新規試験法提案書

皮膚腐食性試験代替法
LabCyte EPI-MODEL24 SCT

令和 3 年12月

国立医薬品食品衛生研究所

新規試験法提案書

令和 3 年 12 月 7 日

No.　2021-02

皮膚腐食性試験代替法LabCyte EPI-MODEL24 SCTに関する提案

令和3年12月7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：本試験法において陽性の結果が得られた場合、被験物質を腐食性物質（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）分類における区分 1）と判定することは可能である。UN GHS 細区分を考慮した評価においても、LabCyte EPI-MODEL24 SCT は、EpiSkin™および EpiDerm™ SCT と同様に評価できる試験法である。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。

この提案書は、皮膚腐食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皮膚腐食性試験代替法 LabCyte EPI-MODEL24 SCT 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 安全性生物試験研究センター/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

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）
高 畑 正 浩　（厚生労働省 医薬・生活衛生局 医薬品審査管理課）
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）
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）
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 *In Vitro* Skin Corrosion: Reconstructed Human Epidermis (RhE) Test Method, LabCyte EPI-MODEL24 SCT

At a meeting held on 7 December 2021 at the National Institute of Health Sciences (NIHS) in Tokyo, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Proposal: Although a positive result in an *in vitro* skin corrosion test using human skin models such as LabCyte EPI-MODEL24 SCT is generally considered sufficient for predicting a test chemical to cause skin corrosion under the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) Category 1, this test as well as EpiSkin™ and EpiDerm™ SCT are considered sufficient for predicting a test chemical to cause skin corrosion under the UN GHS subcategories. Furthermore, thorough consideration must be given to the applicability domain when using this test.

This statement was prepared following a review of the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 431 *In Vitro* Skin Corrosion: Reconstructed Human Epidermis (RhE) Test Method together with other materials prepared by the Skin Corrosion Testing JaCVAM Editorial Committee to acknowledge that the results of a review and study by the JaCVAM Regulatory Acceptance Board have confirmed the usefulness of this assay.

Based on the above, we propose the *In Vitro* Skin Corrosion: RhE Test Method, LabCyte EPI-MODEL24 SCT as a useful means for assessing skin corrosion potential during safety assessments by regulatory agencies.

Akiyoshi Nishikawa
Chairperson
JaCVAM Regulatory Acceptance Board

Yoko Hirabayashi
Chairperson
JaCVAM Steering Committee

December 7, 2021

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 (Center for Biological Safety and Research: CBSR, National Institute of Health Sciences: NIHS / Saiseikai Utsunomiya Hospital) : Chairperson
Ms. Yoko Hirabayashi (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

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)
Mr. Akihiko Hirose (Division of Risk Assessment, CBSR, NIHS)
Mr. Koji Ishii (National Institute of Infectious Diseases)
Mr. Yasunari Kanda (Division of Pharmacology, CBSR, NIHS)
Mr. Satoshi Kitajima (Division of Toxicology, 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. Masahiro Takahata (Ministry of Health, Labour and Welfare)
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

皮膚腐食性試験代替法LabCyte EPI-MODEL24 SCTに関する提案

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評価会議報告書

ヒト表皮モデルを用いた皮膚腐食性試験代替法

JaCVAM 評価会議

令和 3 年(2021 年)12 月 7 日

JaCVAM 評価会議

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/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)

*：資料編纂委員会の委員でもある

任期：令和 2 年 4 月 1 日～令和 4 年 3 月 31 日

略語

EURL ECVAM：European Union Reference Laboratory for alternatives to animal testing
（欧州代替法評価センター）

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）

MTT：3- (4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

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

SCT：Skin Corrosive Test（皮膚腐食性試験）

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、腐食性試験資料編纂委員会により作成された「ヒト表皮モデルを用いた皮膚腐食性試験代替法評価報告書」[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：ヒト表皮モデルを用いた皮膚腐食性試験代替法

代替する対象毒性試験：ウサギを用いる皮膚腐食性試験 [2)]

試験法の概略：

本試験法では、ウサギ皮膚の代わりに角質層を有する 3 次元再構築ヒト表皮モデル（以下、表皮モデル）を用い、被験物質が角質層下の表皮細胞層に対し細胞毒性を示す能力を評価する。表皮モデル表面に被験物質を一定時間適用した後洗浄し、表皮細胞の生存率を MTT の還元量から求め、皮膚腐食性を判定する。

試験法の科学的妥当性：

本試験法は、日本で開発された表皮モデルを用いた LabCyte EPI-MODEL24 SCT であり、JaCVAM によるバリデーション研究とそれに続く第三者評価により、実験動物を用いた皮膚腐食性試験の代替法として科学的に妥当であると報告されている [3,4)]。EURL ECVAM でバリデートされた EpiSkin™および EpiDerm™ SCT と同様 [5,6)]、OECD TG431 にて承認されている [7)]。JaCVAM 皮膚腐食性試験資料編纂委員会では、現在まで公開されている情報 [8-11)] および先に評価された表皮モデルを用いた皮膚腐食性試験代替法と同様に、LabCyte EPI-MODEL24 SCT の科学的妥当性について評価した。その結果、本試験法は、「腐食性物質が角質層の傷害または角質層への吸収を経て下層の表皮細胞に対し毒性を示す」という皮膚腐食性発現機序に基づき、細胞への毒性を指標にしたものであり、原理的にも妥当であると判断された。

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り、OECD TG431で承認された市販されている表皮モデルは他にもあるが、本モデルは国産であり、入手が容易であるとともに、コストも安い。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：

本試験法において陽性の結果が得られた場合、被験物質を腐食性物質（国連 GHS 分類における区分 1）[12)]と判定することは可能である。国連 GHS 細区分を考慮した評価においても、LabCyte EPI-MODEL24 SCT は、EpiSkin™および EpiDerm™ SCT と同様に評価できる

試験法である。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　腐食性試験資料編纂委員会：評価報告書　ヒト表皮モデルを用いた皮膚腐食性試験代替法（2021 年 12 月 7 日）
2) OECD, Guideline for the testing of chemicals. 404, Acute Dermal Irritation/Corrosion 2015.
3) JaCVAM Validation Management Team, Validation Study for *in vitro* skin corrosion test method using reconstructed human epidermal tissue LabCyte EPI-MODEL24. 2019.
4) JaCVAM, Peer Review Panel Evaluation of the Performance-based Validation Study on the LabCyte EPI-Model24 *in vitro* skin corrosion test method as a me-too test method according to OECD GD 219 and falling within the OECD TG 431, 2018.
5) ECVAM, Statement on the scientific validity of the Episkin test (An *in vitro* test for skin corrosivity) 1998.
6) ECVAM, Statement on the Application of the Epidermtm Human Skin Model for Skin Corrosivity Testing 2000.
7) OECD, Guideline for the testing of chemicals. 431, *In Vitro* Skin Corrosion: Human skin model test. 2016.
8) ICCVAM, NIH Publication No.02-4502 ICCVAM Evaluation of Episkin™, EpiDerm™ (EPI-200), and the Rat Skin Transcutaneous Electrical Resistance (TER) Assay: *In Vitro* Test Methods for Assessing Dermal Corrosivity Potential of Chemicals 2002.
9) OECD, Series on Testing and Assessment No. 190 Summary Document on the Statistical Performance of Methods in OECD Test Guideline 431 for Sub-Categorisation, in Series on Testing and Assessment. 2013.
10) OECD, Series on Testing & Assessment No. 203, Guidance Document on an Integrated Approach on Testing and Assessment (IATA) for Skin Corrosion and Irritation. 2014.
11) OECD, Series on Testing & Assessment No. 219 Performance Standards for the Assessment of Proposed Similar or Modified *In Vitro* Reconstructed Human Epidermis (RhE) Test Methods for Skin Corrosion Testing as Described in TG 431. 2015.
12) GHS 関係省庁連絡会議仮訳, 化学品の分類および表示に関する世界調和システム（GHS）改訂 8 版. 化学工業日報社, 東京, 2020.

