を Appendix 2 の図 1 に示す。Phase II 終了後、バリデーション実行委員会で議論した結果、EtOH の受け入れ PL 基準値は≧10%となった。

5-2. 試験法の正確性

Phase III バリデーション研究の結果をもとに、3 施設で行われたデータを用いて正確性が検証された。その結果、感度 75~83%、特異度 42%、正確度 64~69%となった。この値は行政的利用を目指す試験法にとって十分なものではないとバリデーション実行委員会は考えた。

6. 追加検証

開発者は、Appendix1 に示した経緯を経て、OECD 専門家の意見をもとに 158 物質まで追加検討を行い(Appendix 3)、表 2 に示すように、感度 88%(61/69 物質)、特異度 63%(56/89 物質)および正確度 74%(117/158 物質)を得ている。なお、特異度は、phase III 結果と大きく異なるが、バリデーション研究で選択された適用物質は感度の検出を優先していることから、選ばれた陰性物質数の偏りにより、結果として特異度が低くなったと考える。

本追加検討において偽陰性を示した 10 物質のうち 5 物質が酸性であり、調製物の pH は 5 よりも低い値であった。また、調製液に不溶で短時間に相分離する物質も偽陰性となる可能性が示唆された。これらの点を考慮し、pH が 5 以下となる 12 物質および調製後 3 分以内の短

時間に相分離を示す 39 物質を適用除外とした結果、107 物質においてボトムアップ方式での正確性は感度 96%(51/53 物質)、特異度 67%(36/54 物質)、正確度 81%(87/107 物質)へと改善した(表 3)。液体の物質は、感度 100%(34/34 物質)、特異度 71%(22/31 物質)、正確度 86%(56/65 物質)、固体の物質は、感度 89%(17/19 物質)、特異度 61%(14/23 物質)、正確度 74%(31/42 物質)であった。

なお、2 つの偽陰性物質は Camphene と 1,4-Dibutoxybenzene である。いずれも US GHS 区分 2B に分類される。Drivers of classification[12] によると角膜に対する障害は認められず、結膜に対する障害のみ認められていることから、弱い刺激性であるため偽陰性になったものと開発者は考えた。

この適用除外は、バリデーション研究報告書および第三者評価報告書とは異なるが、改定 TG 検討の際に OECD にて合意されたものである。

表 2. 158 物質を用いた開発者によるボトムアップ方式での解析結果

		Vitrigel®-EIT 法		Total
		I	NI	
UN GHS	Cat.1, 2A, 2B	61	8	69
	No Category	33	56	89
Total		94	64	158

感度: 88% (61/69)
特異度: 63% (56/89)
正確度: 74% (117/158)

表 3. 適用除外 39 物質を除く 107 物質を用いた開発者によるボトムアップ方式での解析結果

		Vitrigel®-EIT 法		Total
		I	NI	
UN GHS	Cat.1, 2A, 2B	51	2	53
	No Category	18	36	54
Total		69	38	107

感度: 96% (51/53)
特異度: 67% (36/54)
正確度: 81% (87/107)

7. 試験法の適用範囲および留意点

TG 494 は、試験法の適正と正確性の確保を考慮して Vitrigel®-EIT 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および追加検証において混合物（単一物質の水溶液を除く）、気体（ガス）およびエアゾール様物質は被験物質として用いられていない。したがって、本試験法でこれら物質を評価する場合には、物性や規格要件を把握し、科学的に妥当な結果が得られるかどうかを前もって検討する必要がある。
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。
3) 培養液で 2.5%(w/v)になるように溶解または懸濁した調製物において、pHが 5 以下となる物質および調製後 3 分以内に相分離を示す物質は適用できない。

還元作用や着色のある被験物質ではMTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide）等を用いた細胞毒性評価に支障をきたすが、本試験法の指標であるTEERではそのような被験物質でも評価できる特徴をもつ。

8. 本委員会の結論

Vitrigel®-EIT 法のバリデーション研究の結果、本試験法はバリデーション実行委員会の定めた再現性の基準を満たしていた。また、追加検証の結果、適用除外を理解した上で使用すれば、正確性も十分であると確認された。よって、Vitrigel®- EIT 法 はボトムアップ方式で UN GHS 区分に該当しない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。

謝辞

当該試験法の眼刺激性試験評価報告書を作成するにあたり、国立研究開発法人 農業・食品産業技術総合研究機構（農研機構）の竹澤俊明氏、関東化学株式会社の山口宏之氏、国立医薬品食品衛生研究所の小島肇氏による情報提供等のご協力に深く感謝申し上げます。

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Appendix 1　Vitrigel®-EIT 法　TG494 の改定にいたる経緯

Vitrigel®-EIT 法は、ボトムアップ方式にて UN GHS 区分に該当しない物質の検出をする眼刺激性試験代替法としてバリデーション研究が行われた。3 施設で行われた 36 物質を用いたバリデーション研究の結果、予測性は、感度 75～83%、特異度 42%、正確度 64～69%となった。さらに、開発者のデータを追加した計 93 物質の結果では、感度 83%、特異度 70%、正確度 78%となった。この結果をもとに、以下に示す適用除外条件が提案され、この適用除外条件に当てはまる 17 物質を除いた 76 物質を評価したところ、感度 93%、特異度 69%、正確度 83%となった。

＜適用除外条件＞

1. 培養液で 2.5%(w/v)になるように溶解または懸濁した調製物において、pH が 5 以下となる物質
2. logP 値 2.5 以上、および、密度 0.95 g/ cm^3 以下または 1.10 g/cm^3 以上の固体

上述の適用除外条件を取り入れた Vitrigel®-EIT 法は、第三者評価を経て OECD の TG として提案された。しかしながら、条件 2 については、当てはまる固体が少なく評価できないという判断で OECD 専門家会議により却下された。結果として、固体物質がすべて適用除外になった。その場合の予測性は、この適用除外条件に当てはまる 42 物質を除いた 51 物質を評価したところ、感度 96%、特異度 74%、正確度 84%となった。以下に示す適用除外条件下で、2019 年 6 月 18 日に Vitrigel®-EIT 法は TG494 として OECD で採択された。

＜適用除外条件＞

1. 培養液で 2.5%(w/v)になるように溶解または懸濁した調製物において、pH が 5 以下となる物質
2. 固体物質

TG は採択されたものの、適用可能物質が約 55%(51/93)という少ない状態を改善するため、開発者らは直ちに追加実験の立案に入った。解決すべき問題点は、固体物質を適用するための条件設定であった。

固体物質を適用するための条件設定に関しては、JaCVAM 眼刺激性試験資料編纂委員会の意見も参考に日本薬局方の濁度測定法を用い、被験物質の濃度が 20%変化する指標として、調製直後と 3 分後の吸光度の差が 0.1 を超える場合に培養液中での相分離が起きている、すなわち懸濁液の分散安定性が低いと判断した。

＜適用除外条件＞

1. 培養液で 2.5% (w/v)になるように溶解または懸濁した調製物において、pH が 5 以下となる物質
2. 調製後 3 分以内に相分離を示す物質

あらたな適用除外条件を用いて、158 物質（液体 94、固体 64）の追加検討を行った、そのうち、51 物質（22 の固体のうち、pH5 以下 7 物質、相分離 15 物質；29 の液体のうち、pH5 以下 5 物質、相分離 24 物質）が適用除外となった。残りの 107 物質の適用除外を含む 158 物質の結果と比較すると、感度が 88%から 96%、特異度は 63%から 67%、正確度が 74%から 81%と改善さ

れた。予測性値は以前の TG と大きな変化はないが、適用可能物質が拡大し、適用範囲が科学的に適正となった、この改定案に国際的な合意が得られ、2021 年 6 月 14 日の改定版に反映された（報告書の表 2 および表 3 参照）、107 物質における固体・液体毎の予測性は、TG494 の Table 1 に記載されている。表 3 および Table 1 に示す偽陰性物質はいずれも固体の UN GHS 区分で 2B であった。

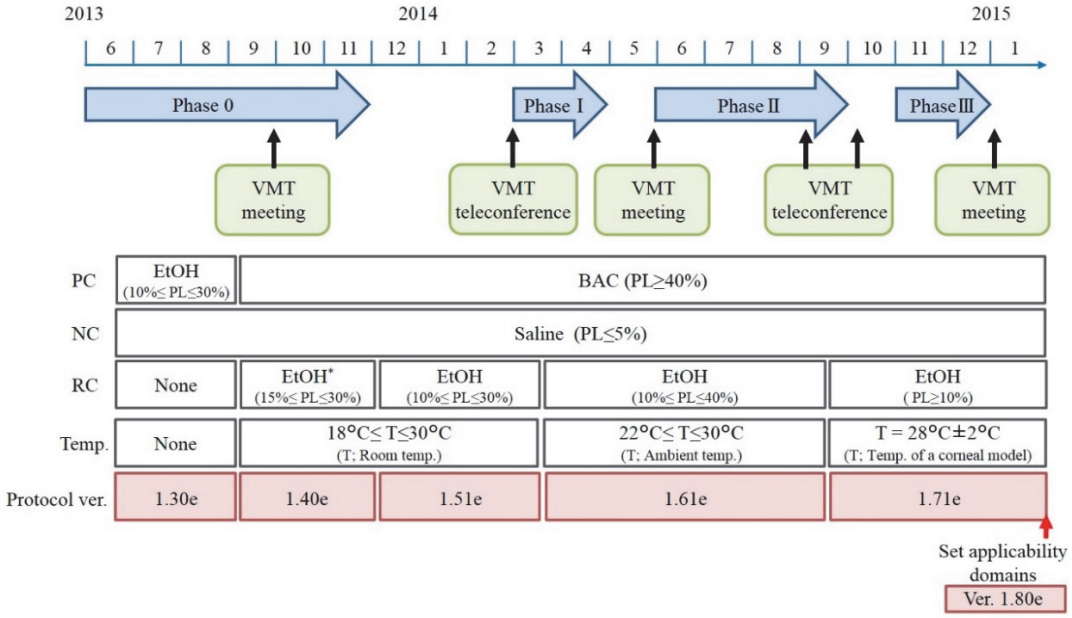
なお、以前の TG では許容されていた液体で相分離を示す 24 物質に関しては、偽陰性が 2 物質（UN GHS 区分 1、区分 2B）含まれており、改定により改善が認められた、ちなみに、固体において相分離を示した 15 物質に関しては、偽陰性が 2 物質（いずれも UN GHS 区分 1）認められた。