評価報告書

ヒト表皮モデルを用いた皮膚腐食性試験代替法

皮膚腐食性試験資料編纂委員会

令和 3 年(2021 年)12 月 7 日

皮膚腐食性試験資料編纂委員会

髙橋　祐次　（委員長：国立医薬品食品衛生研究所 毒性部）
中村るりこ　（独立行政法人 製品評価技術基盤機構　化学物質管理センター）
山城　朋子　（株式会社資生堂　品質・安全性評価室／日本化学工業協会）
小島　　肇　（国立医薬品食品衛生研究所 安全性予測評価部）

List of Abbreviations（略語一覧）

ANOVA：	Analysis of Variance Software（分散分析）
ESAC:	ECVAM Scientific Advisory Committee（ECVAM 科学諮問会議）
EURL ECVAM:	European Union Reference Laboratory for alternatives to animal testing（欧州代替法評価センター）
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）
ICCVAM：	Interagency Coordinating Committee on the Validation of Alternative Methods（米国の代替法に関する省庁間連絡会議）
JaCVAM：	Japanese Center for the Validation of Alternative Methods（日本動物実験代替法評価センター）
MTT：	3- (4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
OECD：	Organisation for Economic Co-operation and Development（経済協力開発機構）
OD：	Optical densities（吸光度）
PBS：	Phosphate buffered saline（リン酸緩衝液）
SCT：	Skin Corrosive Test（皮膚腐食性試験）
SDS：	Sodium dodecylsulphate
TER：	Transcutaneous electrical resistance assay（経皮電気抵抗性試験）
TG：	Test Guideline（試験法ガイドライン）
UN：	United Nations（国際連合）

要旨

ウサギを用いる皮膚腐食性試験の動物実験代替法（以下、代替法と記す）として、経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）にて試験法ガイドライン（TG: Test Guideline）431 として承認されたヒト表皮モデルを用いる試験法の有用性を評価した。当該 TG は 2019 年の改定において、LabCyte EPI-MODEL24 が追加されたことから、他のヒト表皮モデルと信頼性と妥当性という視点において、比較、評価した結果、TG431 に掲載されているモデルの中で、EpiSkin™、EpiDerm™と同様に、LabCyte EPI-MODEL24 が腐食性の有無を評価できるモデルとして推奨できると考えられた。また、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）分類の細区分を考慮した評価においても、LabCyte EPI-MODEL24 SCT（Skin Corrosive Test）は、EpiSkin™、EpiDerm™ SCT と同様に評価できる試験法である。当該モデルは、日本国内で製造されていることから、海外生産のモデルに比較してコスト、入手が容易である点で有用であると考えられた。

1. 試験法の科学的および規制面からの妥当性

皮膚腐食性試験は皮膚刺激性試験の一環として行われ、種々のガイドラインでは Draize らにより提唱されたウサギを用いる方法が推奨されてきた[1)]。この方法は被験物質の刺激性や腐食性を検出する試験として長く使用されてきたものの、判定を肉眼で行うため客観性に乏しく実験間や施設間での再現性が乏しい。更に動物に激しい痛みとストレスを与えることが社会的に問題となり、以前より動物を使用しない動物実験代替法(以下、代替法と記す)の開発が切望されていた。

この代替法として、経済協力開発機構(OECD: Organisation for Economic Co-operation and Development)で試験法ガイドライン(TG: Test Guideline)431 には、皮膚腐食性試験として角質層を有し 3 次元的に再構築されたヒト表皮モデルを使用した評価方法が記載されている[2)]。この試験法は、腐食性物質が角質層の傷害または角質層に吸収された後拡散することにより、下層の細胞に到達して細胞毒性を示すという仮説に基づき、被験物質曝露後の細胞生存率を指標に皮膚腐食性を評価している。EpiSkin™や EpiDerm™等のヒト表皮モデルは欧米では既にバリデーション研究が実施され、欧州では化学物質の皮膚腐食性評価を目的として承認され、化学物質のリスク表示識別等に利用されていることや、昨今、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)」分類に従って評価されるケースが増えていることから、我が国においても腐食性試験の代替法として TG431 の 2016 年改訂版[3)]について、JaCVAM 皮膚腐食性試験資料編纂委員会が信頼性と妥当性という視点から評価を行った。その結果、EpiSkin™、EpiDerm™、SkinEthics™および epiCS®が被験物質の皮膚腐食性を評価する試験法として推奨できるモデルであり、UN GHS 分類の細区分を考慮する場合は EpiSkin™がもっとも有用であるとした評価報告書を作成した[4)]。これはその後、JaCVAM 評価会議においても評価され、適用範囲を十分に配慮した上で使用されるべきであるとされた[4)]。

さらに、UN GHS の最新の改訂版である 8 版(2019 年発行)[5)] においては、皮膚腐食性／刺激性の評価に対し、TG430、431 または 435 に従って実施された試験について一定の判定基準が設けられ、これらが腐食性区分 1(場合によっては細区分にも)に適用可能であることが示されたことから、代替法の行政利用の加速化が期待されているところである。

本評価書では、OECD TG431 の 2019 年改訂版[6)] で新たに追加掲載されたヒト表皮モデル LabCyte EPI-MODEL24 SCT(Skin Corrosive Test)についての腐食性試験法としての有用性を評価した。なお、本評価方法を用いる際の利便性を考慮して、既に評価済みである EpiSkin™ および EpiDerm™ SCT を用いる腐食性試験法の情報についても併記した。

2. 試験プロトコル構成の妥当性

被験物質が角質層を通過して表皮細胞に曝露され、MTT〔3- (4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide〕の還元量から求めた細胞生存率の割合から皮膚腐食性を判定する。それらの概要を表 1 にまとめた。被験物質としては、液体、粘性物、半固体、固体、ロウ様物、粘着性物質を対象にしている。実験操作上の違いは、前培養法および染色液の用量、組織からの抽出法等であり、基本的な曝露時間と判定基準については表 2 を参照されたい。

試験プロトコルとして、EpiSkin™、EpiDerm™ SCT および LabCyte EPI-MODEL24 SCT を例に説明する [6]。

1）EpiSkin™

12well プレートの各 well に培養液 2 mL を加えた後にヒト表皮モデルを置き、被験物質が液状の場合はピペッターで 50 µL 適用、粉末など固形の場合は 20mg を 100µL の NaCl 溶液（9 g/L）と合わせて適用する。被験物質を 3 分間、60 分間、および 240 分間曝露後、25 mL の PBS で被験物質を洗い流す。洗浄後、ペーパータオル等の上にて水分を切るか、表皮の破損に注意しながらピペッターを用い表面に残った PBS を吸い取った後、MTT 色素を含む培養液（0.3 mg/mL）を表皮モデルの下方に 2 mL 添加する。37℃、CO_2 インキュベータ中に 180 分間培養した後、以下の手順にて抽出を行う。MTT 色素を含む培養液を除去後、パンチ生検（biopsy punch）を用いて表皮モデルをくり抜き、酸性化イソプロパノール 0.5 mL 添加したガラスチューブに浸漬し、一晩室温放置する。96 well プレートに抽出液を 200 µL ずつ移し、マイクロプレートリーダーを用いて 545 nm-594 nm の領域での吸光度を測定する。酸性化イソプロパノールのみを加えた well をブランクとし、実測値とブランク値の差を求める。陰性対照である 240 分間曝露の NaCl 溶液（9 g/L）の吸光度を 100%とし、各検体の 3 分間、60 分間および 240 分間曝露時の吸光度を%として算出し細胞の生存率とする。

3 分間曝露したときの生存率が 35%未満を示す物質は“腐食性”と判定する。一方、240 分間曝露したときに 35%以上の結果を示す物質は“非腐食性”と判定する。さらに、腐食性の区分として、3 分間処理したときの生存率が 35%未満の場合は区分 1 A とし、3 分間曝露したときの生存率が 35%以上で、かつ 60 分間曝露したときの生存率が 35%未満の結果を示す物質、あるいは 60 分間曝露したときの生存率が 35%以上で、かつ 240 分間処理したときの生存率が 35%未満の結果を示す物質は、区分 1B/C とする。

2）EpiDerm™ SCT

6 well プレートの各 well に培養液 1 mL を加えた後にヒト表皮モデルを置き、被験物質が液状の場合はピペッターで 50 µL、粉末など固形の場合は 25 mg を 25 µL の水と合わせ、モデル上層に適用する 。被験物質を 3 分間および 60 分間曝露後、プレートから被験物質をデカンテーションにより除去した後、一定の弱流の PBS を用いて 20 回程度洗浄を行う。洗

浄後、ペーパータオル等で水分を切り、破損に注意しながら新たな 24 well プレートに表皮モデルを移動する。MTT 色素を含む培養液（1.0 mg/mL）をヒト表皮モデルの下方に 0.3 mL 添加する。37℃、CO_2 インキュベータ中に 180 分間培養した後、MTT 色素を含む培養液を吸引し PBS を添加、吸引を 2 回程度繰り返すことで、余剰な色素を取り除く。インサートにイソプロパノールを 2 mL 添加し、一晩室温放置後、96 well プレートに抽出液を 200 µL ずつ移し、マイクロプレートリーダーを用いて 540 nm あるいは 570 nm の領域での吸光度を測定する。イソプロパノールのみを加えた well をブランクとし、実測値とブランク値の差を求める。陰性対照である水の吸光度を 100%とし、各検体の 3 分間または 60 分間曝露時の吸光度を%として算出し細胞の生存率とする。