Appendix 2　表１. Phase I バリデーション結果

No.	Test chemical	CASRN	UN GHS	FDSC				BRC				Daicel			
				Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result
Negative control				190 (NI)	0.00 (NI)	0 (NI)	NI	190 (NI)	-0.03 (NI)	0 (NI)	NI	190 (NI)	-0.04 (NI)	0 (NI)	NI
Positive control				0 (I)	0.33 (I)	59 (I)	I	0 (I)	0.23 (I)	42 (I)	I	0 (I)	0.42 (I)	75 (I)	I
Reference control				20 (I)	-	3 (NI)	NG	0 (I)	0.12 (I)	21 (I)	I	0 (I)	0.17 (I)	30 (I)	I
1-1	Imidazole	288-32-4	Cat. 1	190 (NI)	-0.03 (NI)	0 (NI)	NI	160 (I)	0.13 (I)	4 (NI)	I	140 (I)	0.11 (I)	7 (I)	I
1-2	Cyclohexanol	108-93-0		30 (I)	0.16 (I)	25 (I)	I	0 (I)	0.40 (I)	48 (I)	I	0 (I)	0.34 (I)	51 (I)	I
1-3	3,3-Dithiodipropionic acid	1119-62-6	Cat.2A or 2B	190 (NI)	-0.06 (NI)	0 (NI)	NI	190 (NI)	-0.07 (NI)	0 (NI)	NI	190 (NI)	-0.05 (NI)	0 (NI)	NI
1-4	Acetone	67-64-1		10 (I)	0.04 (NI)	10 (I)	I	0 (I)	0.12 (I)	21 (I)	I	0 (I)	0.17 (I)	30 (I)	I
1-5	3-Chloropropionitrile	542-76-7		90 (I)	0.19 (I)	20 (I)	I	10 (I)	0.20 (I)	36 (I)	I	10 (I)	0.21 (I)	39 (I)	I
1-6	Ammonium nitrate	6484-52-2		0 (I)	0.69 (I)	21 (I)	I	0 (I)	0.67 (I)	27 (I)	I	0 (I)	1.05 (I)	52 (I)	I
1-7	n,n-Dimethylguanidine sulfate	598-65-2	No Cat.	190 (NI)	-0.05 (NI)	2 (NI)	NI	0 (I)	0.53 (I)	21 (I)	I	0 (I)	0.54 (I)	27 (I)	I
1-8	Toluene	108-88-3		190 (NI)	-0.01 (NI)	0 (NI)	NI	150 (I)	0.02 (NI)	1 (NI)	I	190 (NI)	0.00 (NI)	0 (NI)	NI
1-9	3-Methoxy-1,2-propanediol	623-39-2		190 (NI)	-0.05 (NI)	0 (NI)	NI	190 (NI)	-0.12 (NI)	0 (NI)	NI	190 (NI)	-0.11 (NI)	0 (NI)	NI
1-10	Gluconolactone	90-80-2		190 (NI)	-0.04 (NI)	0 (NI)	NI	0 (I)	0.28 (I)	8 (I)	I	10 (I)	0.18 (I)	10 (I)	I

Appendix 2　表 2．Phase II バリデーション結果

No.	Test chemical	CASRN	UN GHS	FDSC	BRC	Daicel
2-1	Imidazole	288-32-4	Cat. 1	NI	I	NI
2-2	Cyclohexanol	108-93-0		I	I	I
2-3	Sodium dodecyl sulfate	151-21-3		I	I	I
2-4	Sodium salicylate	54-21-7		I	I	I
2-5	Cyclopentanol	96-41-3	Cat. 2A & 2B	I	I	I
2-6	2-Methyl-1-pentanol	105-30-6		I	I	I
2-7	α-Hexylcinnamaldehyde	101-86-0		NI	NI	NI
2-8	n,n-Dimethylguanidine sulfate	598-65-2	No Category	I	I	I
2-9	Toluene	108-88-3		I	NI	NI
2-10	Gluconolactone	90-80-2		I	I	I



Appendix 2　図 1．プロトコルの変遷

Appendix 2　表 3．Phase III バリデーション結果

No.	Test chemical	CASRN	Physical state	UN GHS	FDSC	BRC	Daicel
3-1	2,5-Dimethyl-2,5-hexanediol	110-03-2	Solid	Cat. 1	I	I	I
3-2	2-Benzyl-4-chlorophenol	120-32-1	Solid		I	I	I
3-3	2,2-Dimethyl butanoic acid	595-379	Liquid		I	I	I
3-4	Captan	133-06-2	Solid		NI	NI	NI
3-5	Tetra-n-Octylammonium bromide	14866-33-2	Solid		I	I	NI
3-6	Butanol	71-36-3	Liquid		I	I	I
3-7	3-(2-Aminoethylaminopropyl) trimethoxysilane	1760-24-3	Liquid		I	I	I
3-8	Sodium dodecyl sulfate	151-21-3	Solid		I	I	I
3-9	*m*-Phenylenediamine	108-45-2	Solid		I	I	I
3-10	Tetraethylene glycol	17831-71-9	Liquid		I	I	I
3-11	Imidazole	288-32-4	Solid		I	I	I
3-12	Sodium salicylate	54-21-7	Solid		I	I	I
3-13	gamma-Butyrolactone	96-48-0	Liquid	Cat. 2A or 2B	I	I	I
3-14	Methyl acetate	79-20-9	Liquid		I	I	I
3-15	Myristyl alcohol	112-72-1	Solid		NI	NI	NI
3-16	2,6-Dichlorobenzoyl chloride	4659-45-4	Liquid*		NI	I	NI
3-17	Dibenzyl phosphate	1623-08-1	Solid		I	I	I
3-18	1-(2-Propoxy-1-methylethoxy)-2-propanol	29911-27-1	Liquid		I	I	I
3-19	Camphene	79-92-5	Solid		I	NI	NI
3-20	Ethyl-2-methylacetoacetate	609-14-3	Liquid		I	I	I
3-21	Propylene glycol propyl ether	1569-01-3	Liquid		I	I	I
3-22	2-Methyl-1-pentanol	105-30-6	Liquid		I	I	I
3-23	α-Hexylcinnamaldehyde	101-86-0	Liquid		NI	NI	NI
3-24	Cyclopentanol	96-41-3	Liquid		I	I	I
3-25	Methyl amyl ketone	110-43-0	Liquid	No Cat.	I	I	I
3-26	2-(n-Dodecylthio)ethanol	1462-55-1	Liquid		NI	NI	NI
3-27	iso-Octylthioglycolate	25103-09-7	Liquid		NI	NI	NI
3-28	2,4-Difluoronitrobenzene	446-35-5	Liquid		I	I	I
3-29	tetra-Aminopyrimidine sulfate	5392-28-9	Solid		NI	NI	NI
3-30	2,4-Pentanediol	625-69-4	Liquid		I	I	I
3-31	iso-Octyl acrylate	29590-42-9	Liquid		NI	NI	NI
3-32	Silicon dioxide n-hydrate	7699-41-4	Solid		NI	NI	NI
3-33	Potassium tetrafluorobroate	14075-53-7	Solid		I	I	I

3-34	*n,n*-Dimethylguanidine sulfate	598-65-2	Solid		I	I	I
3-35	Toluene	108-88-3	Liquid		I	I	I
3-36	Gluconolactone	90-80-2	Solid		I	I	I

*: pH of 2.5% solution ≤ 5.0

Appendix 2　表 4-1. Phase III　FDSC および BRC の正確性

		Vitrigel®-EIT 法		Total
		I	NI	
UN GHS	Cat.1, 2A, 2B	20	4	24
	No Category	7	5	12
Total		27	9	36

感度: 83.3% (20/24)

特異度: 41.7% (5/12)

正確度: 69.4% (25/36)

Appendix 2　表 4-2. Phase III　Daicel の正確性

		Vitrigel®-EIT 法		Total
		I	NI	
UN GHS	Cat.1, 2A, 2B	18	6	24
	No Category	7	5	12
Total		25	11	36

感度: 75.0% (18/24)

特異度: 41.7% (5/12)

正確度: 63.9% (23/36)

Appendix 3 開発者による追加検討（158 物質）

No.	Chemical	Organic functional groups [1]	CAS No.	Form	GHS	Judgment by Vitigel-EIT	Drivers of classification [2] Severity Cut-off values CO mean	Conj mean	IR mean	Number of animals	Persistence Cut-off time CO pers	Conj pers	IR pers	Number of animals	Specific Observations CO=4	Number of animals	A_{660} at 0min	A_{660} at 3min	Absolute differences between the absorbance values of test chemical preparation at 0 and 3 minutes. $\|\Delta A_{660}$ 0-	pH of 2.5% solution	Excluded by pre-test 1(Acidic chemicals)	Excluded by pre-test 2(Briefly phase-separating chemicals)
1	*n*-Octylamine	Aliphatic amine, primary;Amine, primary	111-86-4	Liquid	1	I	n/a				Unknown				= 4	4/4	2.55	0.36	2.19	12		✓
2	Basasol Orange 52L	(n/a)	n/a	Liquid	1	I											3.03	2.63	0.40	4	✓	✓
3	Cyclohexyl isocyanate	Cycloalkane;Isocyanate	3173-53-3	Liquid	1	NI	≥ 3			2/2	21			2/2	= 4	2/2	2.16	1.97	0.19	7		✓
4	Domiphen bromide (10%)	Aryl;Ether moiety;Quaternary ammonium salts;Surfactants - Cationic	538-71-6	Liquid	1	I	≥ 3			2/3	21			at least 2/3	= 4	3/3	0.57	0.54	0.03	7		
5	Tetraethylene glycol	Acrylate;Alkene moiety ;Carboxylic acid ester;Ether moiety	17831-71-9	Liquid	1	I			> 1.5	6/6	21			at least 5/6	= 4	5/6	0.05	0.04	0.01	7		
6	8-Chloro-1-n-octanol	Alcohol;Alkyl halide	23144-52-7	Liquid	1	I	≥ 1	≥ 2		3/3; 3/3	21			3/3			3.07	3.06	0.01	7		
7	Benzethonium chloride (10%)	Alkane, branched with quaternary carbon;Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl;Benzyl;Ether moiety;Quaternary ammonium salts;Surfactants - Cationic;tert-Butyl	121-54-0	Liquid	1	I	≥ 3			2/3	21			at least 2/3	= 4	2/3	0.32	0.32	0.00	9		
8	Cyclohexanol	Alcohol;Cycloalkane	108-93-0	Liquid	1	I	≥ 3			3/4	21			1/4			0.08	0.08	0.00	7		
9	Cetylpyridinium bromide (10%)	Aryl;Pyridine/Pyridinium ion;Surfactants - Cationic	140-72-7	Liquid	1	I	≥ 3		> 1.5	6/6; 4/6	21	21	21	at least 4/6 (max 5); at least 3/6 (max 4); 3/6	= 4	2/6	0.16	0.15	0.00	7		
10	Pyridine	Aryl;Pyridine/Pyridinium ion	110-86-1	Liquid	1	I	≥ 3			2/3	21			at least 1/3	= 4	1/3	0.00	0.00	0.00	7		
11	Lactic acid	Alcohol;Carboxylic acid	50-21-5	Liquid	1	I	≥ 3			3/3	21			2/3	= 4	3/3	-0.01	-0.01	0.00	3	✓	
12	Diethylethanolamine	Alcohol;Aliphatic amine, tertiary;Amine, tertiary	100-37-8	Liquid	1	I	≥ 3			4/6	21	21		5/6; 5/6	= 4	5/6	-0.01	-0.01	0.00	10		
13	Butyl cellosolve	Alcohol;Ether moiety	111-76-2	Liquid	1	I	≥ 1	≥ 2	≥ 1	6/6; 6/6; 5/6	21			1/6			0.01	0.01	0.00	8		
14	Triton X-100	Alcohol;Alkane, branched with quaternary carbon;Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl;Ether moiety;tert-Butyl	9002-93-1	Liquid	1	I			> 1.5	5/6	Jnknown						-0.03	-0.03	0.00	7		
15	Triethanolamine polyoxyethylene (3) lauryl ether sulfate, 40%	Alcohol;Aliphatic amine, tertiary;Amine, tertiary;Ether moiety	27028-82-6	Liquid	1	I											-0.06	-0.06	0.00	7		
16	Sodium polyethylene (3) lauryl ether sulfate, 25.5%	Alkene moiety ;Sulfate	9004-82-4	Liquid	1	I	> 0			3/3; 2/3	21	21		3/3; 3/3			-0.06	-0.06	0.00	7		
17	Polyoxyethylene (10) polyoxypropylene (1.5) lauryl—mystyl ether	Alcohol;Ether moiety	68439-51-0	Liquid	1	I											-0.06	-0.06	0.00	6		
18	3-(2-Aminoethylamino)propyltrimethoxysilane	Aliphatic amine, primary;Aliphatic amine, secondary;Alkoxysilane;Amine, primary;Amine, secondary;Silane	1760-24-3	Liquid	1	I	≥ 1	≥ 2	≥ 1	3/3; 3/3; 2/3	21	21		3/3; 2/3			0.01	0.01	0.00	10		
19	Cetylpyridinium chloride (10%)	Aryl;Pyridine/Pyridinium ion;Surfactants - Cationic	6004-24-6	Liquid	1	I	≥ 1	≥ 2	≥ 1	3/3; 3/3; 3/3	21			at least 2/3	= 4	3/3	0.06	0.06	0.00	7		
20	Stearyltrimethylammonium chloride (10%)	Quaternary ammonium salts;Surfactants - Cationic	112-03-8	Liquid	1	I	≥ 1	≥ 2	≥ 1	3/3; 3/3; 2/3	21			3/3	= 4	3/3	0.30	0.32	0.02	7		
21	Benzyl alcohol	Alcohol;Aryl;Benzyl	100-51-6	Liquid	2	I	≥ 1			2/3	21			at least 1/3	= 4	2/3	-0.02	-0.03	0.01	7		
22	2-Ethoxyethyl acetate	Acetoxy;Carboxylic acid ester;Ether moiety	111-15-9	Liquid	2	I	> 0			1/4							-0.03	-0.03	0.00	7		
23	2,6-Dichlorobenzoyl chloride	Acyl halide;Aryl;Aryl halide	4659-45-4	Liquid	2A	I	≥ 1	≥ 2		6/6; 6/6	7	7		6/6; 2/6			0.86	0.14	0.73	3	✓	✓
24	*n*-Hexanol	Alcohol	111-27-3	Liquid	2A	I	≥ 1	≥ 2		4/4; 4/4		7		4/4			1.93	1.63	0.30	7		✓
25	1-Octanol	Alcohol	111-87-5	Liquid	2A	I	≥ 1	≥ 2	≥ 1	2/3; 2/3; 2/3							2.32	2.20	0.12	7		✓
26	2-Ethyl-1-hexanol	Alcohol;Alkane, branched with tertiary carbon	104-76-7	Liquid	2A	I	≥ 1	≥ 2		4/4; 3/4		7	7	3/4; 1/4			2.24	2.17	0.07	7		
27	Methyl ethyl ketone	Ketone	78-93-3	Liquid	2A	I	≥ 1	≥ 2		3/4; 3/4		7		3/4			-0.03	-0.04	0.00	7		
28	Ethanol	Alcohol	64-17-5	Liquid	2A	I	≥ 2	≥ 1	≥ 1	6/6; 5/6; 5/6	21			1/6			-0.03	-0.03	0.00	7		
29	Acetone	Ketone	67-64-1	Liquid	2A	I	≥ 1	≥ 2	≥ 1	4/4; 4/4; 4/4	7	7	7	4/4; 1/4; 1/4			-0.04	-0.04	0.00	7		

30	Isopropyl alcohol	Alcohol;Alkane, branched with secondary carbon;Isopropyl	67-63-0	Liquid	2A	I		≥ 2		3/4		7	4/4			-0.03	-0.03	0.00	7		
31	Butyrolactone	Lactons;Oxolane	96-48-0	Liquid	2A	I	≥ 1	≥ 2		3/3; 3/3	7	7	2/3; 1/3			-0.02	-0.03	0.00	7		
32	Cyclopentanol	Alcohol;Cycloalkane	96-41-3	Liquid	2A	I	≥ 1	≥ 2		3/3; 3/3	7	7	1/3; 1/3			0.12	0.12	0.00	7		
33	Butanol	Alcohol	71-36-3	Liquid	2A	I	≥ 1	≥ 2	≥ 1	3/3; 3/3; at least 2/3	Jnknown			= 4	1/3	-0.02	-0.02	0.00	8		
34	Isobutyl alcohol	Alcohol;Alkane, branched with tertiary carbon;Isopropyl	78-83-1	Liquid	2A	I	≥ 1	≥ 2		4/4; 4/4	7	7	4/4; 1/4			-0.05	-0.05	0.00	7		
35	Methyl acetate	Acetoxy;Carboxylic acid ester	79-20-9	Liquid	2A	I	≥ 1	≥ 2		3/4; 3/4	7	7	4/4; 1/4			-0.06	-0.06	0.00	7		
36	2-Methyl-1-pentanol	Alcohol;Alkane, branched with tertiary carbon	105-30-6	Liquid	2B	I	≥ 1			2/3						1.32	0.66	0.66	7		✓
37	Pseudoionone	Alkene moiety ;Allyl;Conjugated system;Ketone;Terpenes	141-10-6	Liquid	2B	NI		≥ 2		2/3						2.90	2.78	0.12	7		✓
38	Isobutanal	Aldehyde;Alkane, branched with tertiary carbon;Isopropyl	78-84-2	Liquid	2B	I	≥ 1	≥ 2		2/3; 2/3						0.06	0.00	0.06	6		
39	*n*-Butanal	Aldehyde	123-72-8	Liquid	2B	I	≥ 1	≥ 2		2/3; 2/3						0.02	0.00	0.03	7		
40	Propasol solvent P	Alcohol;Alkane, branched with secondary carbon;Ether moiety	1569-01-3	Liquid	2B	I	≥ 1			5/6	7	7	1/6; 1/6			-0.03	-0.03	0.01	8		
41	Di(propylene glycol) propyl ether	Alcohol;Alkane, branched with secondary carbon;Ether moiety	29911-27-1	Liquid	2B	I	≥ 1	≥ 2		3/3; 3/3						0.00	0.00	0.00	7		
42	3-Chloropropionitrile	Alkyl halide;Nitrile	542-76-7	Liquid	2B	I	≥ 1			2/3						-0.04	-0.05	0.00	5	✓	
43	Ethyl-2-methylacetoacetate	Carboxylic acid ester;Ketone	609-14-3	Liquid	2B	I	≥ 1			3/3						-0.04	-0.04	0.00	7		
44	Monoethanolamine (10%)	Alcohol;Aliphatic amine, primary;Amine, primary	141-43-5	Liquid	2B	I	≥ 1			2/3						-0.04	-0.04	0.00	9		
45	Glycolic acid (10%)	Alcohol;Carboxylic acid	79-14-1	Liquid	2B	NI		≥ 2		2/3						-0.02	-0.02	0.00	4	✓	
46	1,2,3-Trichloropropane	Alkane, branched with secondary carbon;Alkyl halide	96-18-4	Liquid	NC	I	> 0									1.39	0.11	1.28	7		✓
47	Xylene	Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl	1330-20-7	Liquid	NC	I	= 0			Conj 1/4						1.69	0.54	1.16	7		✓
48	Ethyl trimethyl acetate	Carboxylic acid ester;tert-Butyl	3938-95-2	Liquid	NC	I	> 0			CO 1/6						1.49	0.85	0.64	7		✓
49	Styrene	Alkene moiety ;Alkenyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl	100-42-5	Liquid	NC	NI	> 0									1.97	1.33	0.63	7		✓
50	2,4-Difuoronitrobenzene	Aryl;Aryl halide;Nitrobenzene	446-35-5	Liquid	NC	I	= 0									1.50	0.89	0.61	7		✓
51	Diisobutyl ketone	Alkane, branched with tertiary carbon;Isopropyl;Ketone	108-83-8	Liquid	NC	NI	= 0									1.51	1.00	0.51	7		✓
52	Isopropyl myristate	Alkane, branched with secondary carbon;Carboxylic acid ester;Isopropyl	110-27-0	Liquid	NC	NI	= 0									1.82	1.43	0.39	7		✓
53	*n*-Octyl bromide	Alkyl halide	111-83-1	Liquid	NC	NI	= 0									0.40	0.05	0.35	8		✓
54	3, 3-Dimethylpentane	Alkane, branched with quaternary carbon;Alkanes;Hydrocarbons	562-49-2	Liquid	NC	NI	= 0									0.82	0.49	0.33	7		✓
55	Methyl amyl ketone	Ketone	110-43-0	Liquid	NC	I	> 0			Conj 1/4						1.82	1.52	0.29	7		✓
56	2-Methylpentane	Alkane, branched with tertiary carbon;Alkanes;Hydrocarbons;Isopropyl	107-83-5	Liquid	NC	NI	= 0									0.55	0.31	0.24	7		✓
57	1,5-Hexadiene	Alkene moiety ;Alkenes;Allyl;Hydrocarbons	592-42-7	Liquid	NC	NI	= 0									0.28	0.10	0.18	7		✓
58	Methyl isobutyl ketone	Alkane, branched with tertiary carbon;Isopropyl;Ketone	108-10-1	Liquid	NC	I	> 0									1.24	1.06	0.18	7		✓
59	1,2,4-Trimethylbenzene	Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl	95-63-6	Liquid	NC	NI	= 0									1.88	1.71	0.17	7		✓
60	Dodecane	Alkanes;Hydrocarbons	112-40-3	Liquid	NC	NI	= 0									0.75	0.59	0.16	7		✓
61	Isopropyl bromide	Alkane, branched with secondary carbon;Alkyl halide;Isopropyl	75-26-3	Liquid	NC	I	= 0									0.19	0.04	0.15	8		✓
62	Methyl cyclopentane	Alkanes;Cycloalkane;Hydrocarbons	96-37-7	Liquid	NC	NI	= 0									1.69	1.54	0.14	7		✓
63	Hexane	Alkanes;Hydrocarbons	110-54-3	Liquid	NC	NI	= 0									0.55	0.45	0.11	7		✓
64	*iso*-Propyl myristate	Alkane, branched with secondary carbon;Carboxylic acid ester;Isopropyl	110-27-0	Liquid	NC	NI	= 0									1.07	0.99	0.09	7		
65	Petroleum ether	Alkanes;Hydrocarbons	8032-32-4	Liquid	NC	NI	= 0									0.08	0.00	0.08	7		
66	*iso*-Octylthioglycolate	Alkane, branched with tertiary carbon;Carboxylic acid ester;Isopropyl;Thiol	25103-09-7	Liquid	NC	NI	= 0									2.15	2.08	0.07	7		
67	1,4-Dibromobutane	Alkyl halide	110-52-1	Liquid	NC	NI	= 0									3.09	3.02	0.06	7		
68	1,6-Dibromohexane	Alkyl halide	629-03-8	Liquid	NC	Ni	= 0									3.03	2.99	0.04	7		
69	2,2-Dimethyl-3-pentanol	Alcohol;Alkane, branched with quaternary carbon;Alkane, branched with secondary carbon;tert-Butyl	3970-62-5	Liquid	NC	I	> 0			CO 1/3						2.24	2.20	0.04	7		