3 分間曝露したときの生存率が 50%未満、あるいは 3 分間では生存率が 50%以上であるが 60 分間曝露したときに 15%未満の結果を示す物質を“腐食性”と判定する。一方、3 分間曝露したときに生存率が 50%以上で、かつ 60 分間曝露したときに 15%以上の物質は“非腐食性”と判定する。さらに、腐食性の区分として、3 分間曝露したときの生存率が 25%未満の場合は、区分 1 A、25%以上の場合は区分 1 B/C とする。

3）LabCyte EPI-MODEL24 SCT

24well プレートの各 well に培養液 0.5 mL を加えヒト表皮モデルを置き、被験物質が液状の場合はピペッターで 50 µL、粉末など固形の場合は 50 mg を 50 µL の水と合わせ、モデル上層に適用する 。被験物質を 3 分間および 60 分間曝露後、培養カップをピンセットで取り出し、タッピングすることで被験物質を除去した後、一定の強流の PBS を用いて 10 回程度洗浄を行う。洗浄後、滅菌綿棒を用いて水分を取り除き、MTT 色素を含む培養液（0.5 mg/ml）が 0.5 mL 入った新たなプレートにインサートを移動する。37℃、CO_2 インキュベータ中に 180 分間培養した後、以下の手順にて抽出を行う。培養表皮をピンセットで取り出し、イソプロパノール 300 µL の入ったマイクロチューブに浸漬し、一晩冷暗所に放置後、96 well プレートに抽出液を 200 µL ずつ移す。マイクロプレートリーダーを用いて 570 nm および 650 nm の領域での吸光度を測定し、吸光度（570 nm）から吸光度（650 nm）を差し引いた値を測定値とする。イソプロパノールのみを加えた well をブランクとし、実測値とブランク値の差を求める。溶媒対照の水の吸光度を 100%とし各検体の 3 分間または 60 分間曝露時の吸光度を%として算出し細胞の生存率とする。

3 分間曝露したときの生存率が 50%未満、あるいは 3 分間では生存率が 50%以上であるが 60 分間曝露したときに 15%未満の結果を示す物質を“腐食性”と判定する。一方、3 分間処理したときに生存率が 50%以上、かつ 60 分間曝露したときに 15%以上の物質は“非腐食性”と判定する。さらに、腐食性の区分として、3 分間曝露したときの生存率が 15%未満の場合は、区分 1 A、15%以上の場合は区分 1 B/C とする。

表１．皮膚腐食性試験のために確認された RhE 試験方法からなる主な試験方法（OECD TG431 補遺 2　一部改 [6]）

±で示した数字は、ばらつきの許容範囲を示す。

テスト方法 要素	EpiSkin™	EpiDerm™SCT	LabCyte EPI-MODEL24 SCT
モデル表面積	0.38cm^2	0.63 cm^2	0.3cm^2
組織数	曝露時間毎に 2 つ以上	曝露時間毎に 2～3 つ	曝露時間毎に 2 つ以上
使用量と適用	液体および粘性物：50±3 µL（131.6µL/cm^2） 固体：20±2 mg（52.6 mg/cm^2）+ N 塩化ナトリウム溶液(9 g/L) 100 ±5 µL ロウ様/粘着性物質：ナイロンメッシュを用いて 50±2 mg（131.6 mg/cm^2）	液体：ナイロンメッシュを用いる場合も用いない場合でも、50 µL（79.4 µL/cm^2） 被験物質とナイロンメッシュとの親和性は予試験で確認する。 半固体：50 µL（79.4 µL/cm^2） 固体：25 mg（39.7 mg/cm^2）＋25 µL の水(必要であればそれ以上) ロウ様物：15 µL の水で湿らせた直径約 8 mm のフラットなディスク様の片を上に乗せる。	液体および粘性物：50 µL（166.7µL/cm^2） 固体：50±2 mg（166.7 mg/cm^2）+50µL の水 ロウ様物：容積式ピペットを用い、液体および粘性物として処理する。

テスト方法 要素	EpiSkin™	EpiDerm™SCT	LabCyte EPI-MODEL24 SCT
直接 MTT 還元性の事前確認	50 µL(液体)もしくは 20 mg(固体)に 0.3 mg/mL MTT 溶液 2 mL を加えて、37℃、CO_2 濃度 5%、湿度 95%で 180±5 分間培養 →溶液の色が青/紫に変わった場合、水処理でモデル構成細胞を死滅させたものに被験物質を処置する対照もとる。	50 µL(液体)もしくは 25 mg(固体)に 1 mg/mL MTT 溶液 1 mL を加えて、37℃、CO_2 濃度 5%、湿度 95%で 60 分間培養 →溶液の色が青/紫に変わった場合、凍結処理でモデル構成細胞を死滅させたものに被験物質を処置する対照もとる。	50 µL(液体)もしくは 50 mg(固体)+ 0.5 mg/mL の MTT 溶液 500µL を加えて 37°C、CO_2 濃度 5%、湿度 95%で 60 分間培養 →溶液の色が青/紫に変わった場合、凍結処理でモデル構成細胞を死滅させたものに被験物質を処置する対照もとる。
着色障害の事前確認	10 µL(液体)もしくは 10 mg(固体)に 90 µL の水を加えて室温で 15 分間攪拌する。 →溶液が着色した場合、MTT のみを加えない対照をとる。	50 µL(液体)もしくは 25 mg(固体)に 300 µL の水を加えて 37℃、CO_2 濃度 5%、湿度 95%で 60 分間 攪拌する。 →溶液が着色した場合、MTT のみを加えない対照をとる。	50 µL(液体)もしくは 50 mg(固体)に 500 µL の水を加えて 37°C、CO_2 濃度 5%、湿度 95% で 60 分間攪拌する →溶液が着色した場合、MTT のみを加えない対照をとる。
曝露時間と温度	室温(18-28℃)で 3 分間、60 分間(±5 分間)および 240 分間(±10 分間)	室温で 3 分間、および 37℃、CO_2 濃度 5%、湿度 95%で 60 分間	室温で 3 分間、および 37℃、CO_2 濃度 5%、湿度 95%で 60 分間
PBS によるすすぎ	PBS 25 mL(すすぎ一回毎に 2 mL)	PBS を一定した弱流で 20 回	PBS の一定の強流で 10 回以上
陰性対照	50 µL の NaCl 溶液(9g/L) 曝露時間毎に	50 µL の水 曝露時間毎に	50 µL の水 曝露時間毎に
陽性対照	50 µL の氷酢酸で 240 分間曝露時のみ検討	8 N 水酸化カリウム 50 µL で 60 分間曝露時のみで検討	8N 水酸化カリウム 50 µL で 60 分間曝露時のみで検討

テスト方法 要素	EpiSkin™	EpiDerm™SCT	LabCyte EPI-MODEL24 SCT
MTT 溶液	2 mL（0.3 mg/mL）	300 µL（1 mg/mL）	500 µL（0.5 mg/mL）
MTT 溶液での培養時間および温度	37℃、CO_2 濃度 5%、湿度 95%で 180 分間(±15 分間）	37℃、CO_2 濃度 5%、湿度 95%で 180 分間	37°C、CO_2 濃度 5%、湿度 95% で 180 分間（±15 分間）
抽出溶媒	500 µL の酸性化イソプロパノール (0.04 N 塩酸を含むイソプロパノール) (分離した組織を十分に浸漬)	2 mL のイソプロパノール (インサート全体から抽出)	300 µL のイソプロパノール （分離組織を完全に浸漬）
抽出時間および温度	遮光し、室温で一晩	室温で振とうせずに一晩、もしくは室温で振とうした状態で（約 120rpm）120 分間	遮光し、冷暗所で一晩
OD 測定条件	標準フィルターなしで 570nm（545-595nm）	標準フィルターなしで 570nm（もしくは 540nm）	650 nm の標準フィルターにより 570 nm
組織の品質確認	SDS で 18 時間処理 1.0 mg/mL≦IC_{50}≦3.0 mg/mL	1%Triton X-100 で処理 4.08 時間≦ET_{50}≦8.7 時間	SDS で 18 時間処理： 1.4 mg/mL ≤ IC_{50} ≤ 4.0 mg/mL