70	1,5-Dibromopentane	Alkyl halide	111-24-0	Liquid	NC	NI	= 0											2.50	2.46	0.04	7	
71	1,9-Decadiene	Alkene moiety ;Alkenes;Allyl;Hydrocarbons	1647-16-1	Liquid	NC	NI	= 0											0.76	0.74	0.03	7	
72	Cyclohexanone	Cycloketone;Ketone	108-94-1	Liquid	NC	I	> 0			CO 1/3								0.39	0.37	0.02	7	
73	*sec*-Butylbenzene	Alkane, branched with tertiary carbon;Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl	135-98-8	Liquid	NC	NI	= 0											3.52	3.51	0.01	7	
74	2-Ethylhexyl *p*-dimethyl-amino benzoate	Alkane, branched with tertiary carbon;Amine, tertiary;Aromatic amine;Aryl;Carboxylic acid ester	21245-02-3	Liquid	NC	NI	= 0											2.69	2.68	0.01	7	
75	Polyoxyethylene hydrogenated castoroil (60E.O.)	Acylal; Alcohol; Allyl; Ether	61788-85-0	Liquid	NC	NI	= 0											2.63	2.62	0.01	7	
76	1,3-Di-isopropylbenzene	Alkane, branched with tertiary carbon;Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl;Isopropyl	99-62-7	Liquid	NC	NI	= 0											3.06	3.05	0.01	7	
77	Polyoxyethylene 23 lauryl ether (10%)	Alcohol;Ether moiety	9002-92-0	Liquid	NC	NI	= 0											-0.04	-0.05	0.01	7	
78	Piperonyl butoxide	Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl;Benzodioxole;Benzyl;Ether moiety	51-03-6	Liquid	NC	NI	= 0			Conj 2/6								3.24	3.23	0.01	7	
79	Polyethylene glycol 400	Alcohol;Dihydroxyl derivatives;Ether moiety	25322-68-3	Liquid	NC	I	= 0											0.03	0.03	0.00	7	
80	2,4-Pentanedione	Diketone;Ketone	123-54-6	Liquid	NC	I	> 0											0.04	0.04	0.00	6	
81	Plyoxyethylene (160) sorbitan triisostearate	Alcohol;Ether moiety	54392-28-8	Liquid	NC	NI												-0.07	-0.07	0.00	7	
82	Tween20	Acetal;Alcohol;Alkane, branched with secondary carbon;Alkene moiety ;Allyl;Carboxylic acid ester;Ether moiety;Oxolane;Saturated heterocyclic fragment	9005-64-5	Liquid	NC	NI	= 0											-0.01	-0.01	0.00	7	
83	2,4-Pentanediol	Alcohol;Alkane, branched with secondary carbon;Dihydroxyl derivatives	625-69-4	Liquid	NC	I	= 0											-0.05	-0.05	0.00	8	
84	Propylene glycol	Alcohol;Alkane, branched with secondary carbon;Dihydroxyl derivatives	57-55-6	Liquid	NC	NI	= 0											-0.02	-0.02	0.00	7	
85	4-Bromophenetole	Alkoxy moiety;Aryl;Aryl halide;Ether moiety	588-96-5	Liquid	NC	NI	= 0											3.58	3.58	0.00	7	
86	Butyl acetate	Acetoxy;Carboxylic acid ester	123-86-4	Liquid	NC	I	> 0											-0.01	-0.01	0.00	7	
87	Glycerol	Alcohol;Alkane, branched with secondary carbon;Glycerol and derivatives	56-81-5	Liquid	NC	I	= 0											-0.04	-0.04	0.00	7	
88	3-Methoxy-1.2-propanediol	Alcohol;Alkane, branched with secondary carbon;Dihydroxyl derivatives;Ether moiety;Glycerol and derivatives	623-39-2	Liquid	NC	NI	= 0											-0.03	-0.03	0.00	7	
89	Triethanolamine	Alcohol;Aliphatic amine, primary;Aliphatic amine, secondary;Aliphatic amine, tertiary;Amine, primary;Amine, secondary;Amine, tertiary;Dihydroxyl derivatives	102-71-6	Liquid	NC	I	> 0											-0.05	-0.05	0.00	9	
90	Tween80	Acetal;Alcohol;Alkane, branched with secondary carbon;Alkene moiety ;Allyl;Carboxylic acid ester;Ether moiety;Oxolane;Saturated heterocyclic fragment	9005-65-6	Liquid	NC	NI	= 0											-0.07	-0.07	0.00	7	
91	Polyethylene (14) tribenzylated phenyl ether	Alcohol;Ether moiety	116998-28-8	Liquid	NC	NI												-0.06	-0.06	0.00	7	
92	Dimethyl sulfoxide	Sulfoxide	67-68-5	Liquid	NC	NI	> 0											-0.05	-0.05	0.00	7	
93	Polyoxyethylene (13) (mono—, di, tri-) styrenated phenyl ether	Alcohol;Ether moiety	104376-75-2	Liquid	NC	NI												-0.06	-0.06	0.00	7	
94	3-Glycidoxypropyltrimethoxysilane	Alkoxysilane;Epoxide;Ether moiety;Saturated heterocyclic fragment;Silane	2530-83-8	Liquid	NC	I	= 0											0.19	0.19	0.00	7	
95	3,4-Dichlorophenyl isocyanate	Aryl;Aryl halide;Isocyanate	102-36-3	Solid	1	NI		≥ 2		2/3		21			3/3			3.62	3.03	0.59	7	✓
96	Captan	Alkene moiety ;Alkyl halide;Allyl;Cycloalkene moiety;Imide;Pyrrolidine;Pyrrolidone/Pyrrolidinedione;Succinimide/Succinimide, thio;Sulfenamide;Tetrahydrophthalimide;Unsaturated carbocyclic fragment	133-06-2	Solid	1	NI	≥ 1	≥ 2	≥ 1	3/3; 3/3; 3/3		21	21	21	3/3; 2/3; 1/3	= 4	3/3	2.89	2.65	0.24	7	✓
97	(-)-Dibenzoyl-L-tartaric acid	Aryl;Carboxylic acid;Carboxylic acid ester	2743-38-6	Solid	1	I	≥ 3			2/3		Jnknown						3.13	3.11	0.02	7	
98	Promethazine hydrochloride	Alkane, branched with secondary carbon;Amine, tertiary;Ammonium salt;Aryl;Phenothiazine	58-33-3	Solid	1	I	≥ 3			3/3		21			3/3	= 4	3/3	2.63	2.60	0.02	6	
99	2-Benzyl-4-chlorophenol	Aryl;Aryl halide;Phenol;Precursors quinoid compounds	120-32-1	Solid	1	I	≥ 3			6/6		21			6/6	= 4	6/6	3.07	3.05	0.02	7	
100	Distearyldimethylammonium chloride	Quaternary ammonium salts;Surfactants - Cationic	107-64-2	Solid	1	I	≥ 3			3/3		21			3/3	= 4	3/3	2.90	2.89	0.02	7	
101	4-*tert*-Octylphenol	Alkane, branched with quaternary carbon;Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl;Phenol;tert-Butyl	140-66-9	Solid	1	I	≥ 3		> 1.5	6/6; 6/6		Jnknown				= 4	1/6	3.48	3.47	0.00	7	
102	Sodium salicylate	Aryl;Carboxylic acid;Phenol	54-21-7	Solid	1	I	≥ 1	≥ 2		3/3; 3/3		21			2/3	= 4	3/3	0.00	-0.01	0.00	7	
103	Imidazole	Amine, secondary;Aryl;Imidazole	288-32-4	Solid	1	I	≥ 3		> 1.5	3/3; 2/3		21			at least 2/3	= 4	2/3	-0.03	-0.03	0.00	9	
104	Tetrabromophenol blue	Aromatic perhalogencarbons;Aryl;Aryl halide;Phenol;Sulfonate ester	4430-25-5	Solid	1	I	≥ 3			3/3		Jnknown				= 4	3/3	3.80	3.80	0.00	6	
105	*m*-Phenylenediamine	Amine, primary;Aniline;Aryl;Phenylenediamine, meta-	108-45-2	Solid	1	I	≥ 1	≥ 2	≥ 1									-0.01	-0.01	0.00	8	
106	Nonylphenyl-polyethylene glycol	Alcohol;Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl;Ether moiety	9016-45-9	Solid	1	I	≥ 1	≥ 2	≥ 1	6/6; 6/6; 6/6		21			at least 4/6 (max 5)	= 4	5/6	-0.02	-0.02	0.00	7	