テスト方法 要素	EpiSkin™	EpiDerm™SCT	LabCyte EPI-MODEL24 SCT
試験回数	1 回、明確な結果が得られなかった時は 2 回	1 回、明確な結果が得られなかった時は 2 回	1 回、明確な結果が得られなかった時は 2 回
適合判定基準	1. 陰性対照(塩化ナトリウム溶液)で処理された複製組織の OD 値の平均は、いずれの曝露時間についても 0.6 以上 1.5 以下であること。 2. 陽性対照(氷酢酸)に 240 分間曝露された複製組織の生存率の平均値を、陰性対照に対する割合(%)で表した場合、20%以下であること。 3. 生存率の幅が 20~100%の範囲、かつ OD が 0.3 以上の場合、2 つの複製組織間の生存率の差は 30%を超えないこと。	1. 陰性対照(水)で処理された複製組織の OD 値の平均は、全ての曝露時間において 0.8 以上 2.8 以下であること。 2. 陽性対照(8N 水酸化カリウム)に 60 分間曝露された複製組織の生存率の平均値陰性対照に対する割合(%)で表した場合、15%以下であること。 3. 生存率が 20-100%の場合において、複製組織間の変動係数(CV)は 30%を超えないこと。	1. 陰性対照(水)で処理した複製組織の OD 値の平均は、いずれの曝露時間についても 0.7 以上 2.5 以下であること。 2. 陽性対照(8N 水酸化カリウム)に 60 分間曝露された複製組織の生存率の平均値陰性対照に対する割合(%)で表した場合、15%以下であること。 3. 生存率が 20～100%の範囲、かつ OD が 0.3 以上の場合、2 つの複製組織間の生存率の差は 30%を超えないこと。

表 2-1. EpiSkin™ の予測モデル

3 分間、60 分間および 240 分間曝露後の生存率	予測性の評価
3 分間曝露後の生存率が 35%未満	腐食性 ・UN GHS 細区分　1A*
3 分間曝露後の生存率が 35%以上で、かつ 60 分間曝露後の生存率が 35%未満の場合、もしくは 60 分間曝露後の生存率が 35%以上で、かつ 240 分間曝露後の生存率が 35%未満	腐食性 ・UN GHS 細区分 1B あるいは 1C
240 分間曝露後の生存率が 35%以上	非腐食性

*) 腐食性の細区分における RhE 試験法の有用性を評価するために作成したデータによると、EpiSkin™ 試験法により区分 1A に分類された物質／混合物の約 22%が、実際には区分 1B または 1C に属するものである可能性がある（すなわち、過大評価）。

表 2-2. EpiDerm™SCT の予測モデル

3 分間および 60 分間曝露後の生存率	予測性の評価
段階 1	
3 分間曝露後の生存率が 50%未満	腐食性
3 分間曝露後の生存率が 50%以上で、かつ 60 分間曝露後の生存率が 15%未満	腐食性
3 分間曝露後の生存率が 50%以上で、かつ 60 分間曝露後の生存率が 15%以上	非腐食性
段階 2　段階 1 で腐食性と判断された物質／混合物	
3 分間曝露後の生存率が 25%未満	UN GHS 細区分　1A*
3 分間曝露後の生存率が 25%以上	UN GHS 細区分 1B あるいは 1C

*) 腐食性の細区分における RhE 試験法の有用性を評価するために作成したデータによると、EpiDerm™SCT により区分 1A に分類された物質／混合物の約 29%実際には区分 1B または 1C に属するものである可能性がある（すなわち、過大評価）。

表 2-3. LabCyte EPI-MODEL24 SCT 予測モデル

3 分間および 60 分間曝露後の生存率	予測性の評価
段階 1	
3 分間曝露後の生存率が 50%未満	腐食性
3 分間曝露後の生存率が 50%以上で、かつ 60 分間曝露後の生存率が 15%未満	腐食性
3 分間曝露後の生存率が 50%以上で、かつ 60 分間曝露後の生存率が 15%以上	非腐食性
段階 2　　段階 1 で腐食性と判断された物質／混合物	
3 分間曝露後の生存率が 15%未満	UN GHS 細区分　1A*
3 分間曝露後の生存率が 15%以上	UN GHS 細区分 1B あるいは 1C

*）細区分を裏付けるため、RhE 試験法の有用性評価の観点から生成されたデータによると、LabCyte EPI-MODEL24 SCT 試験法での細区分 1A の結果 30%は、実際には細区分 1B または細区分 1C の物質／混合物（すなわち、過大分類）からなる可能性があると示された。

3. 開発および評価に使われた物質の分類、選択理由の妥当性、*in vitro*および参照データの有無

LabCyte EPI-MODEL24 SCT の再現性は、TG431 性能標準に定められた 30 被験物質により調べられている [7]。EpiSkin™との再現性は、60 の被験物質により調べられている [8]。EpiDerm™ SCT の再現性は、24 の被験物質により調べられている [9]。さらに、EpiSkin™と EpiDerm™ SCT の予測性が 80 被験物質で調べられていて [10]、LabCyte EPI-MODEL24 SCT においては 79 被験物質で調べられている [11]。評価に使用された被験物質の多くは欧州代替法評価センター（EURL ECVAM：European Union Reference Laboratory for alternatives to animal testing）主導の皮膚腐食性試験バリデーション研究で使用された物質である。

4. 試験法の正確性（再現性）

LabCyte EPI-MODEL24 SCT においては、TG431 の性能標準に定められた 30 の被験物質を用いた 3 施設での 3 回の実験において、3 施設とも一致しなかった物質や偽陰性物質はなく、施設内再現性は 90%以上（表 3）、および施設間再現性 83.3%（表 4）と性能標準に定められた基準内（施設内一致率：80%、施設間一致率：70%）であった。

EpiSkin™においては、60 物質を用いた 3 施設での 2-way の ANOVA 解析を用い、施設内および施設間の変動については、それらの間に有意な差はないと判断された [8]。60 物質（27 腐食性物質および 33 非腐食性物質）のうち、42 物質は 3 施設とも施設内および施設間再現性が良好であった。残る 18 物質ではいずれかの結果が異なっていたが、EURL ECVAM は本試験法の信頼性と再現性は高いと判断した [12]。この結論は、 ECVAM 科学諮問会議（ESAC: ECVAM Scientific Advisory Committee）および米国の代替法に関する省庁間連絡会議（ICCVAM : Interagency

Coordinating Committee on the Validation of Alternative Methods)[13] での評価においても確認された。

EpiDerm™ SCT においては、24 物質を用いた 3 施設での 2 回の実験において、21 物質の腐食性を 3 施設すべてで正しく予測できた[9]。EURL ECVAM は本試験法の信頼性と再現性は高いと判断した。この結論は、ESAC の評価後[14]、ICCVAM においても確認された[13]。

表 3. LabCyte EPI-MODEL24 SCT の施設内再現性結果

Lab A

No	Run 1	Run 2	Run 3	Concordance
1	NC	NC	NC	✓
2	NC	NC	NC	✓
3	NC	NC	NC	✓
4	NC	NC	NC	✓
5	NC	NC	NC	✓
6	NC	NC	NC	✓
7	NC	NC	NC	✓
8	NC	NC	NC	✓
9	1A	1A	1A	✓
10	1B/C	NC	1B/C	✗
11	1B/C	1B/C	1A	✗
12	1A	1A	1A	✓
13	1B/C	1B/C	1B/C	✓
14	1B/C	1B/C	1B/C	✓
15	1B/C	1B/C	1B/C	✓
16	1A	1A	1A	✓
17	1A	1A	1A	✓
18	1A	1A	1A	✓
19	1A	1B/C	1A	✗
20	1A	1A	1A	✓
21	1A	1A	1A	✓
22	1A	1A	1A	✓
23	1A	1A	1A	✓
24	1A	1A	1A	✓
25	1A	1A	1A	✓
26	1A	1A	1A	✓
27	1A	1A	1A	✓
28	1A	1A	1A	✓
29	1A	1A	1A	✓
30	1B/C	1B/C	1B/C	✓
27/30				**90.00%**

Lab B

No	Run 1	Run 2	Run 3	Concordance
1	NC	NC	NC	✓
2	NC	NC	NC	✓
3	NC	NC	NC	✓
4	NC	NC	NC	✓
5	NC	NC	NC	✓
6	NC	NC	NC	✓
7	NC	NC	NC	✓
8	NC	NC	NC	✓
9	1A	1A	1A	✓
10	NC	NC	NC	✓
11	1A	1B/C	1B/C	✗
12	1A	1A	1A	✓
13	1B/C	1B/C	1B/C	✓
14	1B/C	1B/C	1B/C	✓
15	1B/C	1B/C	1B/C	✓
16	1A	1A	1A	✓
17	1A	1A	1A	✓
18	1A	1A	1A	✓
19	1B/C	1B/C	1B/C	✓
20	1A	1A	1A	✓
21	1A	1A	1A	✓
22	1A	1A	1A	✓
23	1A	1A	1A	✓
24	1B/C	1B/C	1B/C	✓
25	1A	1A	1A	✓
26	1A	1A	1A	✓
27	1A	1A	1A	✓
28	1A	1A	1A	✓
29	1A	1A	1A	✓
30	1B/C	1B/C	1B/C	✓
29/30				**96.70%**