107	Acid red 92	Aromatic perhalogencarbons;Aryl;Aryl halide;Ether moiety;Heterocyclic spiro rings;Lactons;Phenol;Xanthene/ Xanthone	18472-87-2	Solid	2	I	≥ 1			3/3	21		at least 2/3	= 4	3/3	2.65	2.65	0.01	8		
108	Dodecanoic acid	Carboxylic acid;Surfactants - Anionic	143-07-7	Solid	2	I	≥ 1	≥ 2								3.19	3.19	0.00	6		
109	Calcium thioglycolate	Carboxylic acid;Thiol	814-71-1	Solid	2A	I	≥ 3			3/3	21		3/3	= 4	3/3	2.11	2.07	0.04	10		
110	Dibenzyl phosphate	Aryl;Benzyl;Phosphate ester	1623-08-1	Solid	2A	I	≥ 1	≥ 2		3/3; 3/3	7	7	3/3; 1/3			2.75	2.73	0.02	3	✓	
111	Methyl cyanoacetate	Carboxylic acid ester;Nitrile	105-34-0	Solid	2A	I		≥ 2	≥ 1	3/3; 3/3		7	2/3			0.00	0.00	0.00	7		
112	Potassium oleate	Alkene moiety ;Allyl;Carboxylic acid;Surfactants - Anionic	143-18-0	Solid	2A	I										0.51	0.51	0.01	9		
113	3,3'-Dithiodipropionic acid	Carboxylic acid;Disulfide	1119-62-6	Solid	2B	NI	≥ 1			3/3	7		1/3			2.58	1.60	0.97	4	✓	✓
114	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropanoate	Aryl;Aryl halide;Carboxylic acid ester;Ketone;Pyridine/Pyridinium ion	96568-04-6	Solid	2B	I	≥ 1			2/3						1.89	1.49	0.41	5	✓	✓
115	Camphene	Alkene moiety ;Alkenes;Bicycloheptane ;Bridged-ring carbocycles;Cycloalkane;Hydrocarbons;Terpenes	79-92-5	Solid	2B	NI		≥ 2		3/3						2.40	2.39	0.01	7		
116	1,4-Dibutoxybenzene	Alkoxy moiety;Aryl;Ether moiety	104-36-9	Solid	2B	NI		≥ 2		3/3						3.17	3.16	0.00	7		
117	Sodium monochloroacetate	Alkyl halide;Carboxylic acid	3926-62-3	Solid	2B	I		≥ 2		3/3						0.02	0.01	0.00	7		
118	Ammonium nitrate	Organic salts	6484-52-2	Solid	2B	I		≥ 2		3/3	7		1/3			-0.05	-0.05	0.00	8		
119	Methyl *p*-hydroxybenzoate	Aryl;Carboxylic acid ester;Phenol	99-76-3	Solid	NC	I	= 0									2.13	0.92	1.21	7		✓
120	2-Mercaptopyrimidine	Aldimine;Alkene moiety ;Cycloalkene moiety	1450-85-7	Solid	NC	NI	= 0									2.72	1.89	0.83	7		✓
121	Aluminium hydroxide	Inorganic salt	21645-51-2	Solid	NC	NI	= 0									2.19	1.74	0.45	7		✓
122	Theobromine	Amine, tertiary;Aryl;Dihydropurinedione;Fused heterocyclic aromatic;Imidazole;Imide;Urea derivatives	83-67-0	Solid	NC	NI	= 0									3.51	3.28	0.23	7		✓
123	Hydroxyethylcellulose ethoxylate, quaternized	Alcohol; Ammonium salt; Ether	68610-92-4	Solid	NC	NI	= 0									0.27	0.09	0.18	7		✓
124	3,4-Dimethoxybenzaldehyde	Aldehyde;Alkoxy moiety;Aryl;Ether moiety	120-14-9	Solid	NC	I	= 0									2.73	2.59	0.14	7		✓
125	Propylparaben	Aryl;Carboxylic acid ester;Phenol	94-13-3	Solid	NC	NI	> 0									3.43	3.35	0.08	7		
126	Iminodibenzyl	Amine, secondary;Aryl	494-19-9	Solid	NC	NI	> 0									3.69	3.62	0.07	7		
127	Phenothiazine	Amine, secondary;Aryl;Phenothiazine	92-84-2	Solid	NC	NI	= 0									2.74	2.68	0.05	7		
128	Anthracene	Anthracene ;Aryl;Fused carbocyclic aromatic;Naphtalene	120-12-7	Solid	NC	NI	= 0									2.84	2.79	0.05	7		
129	2,3-Indolinedione	Amine, primary;Aniline;Aryl;Phenol;Precursors quinoid compounds	91-65-5	Solid	NC	I	= 0									2.95	2.92	0.03	7		
130	2-Aminophenol	Amine, primary;Aniline;Aryl;Dianilines;Sulfone	95-55-6	Solid	NC	I	= 0									2.95	2.92	0.03	7		
131	4,4'-Sulfonylbisbenzenamide	Amine, primary;Aniline;Aryl;Phenol;Sulfone	80-08-0	Solid	NC	NI	= 0									2.73	2.70	0.02	7		
132	Potassium tetrafluoroborate	Inorganic salt	14075-53-7	Solid	NC	I	= 0									0.04	0.03	0.01	7		
133	Betaine monohydrate	Carboxylic acid;Quaternary ammonium salts	590-47-6	Solid	NC	I	> 0									0.02	0.01	0.00	7		
134	Hexamethylenetetramine	Aliphatic amine, tertiary;Amine, tertiary;Bridged-ring heterocycles;Saturated heterocyclic fragment	100-97-0	Solid	NC	I	= 0									-0.02	-0.02	0.00	7		
135	EDTA,di-potassium	Aliphatic Amine, tertiary; Carboxylic acid	25102-12-9	Solid	NC	I	> 0									-0.01	-0.01	0.00	5	✓	
136	*N,N*-Dimethylguanidine sulfate	Amidine;Ammonium salt	598-65-2	Solid	NC	I	= 0			Conj 1/3						-0.01	-0.01	0.00	7		
137	Gluconolactone	Alcohol;Lactons	90-80-2	Solid	NC	NI	= 0									-0.01	-0.01	0.00	6		
138	1,3,5-Trioxane	Ether moiety;Saturated heterocyclic fragment;Trioxane	110-88-3	Solid	NC	I	> 0									0.00	0.00	0.00	7		
139	Sodium hydrogen sulfite	Inorganic salt	7631-90-5	Solid	NC	I	> 0			CO 1/3; Conj 1/3						0.00	0.00	0.00	5	✓	
140	1-Phenyl-3-pyrazolidinone	Aryl;Hydrazine derivatives;Pyrazolidine;Pyrazolidinedione/Pyrazolidone	92-43-3	Solid	NC	I	> 0									2.69	2.66	0.03	5	✓	
141	Piperazine	Aliphatic amine, secondary;Amine, secondary;Piperazine;Saturated heterocyclic fragment	110-85-0	Solid	NC	I	= 0									0.03	0.03	0.00	14		
142	Famoxadone	Aryl;Carbamate;Ether moiety;Hydrazine derivatives;Imide;Oxazolidine derivatives	131807-57-3	Solid	NC	NI	= 0									2.79	2.76	0.02	7		
143	Thiamethoxam	Aryl;Aryl halide;Ether moiety;Guanidine;N-Nitro;Thiazole/Isothiazole	153719-23-4	Solid	NC	NI	= 0									1.23	0.98	0.24	7		✓
144	Fenamidone	Amidine;Aryl;Hydrazine derivatives;Sulfide	161326-34-7	Solid	NC	NI	= 0									2.71	2.66	0.05	7		
145	Amitraz	Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Amidine;Aryl	33089-61-1	Solid	NC	NI	= 0									3.16	3.08	0.08	7		
146	Pyrimethanil	Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Amine, secondary;Aromatic amine;Aryl;Pyrimidine	53112-28-0	Solid	NC	NI	= 0									2.95	2.95	0.01	7		
147	Fluoxastrobin	Aryl;Aryl halide;Ether moiety;Ketoxime derivatives;Pyrimidine	361377-29-9	Solid	NC	NI	> 0			CO 1/3						2.61	2.54	0.07	7		

148	Tetrabromobisphenol A	Aryl;Aryl halide;Phenol	79-94-7	Solid	NC	NI	= 0		2.96	2.94	0.02	7		
149	4-[3-Chloro-4-(3-fluorobenzyloxy)phenylamino]-6-iodoquinazoline	Amine, secondary;Aromatic amine;Aryl;Aryl halide;Benzyl;Ether moiety;Fused heterocyclic aromatic;Pyrimidine;Quinazoline	231278-20-9	Solid	NC	NI	= 0		2.66	2.64	0.02	7		
150	2,4-Dichloro-5-sulfamoylbenzoic Acid	Aryl;Aryl halide;Carboxylic acid;Sulfonamide	2736-23-4	Solid	NC	NI	> 0		2.73	2.64	0.09	3	✓	
151	1,2-Epoxycyclooctane	Cycloalkane;Epoxide;Fused saturated heterocycles;Saturated heterocyclic fragment	286-62-4	Solid	NC	I	> 0	CO 1/3	3.03	2.83	0.20	7		✓
152	L-Phenylephrine	Alcohol;Aliphatic amine, secondary;Amine, secondary;Aryl;Phenol	59-42-7	Solid	NC	I	> 0		2.65	2.56	0.09	14		
153	1-Phenyl-3-pyrazolidone	Aryl;Hydrazine derivatives;Pyrazolidine;Pyrazolidinedione/Pyrazolidone	92-43-3	Solid	NC	I	> 0		2.55	2.26	0.29	7		✓
154	3,4,4'-Trichlorocarbanilide	Aryl;Aryl halide;Urea derivatives	101-20-2	Solid	NC	NI	= 0		3.26	3.19	0.07	7		
155	N-Phenylthiourea	Aryl;Thiourea derivatives	103-85-5	Solid	NC	NI	= 0		2.58	1.76	0.82	7		✓
156	4,4'-Methylenebis(2,6-di-tert-butylphenol)	Alkyl (hetero)arenes;Alkyl-, alkenyl- and alkynyl (hetero)arenes;Aryl;Phenol;tert-Butyl	118-82-1	Solid	NC	NI	= 0		2.45	1.38	1.06	7		✓
157	2,3-Dimethyl-2,3-dinitrobutane	Alkane, branched with tertiary carbon;Nitroaliphatic compounds	3964-18-9	Solid	NC	NI	= 0		2.34	2.03	0.31	7		✓
158	3',5'-Dihydroxyacetophenone	Aryl;Ketone;Phenol	51863-60-6	Solid	NC	I	= 0		2.13	0.89	1.23	7		✓

[1)] Analyzed by OECD QSAR Toolbox 4.4.1, [2)] Barroso et al., *Arch Toxicol.* 9, 521-547 (2017).

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Vitrigel®-Eye Irritancy 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, which is not fully reversible, occurring after exposure of the eye to a substance or mixture, 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, which are fully reversible, occurring after exposure of the eye to a substance or mixture. Test chemicals that induce serious eye damage are classified as UN GHS Category 1, and those that induce eye irritation are classified as UN GHS Category 2, which includes subcategories 2A or 2B. Test chemicals that are neither Category 1 nor Category 2 do not require classification for eye irritation or serious eye damage and are referred to as UN GHS No Category.

2. The assessment of serious eye damage and eye irritation has historically involved the use of laboratory animals as described in OECD Test Guideline (TG) 405, which was adopted in 1981 and revised in several occasions (2). The choice of the most appropriate test method and the use of this TG should be seen in the context of the OECD Guidance Document (GD) 263 on Integrated Approaches to Testing and Assessment (IATA) for Serious Eye Damage and Eye irritation (3). However, this method does not address the toxicity and reversibility aspects of ocular toxicity. Therefore, consideration would need to be given to all possible mechanisms of ocular toxicity that may be relevant to the test chemical, based on existing data and knowledge as outlined in GD263 (3) when selecting the combination of tests to use for classification purposes.

3. The Vitrigel®-Eye Irritancy Test (EIT) method is an *in vitro* test method that allows the identification of test chemicals not requiring classification and labelling for eye irritation or serious eye damage (UN GHS No Category) as defined by the UN GHS (1) without further testing (4, 5, 6), and therefore is performed in a bottom-up approach, as suggested by Scott et al (7). However, the Vitrigel®-EIT method is not intended to identify nor differentiate between UN GHS Category 1 and UN GHS Category 2. This differentiation will need to be addressed by another tier of a testing strategy (3).