Lab C

No	Run 1	Run 2	Run 3	Concordance
1	NC	NC	NC	✓
2	NC	NC	NC	✓
3	NC	NC	NC	✓
4	NC	NC	NC	✓
5	NC	NC	NC	✓
6	NC	NC	NC	✓
7	NC	NC	NC	✓
8	NC	NC	NC	✓
9	1B/C	1B/C	1B/C	✓
10	NC	NC	1B/C	✗
11	1B/C	1B/C	1B/C	✓
12	1A	1A	1B/C	✗
13	1B/C	1B/C	1B/C	✓
14	1B/C	1B/C	1B/C	✓
15	1B/C	1B/C	1B/C	✓
16	1A	1A	1A	✓
17	1A	1A	1A	✓
18	1A	1A	1A	✓
19	1A	1A	1A	✓
20	1A	1A	1A	✓
21	1A	1A	1A	✓
22	1A	1A	1A	✓
23	1A	1A	1A	✓
24	1B/C	1A	1A	✗
25	1A	1A	1A	✓
26	1A	1A	1A	✓
27	1A	1A	1A	✓
28	1A	1A	1A	✓
29	1A	1A	1A	✓
30	1B/C	1B/C	1B/C	✓
27/30				**90.00%**

表 4. LabCyte EPI-MODEL24 SCT の施設間再現性結果

No	GHS cat.	Lab A	Lab B	Lab C	Concordance
1	NC	NC	NC	NC	✓
2	NC	NC	NC	NC	✓
3	NC	NC	NC	NC	✓
4	NC	NC	NC	NC	✓
5	NC	NC	NC	NC	✓
6	NC	NC	NC	NC	✓
7	NC	NC	NC	NC	✓
8	NC	NC	NC	NC	✓
9	NC	1A	1A	1B/C	✗
10	NC	1B/C	NC	NC	✗
11	1B/C	1B/C	1B/C	1B/C	✓
12	1B/C	1A	1A	1B/C	✗
13	1B/C	1B/C	1B/C	1B/C	✓
14	1B/C	1B/C	1B/C	1B/C	✓
15	1B/C	1B/C	1B/C	1B/C	✓
16	1B/C	1A	1A	1A	✓
17	1B/C	1A	1A	1A	✓
18	1B/C	1A	1A	1A	✓
19	1B/C	1A	1B/C	1A	✗
20	1B/C	1A	1A	1A	✓
21	1A	1A	1A	1A	✓
22	1A	1A	1A	1A	✓
23	1A	1A	1A	1A	✓
24	1A	1A	1B/C	1A	✗
25	1A	1A	1A	1A	✓
26	1A	1A	1A	1A	✓
27	1A	1A	1A	1A	✓
28	1A	1A	1A	1A	✓
29	1A	1A	1A	1A	✓
30	1A	1B/C	1B/C	1B/C	✓

施設間再現性 83.3％(25/30)

5. 試験法の信頼性 [7, 10, 15)]

LabCyte EPI-MODEL24 SCT の 3 施設における細区分の予測性を表 5 にまとめた。3 施設の予測性はほぼ同等であったが、1-B および 1-C を 1-A とする場合は多く、正確に評価できた値は表 5 に示す LabCyte EPI-MODEL24 requirement の基準である 55%を 43.3%と下回った。一致しなかった物質は(表 3 または表 4 の No.9、12、16～20)であった。非腐食性物質を 1-A とする結果(表 3 または表 4 の No.9)から、5%の上記基準を上回り、平均 6.7%であった。

なお、LabCyte EPI-MODEL24 SCT を含む既存のモデルとの比較として、細区分の予測性を表 6 にまとめた。79 物質を 2、3 回実験して得られた値を見る限り、いずれの値も基準を満たしており、モデル間の予測性にほとんど差はなかった。

これらモデルは MTT 還元物質への対応方法も TG431[6)] に記載されている。適用できない物質としてはガス、エアロゾールのみが記載されている(バリデーション未実施)。これらモデルは物理状態(液体・固体等)および水溶性の有無にかかわらず適用可能であり、ガス、エアロゾールを除く混合物でも適用可能とされている。なお、特定の種類の物質や混合物においてこれらモデルの適用性を否定するような明確な根拠が得られた場合には、適用範囲から除外するべきであるとされている。

表 5. OECD で集計した全セットの化学物質を用いた予測性の計算結果
(細区分：UN GHS 区分 1A、1B/1C、非腐食性)

	Lab A	Lab B	Lab C	All Labs	LabCyte EPI-MODEL24 requirements
Sensitivity (for predictions C vs. NC)	100%	100%	100%	100%	≥ 95.0%
Correctly classified 1A	90.0%	80.0%	86.7%	85.6%	≥ 80.0%
1A underclassified 1B-and-1C	10.0%	20.0%	13.3%	14.4%	≤ 20.0%
1A underclassified NC	0%	0%	0%	0%	0%
Correctly classified 1B-and-1C	*40.0%*	*46.7%*	*43.3%*	*43.3%*	≥ 55.0%
1B-and-1C overclassified 1A	*60.0%*	*53.3%*	*56.7%*	*56.7%*	≤ 45.0%
1B-and-1C underclassified NC	0%	0%	0%	0%	≤ 5.0%
Specificity (C vs NC)	83.3%	90.0%	86.7%	86.7%	≥ 70.0%
NC overclassified 1A	*10.0%*	*10.0%*	0%	*6.7%*	≤ 5.0%
NC overclassified 1B-and-1C	6.7%	0%	13.3%	6.7%	≤ 30.0%
Accuracy (C vs. NC)	94.4%	96.7%	95.6%	95.6%	≥ 87.0%
Accuracy (1A vs. 1B-and-1C vs. NC)	*71.1%*	72.2%	72.2%	*71.9%*	≥ 72.0%

Numbers in italic: rates that do not meet the required values.

表 6. OECD で集計した全セットの 79 化学物質を用いた予測性の計算結果

（細区分：UN GHS 区分 1A、1B/1C、非腐食性）

	EpiSkin™	EpiDerm™ SCT	LabCyte EPI-MODEL24 SCT	Criteria requirements
Sensitivity (for predictions C vs. NC)	98.5%	100%	100%	≥ 95%
Correctly classified 1A	83.3%	83.3%	86.1%	≥ 80%
1A underclassified 1B-and-1C	16.7%	16.7%	13.9%	≤ 20%
1A underclassified NC	0%	0%	0%	0%
Correctly classified 1B-and-1C	76.3%	71.0%	70.0%	≥ 55%
1B-and-1C overclassified 1A	21.5%	29.0%	30.0%	≤ 45%
1B-and-1C underclassified NC	2.2%	0%	0%	≤ 5%
Specificity (C vs NC)	79.3%	73.9%	78.4%	≥ 70%
NC overclassified 1A	0%	2.7%	2.7%	≤ 5%
NC overclassified 1B-and-1C	20.7%	23.4%	18.9%	≤ 30%
Accuracy (C vs. NC)	89.6%	87.9%	89.9%	≥ 87%
Accuracy (1A vs. 1B-and-1C vs. NC)	78.8%	74.2%	76.4%	≥ 72%

6. 他の科学的な報告との比較の有無

OECD の腐食性試験代替法ガイドラインとして、TG431 の他に「TG430 TER（Transcutaneous Electrical Resistance）Test Methods: 経皮電気抵抗試験」[16] および「TG435 *In Vitro* Membrane Barrier Test Method for Skin Corrosion: *in vitro* 膜バリア試験」[17] が承認されている。これらはいずれも EURL ECVAM にてバリデーション研究が実施され、ICCVAM はこれらの試験法（Rat Skin TER, EpiSkin™、EpiDerm™ SCT および Corrositex®）の正確度、感度および特異度について比較している（表 7）[10, 13]。

これらの比較においては同じ物質を用いて評価されておらず、試験物質の数量や選択物質の種類が異なっているため、結果の数値だけをもって単純にヒト表皮モデルの優越性を比較評価することは困難であるが、いずれの試験法も同等の予測性を有すると思われる。LabCyte EPI-MODEL24 SCT は EpiSkin™並びに EpiDerm™ SCT と比較されており、ヒト皮膚三次元モデルとしては十分な予測性を有していると考えられる。

表 7. 試験法の比較結果 [18)]

	TER	EpiSkin™	EpiDerm™ SCT	LabCyte EPI-MODEL24 SCT	Corrositex
物質数	122	79*	79*	79	163
正確度	81%	89.6%	87.9%	89.9%	79%
感度	94%	98.5%	100%	100%	85%
特異度	71%	79.3%	73.9%	78.4%	72%

*:OECD EWG によって選択された 80 化学物質から商業的に入手不可の 1 化合物を除外

7. 3Rs 原則との関係(動物福祉面からの妥当性)

LabCyte EPI-MODEL24 SCT は他の表皮モデルと同様に動物を使用しておらず、動物福祉面から代替法として妥当である。

8. 試験法の有用性と限界(コスト、時間からの妥当性など)