4. This TG is based on a protocol developed by Yamaguchi and Takezawa (8), which was subjected to a validation study by a validation management team (VMT) organized by the Japanese Centre for the Validation of Alternative Methods (JaCVAM) in cooperation with the International Collaboration on Alternative Test Methods (ICATM) (9). The validation study was carried-out by three participating Japanese laboratories. The validation report was evaluated by an independent peer-review panel composed of

international experts (10). Further, the OECD Expert Group concluded that the Vitrigel®-EIT method is valid for use as an initial step in a bottom-up approach for identification of test chemicals not requiring classification and labelling for eye irritation or serious eye damage (UN GHS No Cat. chemicals).

5. This TG describes a procedure for assessing the eye irritation potential of a test chemical based on its ability to induce damage to the barrier function of the human corneal epithelium (hCE) models used in the Vitrigel®-EIT method. In existing *in vitro* test methods, the viability of cells in culture *in vitro* or the corneal opacity of isolated eyeballs *ex vivo* have been utilized as an endpoint. It is known that chemicals that are irritating to the eye first destroy tear film and epithelial barrier function of the eye, subsequently induce epithelial cell death, and finally produce stromal degeneration and endothelial cell death, resulting in corneal opacity (11, 12). Therefore, the change of the epithelial barrier function is a relevant endpoint for detecting eye irritation (13, 14). In the Vitrigel®-EIT method, time-dependent changes in the Transepithelial Electrical Resistance (TEER) values are indicative of damage to the barrier function of the corneal epithelium following exposure to a test chemical; this situation is similar to the observed damage of the rabbit cornea following exposure to a test chemical, which is an important mode of action leading to damage of the corneal epithelium and eye irritation (4, 13).

6. The term “test chemical” is used in this TG to refer to the chemicals being tested and is not a reference to the applicability of the Vitrigel®-EIT method to the testing of chemicals. The term “test chemical preparation” is used to describe the mixture of the test chemical with a culture medium (see paragraph 27).

7. Definitions are provided in ANNEX 1 - .

PRINCIPLE OF THE TEST

8. The Vitrigel®-EIT method is an *in vitro* assay using hCE models fabricated in a collagen vitrigel® membrane (CVM) chamber (5). The eye irritation potential of the test chemical is predicted by analyzing time-dependent changes in TEER values using the score of three indexes (see the prediction model in Table 2).

9. The Vitrigel®-EIT method makes use of the destructive activity of the test chemical against the barrier function of hCE models as an endpoint to assess the extent of damage to the hCE model. The test chemical is dissolved or suspended in the culture medium before exposure of the hCE model to prevent a delay of the reaction due to slow dissolution. In a previous study it was observed that the TEER values of the hCE models decreased immediately after exposing the test chemical preparations and became constant within 3 minutes (4). Therefore, the exposure period of the hCE model to the test chemical preparation was limited to 3 minutes.

INITIAL CONSIDERATIONS AND LIMITATIONS

10. The Vitrigel®-EIT can be applied to test chemicals absorbing light in the same range as formazan dye, and test chemicals able to directly reduce the tetrazolium dye (see paragraph 16). However, test chemical preparations of both solids and liquids showing acidity (pH ≤ 5) and rapid phase separation are not in the applicability domain of the test method as explained below. When the absolute difference of the absorbance values of the 2.5% weight/volume (w/v) test chemical preparation at 0 and 3 minutes is greater than 0.1, the chemical should not be tested. See paragraph 28 for further details.

11. The results of the validation study showed within-laboratory reproducibility to be 80–100% at all three laboratories and a between-laboratory reproducibility of 92%. The predictive capacity was evaluated based on validation and the developer's in-house data for 93 chemicals (9, 15, 16, 17). The Vitrigel®-EIT method achieved a sensitivity of 83% (50/60), a specificity of 70% (23/33), and an accuracy of 78% (73/93). The assay, as described in this paragraph, would not be the method of choice because of its limited predictivity (i.e., low specificity).

12. Analysis of the false-negative reactions showed that five of the 10 false-negative chemicals were acidic, and the 2.5% w/v preparations used for exposure had a pH level lower than five. Typically, the TEER values of the hCE model after exposure to UN GHS No Category chemicals changed little from their initial TEER values. The TEER values of the hCE models increased after exposure to the five acidic test chemicals that yielded false-negatives as previously reported (18, 19). Also, water-insoluble solids that readily separate from the culture medium may yield false-negative results. It should be noted that these chemicals lead to variable and extreme exposure conditions in the *in vivo* Draize eye irritation test, which may result in irrelevant predictions of their true irritation potential (2). The Vitrigel®-EIT method can be applied not only to liquids but also to solids by performing a pre-test to exclude solid test chemical preparations showing pH ≤ 5 and/or rapid phase separation during the testing time of three minutes (see paragraph 28).

13. Following these considerations, 158 test chemicals comprising 94 liquids and 64 solids were tested. Among these 158 tested chemicals, 22 solids were excluded on the basis of the pre-test: 7 showed a pH≤ 5 (2 of which also showed rapid phase separation), and 15 showed rapid phase-separation only; 29 liquids were excluded: 5 showed acidity (2 of which also showed rapid phase- separation) and 24 showed rapid phase-separation only as well. Accounting for these exclusions, the sensitivity, specificity and accuracy of the assay for the 107 test chemicals remaining in-domain is 96% (51/53), 67% (36/54) and 81% (87/107), respectively. Table 1 shows the predictive capacity for the test liquids and solids separately.

Table 1. Predictive Capacity

Liquids	Solids
Sensitivity: 100% (34/34) Specificity: 71% (22/31) Accuracy: 86% (56/65)	Sensitivity: 89% (17/19) Specificity: 61% (14/23) Accuracy: 74% (31/42)

The two false negative chemicals were GHS Cat. 2B (mild eye irritants).

14. The test method shows a high percentage of false positive results. The false positive rates obtained with the Vitrigel®-EIT method are not critical in the context of this TG since all test chemicals that result in "No Stand-alone Prediction Can Be Made" will require further information and/or testing, depending on regulatory requirements, according to the OECD GD 263 (3). The appropriate regulatory authorities should be consulted before using the test method under classification schemes other than UN GHS.

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

(3).

16. This TG is technically applicable to mono-constituent substances, multi-constituent substances, substances of unknown or variable composition, complex reaction products or biological materials (UVCBs). Mixtures, gases and aerosols, however, have not been assessed in the validation study. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable or water reactive), or test chemicals not clearly within the applicability domain described in this TG , upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture. Test chemicals absorbing light in the same range as formazan dye and test chemicals able to directly reduce the tetrazolium dye MTT can also be tested using the Vitrigel®-EIT method.

17. Any test chemical satisfying the criteria of the pre-test described above (i.e., test chemical preparations showing pH > 5 and keeping dissolution or homogeneous dispersion for at least three minutes) in a 2.5% w/v concentration in culture medium can be tested with the Vitrigel®-EIT method. Test chemicals that do not dissolve readily can be tested after using one of the following techniques: a) mix mechanically using a vortex mixer, b) sonication, and/or c) heating to a maximum temperature of 70°C (See PROCEDURE).

DEMONSTRATION OF PROFICIENCY

18. Prior to routine use of the Vitrigel®-EIT method described in this TG, laboratories should demonstrate technical proficiency by correctly classifying the 10 substances recommended in Table 32 in ANNEX 2 These substances were selected to represent the full range of responses for serious eye damage or eye irritation based on results of TG 405 on *in vivo* rabbit eye tests (2) and the UN GHS classification system (1). Other selection criteria stipulated that the proficiency substances should be commercially available, with high-quality *in vivo* reference data and high-quality *in vitro* data from the Vitrigel®-EIT method available (9, 10). In situations where a listed substance is unavailable or cannot be used for other justifiable reason, it should be substituted with another proficiency substance for which adequate *in vivo* and *in vitro* reference data are available.

PROCEDURE

19. The following paragraphs describe the main components and procedures of the Vitrigel®-EIT method, being referred as the Validated Reference Method (VRM)(8). Testing should be performed in accordance with Good In Vitro Method Practices (20). Values specified in this protocol as integers are considered to be accurate to one additional significant digit. Thus, “37°C” indicates an acceptable range from 36.5°C to 37.4°C.

Culture of hCE cells

20. Immortalized hCE cells[1] are maintained in a culture medium comprising a 1:1

[1] HCE-T cells, RCB no. 2280 obtained from RIKEN BioResource Research Center, Tsukuba, Japan. MTA with RIKEN BRC are required to perform this Test Guideline.

mixture of Dulbecco's modified Eagle medium and nutrient mixture F-12 supplemented with 5% heat-inactivated fetal bovine serum, 5 µg/mL recombinant human insulin, 10 ng/mL recombinant human epidermal growth factor, 0.5% dimethyl sulfoxide, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells are grown at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells should be free of contamination by bacteria, viruses, mycoplasma, and fungi except for the application of test chemical preparations to hCE models.

Preparation of CVM chambers

21. A collagen xerogel membrane chamber (i.e. ad-MED Vitrigel®, as used for the VRM[2]) is set in the well of a 12-well plate and immersed in the culture medium by pouring 1.5 mL outside and 0.5 mL inside the chamber in the well and waiting for 10 minutes to allow the xerogel to convert into vitrigel® immediately before use. If alternative chambers are used, hCE models prepared in the chamber should show the appropriate TEER values.

Fabrication of a hCE model

Culture procedure

22. The culture medium outside the chamber in the well of the 12-well plate is replaced with 1.5 mL of the fresh medium. The medium inside the chamber is carefully removed by using a micropipette and 0.5 mL of the cell suspension in the culture medium at a density of 1.2×10^5 cells/mL is poured onto the CVM in the chamber and cultured for two days at 37°C. After carefully removing the inside medium by using a micropipette and changing the outside medium to fresh medium, the cells are cultured for four more days at the air-liquid interface to obtain the hCE model. On the third day of culture at the air-liquid interface, the medium outside the chamber is changed.

Quality check

23. The hCE model should possess sufficient robustness equivalent to hCE in order to avoid rapid disruption after chemical exposure. The barrier function of each hCE model is checked by measuring its TEER value. First, 500 µL of fresh culture medium is poured in the chamber of the hCE models and the temperature of the culture medium is adjusted to 28±2°C. Next, the longer electrode of a TEER Measuring System (refer to the section "Measurement of TEER value in a hCE model") is set into the culture media outside the chamber, and the shorter electrode is set into the culture media inside the chamber, after which the TEER value of each hCE model (pre-exposure TEER values) is measured. Only hCE models with a TEER value within adequate range are acceptable for the testing of chemicals conducted on the same day. For the VRM, hCE models with a TEER value between 140 $\Omega \cdot cm^2$ and 220 $\Omega \cdot cm^2$ are acceptable for the testing.

Measurement of TEER value in a hCE model

24. TEER values of the hCE model should be measured by using an electrical resistance meter with low-voltage and alternating current. General specifications of the instrument are an alternating current of 50–1,000 Hz and a measuring range of at least

[2] ad-MED Vitrigel® (Kanto Chemical Co., Inc., Tokyo, Japan.)

0.1–3 kΩ. Photographic images of the TEER measuring system for the VRM[3] are shown in Annex 3. If an alternative apparatus is used, it should be demonstrated that it produces the same results. The electrode unit is 23 mm in diameter and 35 mm in height. The inner electrode is positioned inside the chamber, and the outer electrode is positioned outside the chamber. The distance between the inner and outer electrode is fixed, because this distance affects the electrical resistance value obtained. Also, during resistance measurement, the depth to which the electrodes are submerged in the medium or buffer solution inside and outside of the chamber is also fixed. The electrical resistance value of the hCE model cultured in the CVM chamber (Rmodel) and that of a blank empty CVM chamber (Rblank) are measured. The TEER value of a hCE model is calculated as follows:

$$\text{TEER value of a hCE model } (\Omega \cdot cm^2) =$$

$$\{\text{Rmodel } (\Omega) - \text{Rblank } (\Omega)\} \times \text{effective surface area } (cm^2)$$

25. The sensitivity of the TEER Measuring System should be checked before testing, and adequate ranges should be provided. This can be achieved by measuring the electrical resistance of two or more solutions having different conductivities, thereby confirming that the differences of these conductivities are within the predetermined value. For the VRM, pre-operation check of the TEER Measuring System is performed as follows. The individual CVM-free chamber (ad-MED Vitrigel® without a CVM) is set in two wells of a 12-well plate, and subsequently one well is filled with 3.0 mL of 0.90% NaCl aqueous solution and another well is filled with 0.45% NaCl aqueous solution at 25±5°C. Then, the TEER values in both wells are measured using the TEER Measuring System. The TEER measurement is functioning normally when the measured TEER values satisfy the following conditions.

$$\text{(TEER value of 0.45\% NaCl aqueous solution)}$$

$$- \text{(TEER value of 0.90\% NaCl aqueous solution)} \geq 60\ \Omega \cdot cm^2$$

Preparation of Control Substances

26. The Vitrigel®-EIT method uses saline as a negative control, benzalkonium chloride as a positive control, and ethanol which induces a response in the medium range as a reference control. The reference control is used to check the quality of the hCE models. Control substance solutions are prepared in the culture medium at a concentration of 2.5% w/v by adding 0.1–0.2 g of saline, benzalkonium chloride, or ethanol to a 15-ml tube, pouring an appropriate volume of the culture medium into the tube, and mixing until dispersed uniformly. In addition, benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals, or for evaluating the relative ocular irritancy potential of a chemical within a specific range of irritant responses.

[3] e.g., TEER Measuring System (Kanto Chemical Co., Inc., Tokyo, Japan.)

Preparation of Test Chemicals

27. A test chemical solution or suspension is prepared in the culture medium at a concentration of 2.5% w/v, as the electrical resistance value of 2.5% w/v preparation usually has little or no effect on the electrical resistance of the culture medium irrespective of the test chemical conductivity. The test chemical is manually mixed in the medium until dissolved or for a maximum of one minute. If the test chemical does not dissolve readily, one of the following techniques is used, listed here in order of preference:

a) mix mechanically for a maximum of one minute using a vortex mixer,

b) sonication for a maximum of 20 minutes, or

c) heating to a maximum temperature of 70°C.

After mixing, the temperature of the test chemical preparation is adjusted to 28±2°C using a hot plate, a water bath, or an air conditioner, and the solubility of the test chemical is checked by visual inspection. The next step is taken only once the test chemical preparation is well dissolved or homogeneously dispersed. For test chemicals that prove to be insoluble or immiscible using the above techniques, the test chemical preparation is prepared as a homogeneous suspension by vortexing the test chemical in the medium for up to one minute immediately before use.

28. The pH of each 2.5% w/v test chemical preparation is measured using a universal pH test paper covering a range from pH 1 to 11 or a pH meter. If pH of a 2.5% w/v preparation is ≤ 5 the chemical should not be tested. Also, absorbance at 660 nm of the 2.5% w/v test chemical preparation is measured by a UV-VIS spectrophotometer at 0 and 3 minutes after the preparation. However, a different wavelength has to be used in case the absorption of the test chemical at this wavelength disturbs the measurement. In the case where the absolute difference measured is above 0.1, the chemical should not be tested.

Application of the Test Chemicals and Control Substances

29. The hCE models that pass the quality check can be used for exposure to the test chemical. The hCE model should be subjected to the chemical exposure experiment within two hours after drawing it from a CO_2 incubator. The medium inside the chamber is replaced with 500 µL of test chemical preparation, and Rmodel values are measured at intervals of 10 seconds for a period of three minutes after exposure to the test chemical preparation. At least three hCE models should be used for each control substance solution and each test chemical preparation in each run. The eye irritation potential of the test chemical is predicted using the result of one run.

30. To ensure reproducibility, it is essential that measurements begin between two to five seconds after adding the test chemical preparation. A minimum of a two-second wait before beginning measurements is necessary, because the liquid around the electrode is often unstable for up to two seconds after adding the test chemical preparation. However, it should begin before 5 seconds after adding the test chemical preparation, as the TEER value of the hCE model changes in the presence of the test chemical for over five seconds.

31. The temperature of the hCE models and the test chemical preparations should be maintained at 28±2°C during the chemical exposure tests. This can be done using a hot plate, a water bath, or an air conditioner. The temperature of the hCE model can be checked by measuring the actual temperature of culture medium outside the hCE model.

Prediction Model

32. The TEER values of the hCE model after exposure to a test chemical is calculated using the formula given above in the section "Measurement of TEER value in a hCE model". The mean TEER values for all three tests are analyzed by using the following three indexes: time lag (t1), intensity (− [P2 − P1] / [t2 − t1]), and plateau level (100 − P2). Annex 4 provides a graph showing an analysis of a TEER profile after exposure of an hCE model to a test chemical. The score of each index is calculated. The test chemical is identified as not requiring classification and labelling according to UN GHS (No Category) if the scores of the indexes are Time lag > 180 seconds and Intensity < 0.05 %/seconds and Plateau level ≤ 5.0 %, as shown in Table 2. In this case, no further testing with another test method is considered necessary. If the scores of the indexes are Time lag ≤ 180 seconds or Intensity ≥ 0.05 %/seconds or Plateau level > 5.0 %, then no stand-alone prediction can be made from this result in isolation, as shown in Table 2. This is because in case of a true positive, the method cannot resolve between UN GHS Categories 1 and 2. Furthermore, the Vitrigel®-EIT method shows a high percentage of false positive results (see paragraph 14). In both cases, further information and or testing will be required for classification purposes according to GD 263 (3).

Table 2. Prediction Models according to UN GHS classification*

Criteria	Prediction
Time lag ≤ 180 seconds or Intensity ≥0.05 %/seconds or Plateau level >5.0 %	No Stand-alone Prediction Can Be Made[1]
Time lag > 180 seconds and Intensity <0.05 %/seconds and Plateau level ≤ 5.0 %	No Category[2]

Notes:
[1]No Stand-alone Prediction Can Be Made corresponds to chemicals that require further information for classification purposes according to the IATA guidance document (3).
[2]No Category corresponds to chemicals that do not require classification for serious eye damage or eye irritation according to UN GHS
* Consideration would need to be given to all possible mechanisms of ocular toxicity that may be relevant to the test chemical, based on existing data and knowledge as outlined in GD263 (3), when deriving a classification.

Acceptance Criteria

33. Test run is judged to be acceptable when the following four criteria are all satisfied:

a) Negative control: The plateau level is ≤ 5%.

b) Positive control: The plateau level is ≥ 40%

c) Reference control: The plateau level is ≥ 10%.

d) The average standard deviation of the overall TEER profile for each test chemical is ≤ 15%.

The range of historical results for the positive control in the validation study is from 65% to 90%.

DATA AND REPORTING

Data

34. TEER values obtained for each individual hCE model, the scores of each index, and the final prediction by the Vitrigel®-EIT method should be reported.

Test Report

35. The test report should include the following information:

Test Chemical and Control Substances

- 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
- Multi-constituent substance, UVCB and mixture: Characterization 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 possible
- Physical state, pH, volatility, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent possible
- Purity, chemical identity of impurities as appropriate and practically feasible, etc.
- Treatment prior to testing, if applicable (e.g., warming)
- Storage conditions and stability to the extent possible

Test Method Conditions and Procedures

- Name and address of the sponsor, test facility and study director
- Description of the test method used
- Details of test procedure used
- Cell line used, its source, passage number and confluence of cells used for testing
- Supplier, catalog number and lot number of a reagent
- Time and date of sub-culturing hCE cells, duration of tripsinization, dilution ratio of the cells
- Duration of each step for preparation of hCE models
- Data of QC check for TEER measuring system
- Record of test chemical preparation (e.g. weight of test chemical, volume of medium, mixing method and solubility of test chemical, pH of the test chemical preparation, and absorbance values at 660 nm values at 0 and 3 minutes of the test chemical preparation)
- Temperature of the hCE models and test chemical preparation at the start of exposure test

- Lot number of a hCE model
- Time of removal of the hCE model from CO_2 incubator, and exposure of the hCE model to the test chemical
- Time of starting TEER measurement by a TEER Measuring System
- Test chemical concentrations used (if different than the ones recommended)
- Duration of exposure to the test chemical (if different than the one recommended)
- Number of runs and number of hCE models used within each run (if different than recommended)
- Description of any modifications of the test procedure
- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals

Results

- For each test chemical and control substance, tabulation should be given for preexposure TEER values and time dependent TEER values after exposing test chemicals for three minutes for each hCE model used, scores of three indexes, and the *in vitro* prediction of the test chemical.
- Description of other effects observed

Discussion of the Results

Conclusions

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ANNEX 1 - DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with “concordance” to mean the proportion of correct outcomes of a test method (21).

Applicability domain: A description of the physicochemical or other properties of the chemicals for which a test method is applicable for use (21).

Bottom-Up Approach: A step-wise approach used for a test chemical suspected of not requiring classification for eye irritation or serious eye damage, which starts with the determination of chemicals not requiring classification (negative outcome) from other chemicals (positive outcome) (3).

Chemical: means a substance or mixture.

Collagen vitrigel membrane (CVM): A membrane composed of high density collagen fibrils modelling the connective tissues *in vivo* and is easily handled with tweezers. Also, it possesses excellent transparency and permeability of protein with high molecular weight and consequently provides an ideal cell culture scaffold (22-26). The CVM chamber is prepared from a commercially available collagen xerogel membrane chamber.

Effective surface area: The bottom surface area of the CVM chamber.

Eye irritation: The production of changes in the eye, which are fully reversible, occurring after the exposure of the eye to a substance or mixture (1).

False negative rate: The proportion of all positive chemicals falsely identified by a test method as negative. It is one indicator of test method performance (21).

False positive rate: The proportion of all negative (non-active) chemicals that are falsely identified as positive. It is one indicator of test method performance (21).