化学物質の皮膚腐食性は、その物理化学的性質に強く依存したものであり、強い酸性(pH 2.0 以下)またはアルカリ性(pH 11.5 以上)物質は、強い局所作用を有する可能性が高いことから、皮膚腐食性と判断しても良いことになっている [19)]。しかしながら、これは腐食性についての情報が他にない場合に行われるワーストケースとしての判断であり、例えば酸や塩基の添加により pH が変わり易い物質や混合物の場合は偽陽性の判断となる可能性も考えられる [20)]。このため、このような物質に対して動物を用いる必要のない TG431 で皮膚腐食性を評価することは有用である。

各試験実施施設で本モデルを導入する際には、正しく評価できることを予め確認する必要がある。TG431 には、習熟度確認物質を正しく分類できるか否か試験することにより、専門技術の習熟について確認することができるとの記載がある(表 8)。

LabCyte EPI-MODEL24 は、日本国内で製造されていることから海外で開発された製品に比較して入手が容易であり、試験計画への柔軟な対応が可能になると考えられる。例えば、EpiDerm™ が 2 回／月の発送であるのに対し LabCyte EPI-MODEL24 は 1 回／週、モデルの入手コストは EpiDerm™の約半分である。

表 8. 習熟度確認物質　　OECD Test Guideline No 431 Table 1[6] 一部改

化学物質 1	CASRN	化学物質分類	UN GHS (*in vivo* 試験) による区分	VRM (*in vitro* 試験) による区分	物理的状態
区分 1A の *in vivo* 腐食性物質					
ブロモ酢酸	79-08-3	有機酸	1A	1A	固体
3 フッ化ボロン二水和物	13319-75-0	無機酸	1A	1A	液体
フェノール	108-95-2	フェノール類	1A	1A	固体
ジクロロアセチルクロリド	79-36-7	求電子剤	1A	1A	液体
区分 1B/1C の *in vivo* 腐食性物質					
グリオキシル酸一水和物	563-96-2	有機酸	1B/1C	1B/1C	固体
乳酸	598-82-3	有機酸	1B/1C	1B/1C	液体
エタノールアミン	141-43-5	有機塩基	1B	1B/1C	粘稠性
塩酸（14.4%）	7647-01-0	無機酸	1B/1C	1B/1C	液体
***in vivo* 非腐食性物質**					
臭化フェネチル	103-63-9	求電子剤	NC	NC	液体
4-アミノ-1,2,4-トリアゾール	584-13-4	有機塩基	NC	NC	固体
4-（メチルチオ）ベンズアルデヒド	3446-89-7	求電子剤	NC	NC	液体
ラウリン酸	143-07-7	有機酸	NC	NC	固体

＜略語＞

CASRN：CAS 登録番号

UN GHS：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」

VRM：バリデーション済み標準試験法

NC：非腐食性

9. その他

前回の皮膚腐食性試験代替法の評価後[4]、現在までの間に、UN GHS 専門家小委員会において、動物実験代替法の導入に関する議論が進み、UN GHS の最新の改訂版である 8 版（2019 年発行）[5]では、第 3.2 章　皮膚腐食性／刺激性に「3.2.2.3　*in vitro*（試験管内）/*ex vivo*（生体外）のデータに基づく分類」という項目が新たに追記された（Annex 1 参照）。

UN 文書においては商標登録された情報を記述することができないため、具体的なヒト表皮モデル名はないものの、それらの区分／細区分への判定基準が明記された。

これらが掲載された背景には JaCVAM をはじめ、EURL ECVAM や ICCVAM といった組織による動物実験代替法の検証結果が貢献するところが大きいと考えられる。

10. 結論

LabCyte EPI-MODEL24 を信頼性と妥当性という視点において評価した結果、他のヒト表皮モデルを用いた皮膚腐食性試験（EpiSkin™、EpiDerm™ SCT）と同様に、被験物質の皮膚腐食性を評価する試験法として推奨できるモデルであると結論された。UN GHS 細区分を考慮した評価においても、LabCyte EPI-MODEL24 SCT は、EpiSkin™、EpiDerm™ SCT と同様に皮膚腐食性を評価できる試験法である。

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20) Scheel, J. et al.: Toxicology *In Vitro*, 25: 1435-1447, 2011.

3.2.2.3 *in vitro*（試験管内）/*ex vivo*（生体外）のデータに基づく分類

3.2.2.3.1 現在入手できるそれぞれの *in vitro/ex vivo* 試験方法は皮膚刺激性または皮膚腐食性のどちらかを評価するが、一つの試験で両方の影響は評価しない。従って *in vitro/ex vivo* 試験結果のみに基づいた分類は二つ以上の方法で得られたデータを必要とするであろう。区分 3 を導入する所管官庁は、現在入手可能な国際的に検証され受け入れられている *in vitro/ex vivo* 試験方法では、区分 3 と分類される物質を識別することはできないことを認識することが重要である。

3.2.2.3.2 可能な限り分類は国際的に検証され受け入れられている *in vitro/ex vivo* 試験方法を用いて取られたデータに基づくべきであり、これらの試験方法に記載されている分類判定基準が適用される必要がある。試験物質が用いられた試験方法の適用範囲内にある場合にのみ、*in vitro/ex vivo* のデータは分類に使用することができる。公表された文献に記載されている追加的な制限も考慮されるべきである。

3.2.2.3.3 皮膚腐食性

3.2.2.3.3.1 OECD TG430、431 または 435 に従って試験が実施された場合、表 3.2.6 の判定基準に基づいて、物質は皮膚腐食性区分 1（また可能であり要求されていれば細区分 1A、1B または 1C）に分類される。

3.2.2.3.3.2 いくつかの *in vitro/ex vivo* 試験方法では細区分 1B および 1C（表 3.2.6 参照）を区別することはできない。細区分が所管官庁によって要求されており、既存の *in vitro/ex vivo* データが細区分を区別することができない場合には、これら二つの細区分を区別するための追加的な情報を考慮しなければならない。追加的な情報が無いまたは不十分である場合には、区分 1 が適用される。

3.2.2.3.3.3 腐食性とは同定されない物質は皮膚刺激性としての分類が検討されるべきである。

3.2.2.3.4 皮膚刺激性

3.2.2.3.4.1 腐食性に関する分類が除外され、しかも OECD TG439 に従った試験が実施された場合には、物質は表 3.2.7 の判定基準に基づいて皮膚刺激性区分 2 としての分類が検討されるべきである。

3.2.2.3.4.2 所管官庁が区分 3 を採用している場合、現在入手可能な皮膚刺激性に関する *in vitro/ex vivo* 試験方法（例えば OECD TG439）では、物質を区分 3 に分類することはできないことを認識することが重要である。このような場合、区分 1 または区分 2 に関するどちらの分類判定も実行されない場合、区分 3 または区分に該当しないを区別するために追加的な情報が必要とされる。

3.2.2.3.4.3 所管官庁が区分 3 を採用しない場合、皮膚刺激性に関して国際的に受け入れられ検証されている *in vitro/ex vivo* 試験、例えば OECD TG439 における陰性結果は皮膚刺激性の区分に

該当しないと結論するために使用することができる。

表 3.2.6.　*in vitro/ex vivo* 方法に関する皮膚腐食性判定基準（TG431 部分のみ抜粋）

<table>
<tr><td rowspan="2">区分</td><td colspan="4">再構築ヒト表皮モデル試験： OECD TG431、
附属書 2、方法 1、2、3、4</td></tr>
<tr><td colspan="4">試験化学品を、ヒトの皮膚の上層部に性質がよく似た 3 次元再構築ヒト表皮（RhE）に局所的に適用する 4 つの方法がある。試験方法は、腐食性化学品は拡散または浸食により角質層を貫通し、かつ下層の細胞に対して毒性があるという前提に基づく。組織の生存率は、MTT 染料の酵素反応による青色ホルマザン塩への変換を、青色ホルマザン塩を組織から抽出して定量することで評価する。腐食性化学品は、定められた閾値以下の組織の生存率を低下させる能力で決定される。
判定基準は、定義された曝露期間による組織の生存率に基づく。</td></tr>
<tr><td>1</td><td>方法 1
3 分間、60 分間または 240 分間曝露後 35%未満</td><td colspan="3">方法 2、3、4
3 分間曝露後 50%未満；または
3 分間曝露後 50%以上かつ 60 分間曝露後 15%未満</td></tr>
<tr><td>1A</td><td>方法 1
3 分間曝露後 35%未満</td><td>方法 2
3 分間曝露後 25%未満</td><td>方法 3
3 分間曝露後 18%未満</td><td>方法 4
3 分間曝露後 15%未満</td></tr>
<tr><td>1B</td><td rowspan="2">3 分間曝露後 35%以上かつ 60 分間曝露後 35%未満または 60 分間曝露後 35%以上かつ 240 分間曝露後 35%未満</td><td rowspan="2">3 分間曝露後 25%以上かつ区分 1 の判定基準を満足</td><td rowspan="2">3 分間曝露後 18%以上かつ区分 1 の判定基準を満足</td><td rowspan="2">3 分間曝露後後 15%以上かつ区分 1 の判定基準を満足</td></tr>
<tr><td>1C</td></tr>
<tr><td>皮膚腐食性とは分類されない</td><td>240 分間曝露後 35%以上</td><td colspan="3">3 分間曝露後 50%以上、かつ 60 分間曝露後 15%以上</td></tr>
</table>

Adopted:
14 June 2019

OECD GUIDELINE FOR TESTING OF CHEMICALS

In Vitro Skin Corrosion: Reconstructed Human Epidermis (RhE) Test Method

INTRODUCTION

1. Skin corrosion refers to the production of irreversible damage to the skin manifested as visible necrosis through the epidermis and into the dermis, following the application of a test chemical [as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS)] (1). This updated Test Guideline 431 provides an in vitro procedure allowing the identification of non-corrosive and corrosive substances and mixtures in accordance with UN GHS (1). It also allows a partial sub-categorisation of corrosives.

2. The assessment of skin corrosion potential of chemicals has typically involved the use of laboratory animals (OECD Test Guideline 404 (TG 404); originally adopted in 1981 and revised in 1992, 2002 and 2015) (2). In addition to the present TG 431, two other in vitro test methods for testing corrosion potential of chemicals have been validated and adopted as OECD Test Guidelines 430 (3) and 435 (4). Furthermore the in vitro OECD TG 439 (5) has been adopted for testing skin irritation potential. A document on Integrated Approaches to Testing and Assessment (IATA) for Skin Corrosion and Irritation describes several modules which group information sources and analysis tools, and provides guidance on (i) how to integrate and use existing testing and non-testing data for the assessment of skin irritation and skin corrosion potentials of chemicals and (ii) proposes an approach when further testing is needed (6).

3. This Test Guideline addresses the human health endpoint skin corrosion. It makes use of reconstructed human epidermis (RhE) (obtained from human derived non-transformed epidermal keratinocytes) which closely mimics the histological, morphological, biochemical and physiological properties of the upper parts of the human skin, i.e. the epidermis. This Test Guideline was originally adopted in 2004 and updated in 2013, 2016 and 2019 to include additional test methods using the RhE models. The Test Guideline was also updated in 2015 to introduce the possibility to use the methods to support the sub-categorisation of corrosive chemicals, and to refer to the IATA guidance document, and introduce the use of an alternative procedure to measure viability.

4. Five validated test methods using commercially available RhE models are included in this Test Guideline, as described below. Prevalidation studies (7), followed by a formal validation study for assessing skin corrosion (8) (9) (10) have been conducted (11) (12) for two of these commercially available test methods, EpiSkin™ Standard Model (SM), and EpiDerm™ Skin Corrosivity Test (SCT) (EPI-200) (referred to in the following text as the Validated Reference Methods – VRMs, EpiSkin^{TM}=VRM1, EpiDerm^{TM}= VRM2). The outcome of these studies led to the recommendation that the two VRMs mentioned above could be used for regulatory purposes for distinguishing corrosive (C) from non-corrosive (NC) substances, and that the EpiSkin™ could moreover be used to support sub-categorisation of corrosive substances (13) (14) (15). Two other commercially available in vitro skin corrosion RhE test methods have subsequently shown similar results to the EpiDerm™ SCT according to PS-based Validation (16) (17) (18). These are the SkinEthicTM RHE1 and epiCS® (previously named EST-1000) that can also be used for regulatory purposes for distinguishing corrosive from non-corrosive substances (19) (20). Post validation studies performed by the RhE model producers in the years 2012 to 2014 with a refined protocol correcting interferences of unspecific MTT reduction by the test chemicals improved the performance of both discrimination of C/NC as well as supporting sub-categorization of corrosives (21) (22). Further statistical analyses of the post-validation data generated with Epiderm™ SCT, SkinEthic™ RHE and epiCS® have been performed to identify alternative predictions models that improved the predictive capacity for sub-categorisation (23). Finally, the LabCyte EPI-MODEL24 is another commercially available in vitro skin corrosion RhE test that was shown to be scientific similar to the VRMs and can therefore be used for regulatory purposes to distinguish corrosive from non-corrosive substances as well as support sub-categorization of corrosives (40) (41) (42)(43).

5. Before a proposed similar or modified in vitro RhE test method for skin corrosion other than the VRMs can be used for regulatory purposes, its reliability, relevance (accuracy), and limitations for its proposed use should be determined to ensure its similarity to the VRMs, in accordance with the requirements of the Performance Standards (PS) (24) set out in accordance with the principles of Guidance Document No.34 (25). The Mutual Acceptance of Data will only be guaranteed after any proposed new or updated test method following the PS have been reviewed and included in this Test Guideline. The test methods included in this Test Guideline can be used to address countries' requirements for test results on in vitro test method for skin corrosion, while benefiting from the Mutual Acceptance of Data.

DEFINITIONS

6. Definitions used are provided in Annex I.

INITIAL CONSIDERATIONS

7. This Test Guideline allows the identification of non-corrosive and corrosive substances and mixtures in accordance with the UN GHS (1). This Test Guideline further supports the sub-categorisation of corrosive substances and mixtures into optional Sub-category 1A, in accordance with the UN GHS (1), as well as a combination of Sub-categories 1B and 1C (21) (22) (23). A limitation of this Test Guideline is that it does not allow discriminating between skin corrosive Sub-category 1B and Sub-category 1C in accordance with the UN GHS (1) due to the limited set of well-known in vivo corrosive Sub-category 1C chemicals. The five test methods under this test guideline are able to discriminate sub-categories 1A versus 1B-and-1C versus NC.

8. A wide range of chemicals representing mainly individual substances has been tested in the validation studies supporting the test methods included in this Test Guideline. The original database of the validation study conducted for identification of non-corrosives versus corrosives amounted to 60 chemicals covering a wide range of chemical classes (8) (9) (10). Testing to demonstrate sensitivity, specificity, accuracy and within-laboratory-reproducibility of the assay for sub-categorisation was further performed by the test method developers using 79 to 80 chemicals also covering a wide range of chemical classes, and results were reviewed by the OECD (21) (22) (23). On the basis of the overall data available, the Test Guideline is applicable to a wide range of chemical classes and physical states including liquids, semi-solids, 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 prior treatment of the sample is required. In cases where evidence can be demonstrated on the non-applicability of test methods included in the Test Guideline to a specific category of test chemicals, these test methods should not be used for that specific category of test chemicals. In addition, this Test Guideline is assumed to be applicable to mixtures as an extension of its applicability to substances. However, due to the fact that mixtures cover a wide spectrum of categories and composition, and that only limited information is currently available on the testing of mixtures, in cases where evidence can be demonstrated on the non-applicability of the Test Guideline to a specific category of mixtures (e.g. following a strategy as proposed in (26)), the Test Guideline should not be used for that specific category of mixtures. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, 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. Gases and aerosols have not been assessed yet in validation studies (8) (9) (10). While it is conceivable that these can be tested using RhE technology, the current Test Guideline does not allow testing of gases and aerosols.

9. Test chemicals absorbing light in the same range as MTT formazan and test chemicals able to directly reduce the vital dye MTT (to MTT formazan) may interfere with the tissue viability measurements and need the use of adapted controls for corrections. The type of adapted controls that may be required will vary depending on the type of interference produced by the test chemical and the procedure used to measure MTT formazan (see paragraphs 25-31).

10. While this Test Guideline does not provide adequate information on skin irritation, it should be noted that OECD TG 439 specifically addresses the health effect skin irritation in vitro and is based on the same RhE test system, though using another protocol (5). For a full evaluation of local skin effects after a single dermal exposure, the Guidance Document No. 203 on Integrated Approaches for Testing Assessment should be consulted (6). This IATA approach includes the conduct of in vitro tests for skin corrosion (such as described in this Test Guideline) and skin irritation before considering testing in living animals. It is recognized that the use of human skin is subject to national and international ethical considerations and conditions.

PRINCIPLE OF THE TEST

11. The test chemical is applied topically to a three-dimensional RhE model, comprised of non-transformed, human-derived epidermal keratinocytes, which have been cultured to

form a multi-layered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multi-layered stratum corneum containing intercellular lamellar lipid layers representing main lipid classes analogous to those found in vivo.

12. The RhE test method is based on the premise that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion, and are cytotoxic to the cells in the underlying layers. Cell viability is measured by enzymatic conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue tetrazolium bromide; CAS number 298-93-1], into a blue formazan salt that is quantitatively measured after extraction from tissues (27). Corrosive chemicals are identified by their ability to decrease cell viability below defined threshold levels (see paragraphs 35 and 36). The RhE-based skin corrosion test methods have shown to be predictive of in vivo skin corrosion effects assessed in rabbits according to the OECD guideline 404 (2).