Hazard: The potential for an adverse health or ecological effect. The adverse effect is manifested only if there is an exposure of sufficient level (21).

hCE: human corneal epithelium.

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

MoA: mode of action.

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.

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 check the durability of the hCE models.

Performance standards: Standards, based on a validated test method, 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. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Reference control: A sample containing all components of a test system and treated with a substance known to induce a middle class response in the system. This sample is processed with test chemical-treated samples and other control samples and is used to check the quality of the hCE models.

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 (21).

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).

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

Sensitivity: The proportion of all positive/active substances 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: The production of tissue damage in the eye, or serious physical decay of vision, which is not fully reversible, occurring after exposure of the eye to a substance or mixture (1).

Specificity: The proportion of all negative/inactive substances 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, inducing 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 chemical: The term "test chemical" is used to refer to what is being tested.

Tiered testing strategy: A stepwise testing strategy where all existing information on a test chemical is reviewed, in a specified order, using a weight of evidence process at each tier to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier. If the irritancy potential

of a test chemical can be assigned based on the existing information, no additional testing is required. If the irritancy potential of a test chemical cannot be assigned based on the existing information, a stepwise sequential animal testing procedure is performed until an unequivocal classification can be made (21).

Top-Down Approach: step-wise approach used for a test 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).

Transepithelial electrical resistance (TEER): The electrical resistance of an epithelium or epithelial cell layers. It is considered a suitable means (index) for evaluating the integrity of the tight junction of corneal epithelium.

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

UN GHS Category 1: Serious eye damage/irreversible effects on the eye (1).

UN GHS Category 2: Eye irritation/reversible effects on the eye (1).

UN GHS Category 2A: Irritating to eyes (1).

UN GHS Category 2B: Mildly irritating to eyes (1).

UN GHS No Category: Chemicals that are not classified as UN GHS Category 1 or 2 (2A or 2B) (1).

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

Vitrigel: The ad-MED Vitrigel® collagen xerogel membrane chamber was used for the VRM. The definition of vitrigel is a gel in a stable state produced by rehydration after the vitrification of a traditional hydrogel (22, 23, 24, 25, 26). The literal element for “VITRIGEL” is a trademark of National Agriculture and Food Research Organization (NARO) and registered in Japan, USA, EU and China.

VRM: Validated Reference Method.

ANNEX 2 - PROFICIENCY CHEMICALS FOR THE VITRIGEL®-EYE IRRITANCY TEST METHOD

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly identifying the eye hazard classification of the 10 chemicals recommended in Table 3. The Vitrigel®-Eye Irritancy Test Method outcomes provided represent examples of the results observed during its validation study (9, 10). The selection includes, insofar as possible, chemicals that

(i) cover the full range of *in vivo* serious eye damage/eye irritation responses based on the UN GHS classification system (i.e., Categories 1, 2A, 2B or No Category),

(ii) are based on high quality results obtained in the reference *in vivo* rabbit eye test (OECD TG 405), (2)

(iv) cover a broad range of the chemical classes and organic functional groups, representative of those used in the validation study, (10)

(v) cover the range of *in vitro* responses based on high quality Vitrigel®-EIT data,

(vi) produced correct and reproducible predictions in the VRM,

(vii) are commercially available, and

(viii) are not prohibitively expensive either to acquire or dispose of.

In situations where a listed chemical is unavailable or cannot be used for other justifiable reason, it should be substituted with another chemical that fulfills the criteria described above, e.g. from the chemicals used in the validation of the Vitrigel®-Eye Irritancy Test Method or listed as a reference chemical within the Performance Standards (27).

Table 3. Recommended chemicals for demonstrating technical proficiency with the Vitrigel®-Eye Irritancy Test Method

1) CASRN and physicochemical properties

	Chemical Name	CASRN	Organic Functional Group	Physical State	pH
***In vivo* UN GHS Category 1**[1]					
1	3-(2-Aminoethylamino) propyl]trimethoxysilane	1760-24-3	Silicon compound	Liquid	10
2	Tetraethylene glycol diacrylate	17831-71-9	Acrylate, Ester	Liquid	7
3	Sodium salicylate	54-21-7	Organic salts	Solid	7
***In vivo* UN GHS Category 2A**[1]					
4	Cyclopentanol	96-41-3	Alcohols	Liquid	7
5	Methyl cyanoacetate	105-34-0	Esters, Nitrile compounds	Solid	7
***In vivo* UN GHS Category 2B**[1]					
6	Ethyl-2-methylacetoacetate	609-14-3	Esters	Liquid	7
7	Ammonium nitrate	6484-52-2	Inorganic salts	Solid	8
***In vivo* UN GHS No Category**[1]					
8	*iso*-Octylthioglycolate	25103-09-7	Thiocompound, Ester	Liquid	7
9	Tetrabromobisphenol A	79-94-7	Aryl; Aryl halide Phenol	Solid	7
10	4,4'-Sulfonylbisbenzenamide	80-08-0	Dianilines, Sulfone	Solid	7

2) Test results

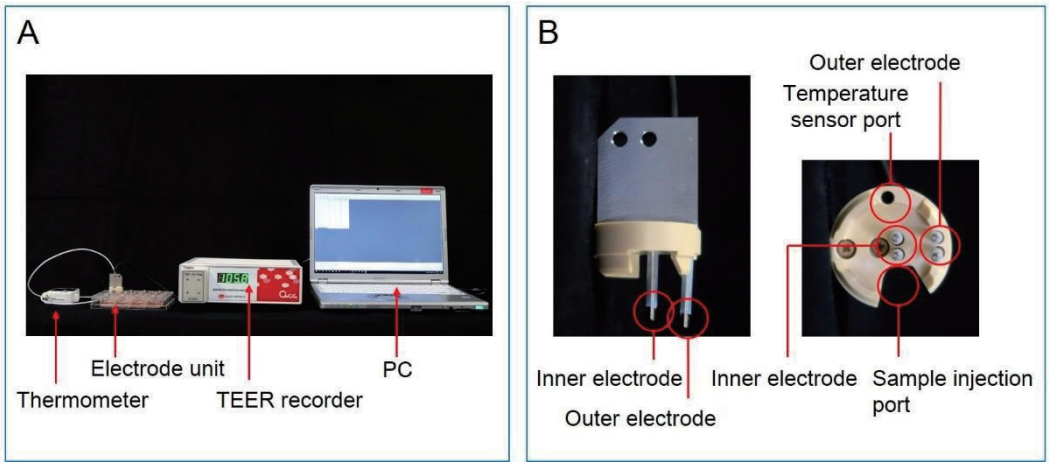
	Chemical Name	Time lag (seconds)[2]			Intensity (%/seconds)[2]			Plateau level (%)[2]			Prediction
		Mean± SD	Min.	Max	Mean± SD	Min.	Max	Mean± SD	Min.	Max	
***In vivo* UN GHS Category 1[1]**											
1	3-(2-Aminoethylamino) propyltrimethoxysilane	0 ± 0	0	0	0.36 ± 0.05	0.33	0.41	64 ± 8	60	73	No Stand-alone Prediction Can Be Made
2	Tetraethylene glycol diacrylate	7 ± 12	0	20	0.23 ± 0.02	0.20	0.24	40 ± 5	35	43	No Stand-alone Prediction Can Be Made
3	Sodium salicylate	0 ± 0	0	0	0.48 ± 0.11	0.35	0.54	41 ± 3	38	43	No Stand-alone Prediction Can Be Made
***In vivo* UN GHS Category 2A[1]**											
4	Cyclopentanol	0 ± 0	0	0	0.29 ± 0.01	0.28	0.30	52 ± 2	51	55	No Stand-alone Prediction Can Be Made
5	Methyl cyanoacetate	10 ± 10	0	20	0.10 ± 0.06	0.06	0.17	18 ± 11	11	30	No Stand-alone Prediction Can Be Made
***In vivo* UN GHS Category 2B[1]**											
6	Ethyl-2-methylacetoacetate	3 ± 6	0	10	0.22 ± 0.03	0.19	0.25	41 ± 6	34	46	No Stand-alone Prediction Can Be Made
7	Ammonium nitrate	0 ± 0	0	0	0.69 ± 0.12	0.58	0.82	29 ± 17	18	49	No Stand-alone Prediction Can Be Made
***In vivo* UN GHS No Category[1]**											
8	*iso*-Octylthioglycolate	>180	>180	>180	-0.01 ± 0.01	-0.02	0.00	1 ± 1	0	2	No Category
9	Tetrabromobisphenol A	>180	>180	>180	-0.02 ± 0.01	-0.02	-0.01	0 ± 0	0	0	No Category
10	4,4'-Sulfonylbis benzenamide	>180	>180	>180	-0.02 ± 0.01	-0.03	-0.01	0 ± 0	0	0	No Category

Abbreviations: CASRN, Chemical Abstracts Service Registry Number; UN GHS, United Nations Globally Harmonized System of Classification and Labelling of Chemicals; VRM, Validated Reference Method.

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

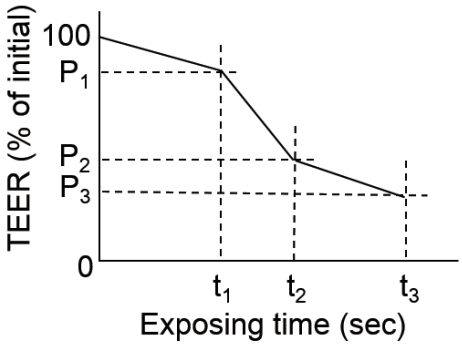
[2] Based on results obtained with validation Study of the Vitrigel®-EIT method (9,10). Data for chemical No. 5, 7, 9 and 10 are based on results obtained with the developer's in-house data. Each score was calculated from the data of three runs. Three hCE models were used for each run.

ANNEX 3 - PHOTOGRAPHIC IMAGES OF THE TEER MEASURING SYSTEM



The TEER measuring system (A) and the electrode unit (B).

ANNEX 4 - GRAPH SHOWING AN ANALYSIS OF A TEER PROFILE AFTER EXPOSURE OF A MODEL TO A TEST CHEMICAL



Time lag (second) = t_1

Intensity (% / second)= $-(P_2 - P_1)/(t_2 - t_1)$

Plateau level (%)= $100 - P_2$

Notes:

t1 (second); The maximum time at which a profile is maintained at $0 \geq dP/dT > -0.03\%$/second.
t2 (second); The initial time at which the profile is maintained at $0 \geq dP\ (P3 - P2)/dT\ (t3 - t2) > -0.03\%$/second after the profile is maintained at $dP/dT \leq -0.03\%$/second.
t3 (second); t2 + 30 seconds because the plateau level is evaluated by the profile for 30 seconds.
P1 (%); The percentage of TEER value at t1 against the TEER value at 0 second.
P2 (%); The percentage of TEER value at t2 against the TEER value at 0 second.
P3 (%); The percentage of TEER value at t3 against the TEER value at 0 second.
dP/dT; The derivative of P with respect to t.