DEMONSTRATION OF PROFICIENCY

13. Prior to routine use of any of the five validated RhE test methods that adhere to this Test Guideline, laboratories should demonstrate technical proficiency by correctly classifying the twelve Proficiency Substances listed in Table 1. In case of the use of a method for sub-classification, also the correct sub-categorisation should be demonstrated. In situations where a listed substance is unavailable or where justifiable, another substance for which adequate in vivo and in vitro reference data are available may be used (e.g. from the list of reference chemicals (24)) provided that the same selection criteria as described in Table 1 are applied.

Table 1. List of Proficiency Substances[1]

Substance	CASRN	Chemical Class[2]	UN GHS Cat. Based on *In Vivo* results [3]	Cat. Based on *In Vitro* results[4]	Mean cell viability for VRMs				Physical State
					VRM1		VRM2		
					3 min	60 min.	3 min.	60 min	
Sub-category 1A *In Vivo* Corrosives									
Bromoacetic acid	79-08-3	Organic acid	1A	(3) 1A	3	2.8	3.2	2.8	S
Boron trifluoride dihydrate	13319-75-01	Inorganic acid	1A	(3) 1A	2.4	4.2	4.4	10.1	L
Phenol	108-95-2	Phenol	1A	(3) 1A	29.8	21.8	22.6	13.5	S
Dichloroacetyl chloride	79-36-7	Electrophile	1A	(3) 1A	5.6	6.3	1.3	1.4	L
Combination of sub-categories 1B-and-1C *In Vivo* Corrosives									
Glyoxylic acid monohydrate	563-96-2	Organic acid	1B-and-1C	(3) 1B-and-1C	110.4	22.5	90.4	3.1	S
Lactic acid	598-82-3	Organic acid	1B-and-1C	(3) 1B-and-1C	80.2	9.4	90	3.5	L
Ethanolamine	141-43-5	Organic base	1B	(3) 1B-and-1C	66.2	40.3	69.7	9.3	Viscous
Hydrochloric acid (14.4%)	7647-01-0	Inorganic acid	1B-and-1C	(3) 1B-and-1C	69.3	5.7	80.8	9	L

***In Vivo* Non Corrosives**									
Phenethyl bromide	103-63-9	Electrophile	NC	(3) NC	141	117.2	112.5	71.2	N
4-Amino-1,2,4-triazole	584-13-4	Organic base	NC	(3) NC	116.8	120.6	105.7	88.2	N
4-(methylthio)-benzaldehyde	3446-89-7	Electrophile	NC	(3) NC	136.7	150.4	85.4	81.6	N
Lauric acid	143-07-7	Organic acid	NC	(3) NC	102	117.4	90.7	64.4	N

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonized System (1); VRM = Validated Reference Method, EpiSkin™=VRM1, EpiDerm™= VRM2; NC = Not Corrosive

[1]The proficiency substances, sorted first by corrosives versus non-corrosives, then by corrosive sub-category and then by chemical class, were selected from the substances used in the ECVAM validation studies EpiSkin™ and EpiDerm™ (8) (9) (10) and from post-validation studies based on data provided by EpiSkin™ (22), EpiDerm™, SkinEthic™ and epiCS® developers (23). Unless otherwise indicated, the substances were tested at the purity level obtained when purchased from a commercial source (8) (10). The selection includes, to the extent possible, substances that: (i) are representative of the range of corrosivity responses (e.g. non-corrosives; weak to strong corrosives) that the VRMs are capable of measuring or predicting; (ii) are representative of the chemical classes used in the validation studies; (iii) have chemical structures that are well-defined; (iv) induce reproducible results in the VRM; (v) induce definitive results in the in vivo reference test method; (vi) are commercially available; and (vii) are not associated with prohibitive disposal costs.

[2]Chemical class assigned by Barratt et al. (8).

[3]The corresponding UN Packing groups are I, II and III, respectively, for the UN GHS 1A, 1B and 1C.

[4]The in vitro predictions reported in this table were obtained with all five test methods covered in TG 431; for phenol though the LabCyte EPI-MODEL24 had slightly discordant results across runs, i.e. 1A-1BC-1BC; other methods achieved these classifications in validation or post-validation testing performed by the test method developers.

[5]The viability values obtained in the ECVAM Skin Corrosion Validation Studies were not corrected for direct MTT reduction (killed controls were not performed in the validation studies). However, the post-validation data generated by the test method developers that are presented in this table were acquired with adapted controls (23).

14. As part of the proficiency exercise, it is recommended that the user verifies the barrier properties of the tissues after receipt as specified by the RhE model manufacturer. 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 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 in regular intervals.

PROCEDURE

15. The following is a generic description of the components and procedures of the RhE test methods for skin corrosion assessment covered by this Test Guideline. The RhE models endorsed as scientifically valid for use within this Test Guideline, i.e. the EpiSkin™ (SM), EpiDerm™ (EPI-200), SkinEthic™ RHE, epiCS® and LabCyte EPI-MODEL24 (16) (17) (19) (28) (29) (30) (31) (32) (33) (40) (41), can be obtained from commercial sources. Standard Operating Procedures (SOPs) for these five RhE models are available (34) (35) (36) (37) (42), and their main test method components are summarised in Annex 2. It is recommended that the relevant SOP be consulted when implementing and using one of these methods in the laboratory. Testing with the five RhE test methods covered by this Test Guideline should comply with the following:

RHE TEST METHOD COMPONENTS

General conditions

16. Non-transformed human keratinocytes should be used to reconstruct the epithelium. Multiple layers of viable epithelial cells (basal layer, stratum spinosum, stratum granulosum) should be present under a functional stratum corneum. The stratum corneum should be multi-layered containing the essential lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic benchmark chemicals, e.g. sodium dodecyl sulphate (SDS) or Triton X-100. The barrier function should be demonstrated and may be assessed either by determination of the concentration at which a benchmark chemical reduces the viability of the tissues by 50% (IC50) after a fixed exposure time, or by determination of the exposure time required to reduce cell viability by 50% (ET50) upon application of the benchmark chemical at a specified, fixed concentration (see paragraph 18). The containment properties of the RhE model should prevent the passage of material around the stratum corneum to the viable tissue, which would lead to poor modelling of skin exposure. The RhE model should be free of contamination by bacteria, viruses, mycoplasma, or fungi.

Functional conditions

Viability

17. The assay used for quantifying tissue viability is the MTT-assay (27). The viable cells of the RhE tissue construct reduce the vital dye MTT into a blue MTT formazan precipitate, which is then extracted from the tissue using isopropanol (or a similar solvent). The OD of the extraction solvent alone should be sufficiently small, i.e., OD < 0.1. The extracted MTT formazan may be quantified using either a standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure (38). The RhE model users should ensure that each batch of the RhE model used meets defined criteria for the negative control. An acceptability range (upper and lower limit) for the negative control OD values should be established by the RhE model developer/supplier. Acceptability ranges for the negative control OD values for the five validated RhE test methods included in this Test Guideline 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 that the tissues treated with negative control are stable in culture (provide similar OD measurements) for the duration of the exposure period.

Table 2. Acceptability ranges for negative control OD values to control batch quality

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM)	≥ 0.6	≤ 1.5
EpiDerm™ SCT (EPI-200)	≥ 0.8	≤ 2.8
SkinEthic™ RHE	≥ 0.8	≤ 3.0
epiCS	≥ 0.8	≤ 2.8
LabCyte EPI-MODEL24 SCT	≥ 0.7	≤ 2.5

Barrier function

18. The stratum corneum and its lipid composition should be sufficient to resist the rapid penetration of certain cytotoxic benchmark chemicals (e.g. SDS or Triton X-100), as estimated by IC50 or ET50 (Table 3). The barrier function of each batch of the RhE model used should be demonstrated by the RhE model developer/vendor upon supply of the tissues to the end user (see paragraph 21).

Morphology

19. Histological examination of the RhE model should be performed demonstrating multi-layered human epidermis-like structure containing stratum basale, stratum spinosum, stratum granulosum and stratum corneum and exhibits lipid profile similar to lipid profile of human epidermis. Histological examination of each batch of the RhE model used demonstrating appropriate morphology of the tissues should be provided by the RhE model developer/vendor upon supply of the tissues to the end user (see paragraph 21).

Reproducibility

20. Test method users should demonstrate reproducibility of the test methods over time with the positive and negative controls. Furthermore, the test method should only be used if the RhE model developer/supplier provides data demonstrating reproducibility over time with corrosive and non-corrosive chemicals from e.g. the list of Proficiency Substances (Table 1). In case of the use of a test method for sub-categorisation, the reproducibility with respect to sub-categorisation should also be demonstrated.

Quality control (QC)

21. The RhE model should only be used if the developer/supplier demonstrates that each batch of the RhE model used meets defined production release criteria, among which those for viability (paragraph 17), barrier function (paragraph 18) and morphology (paragraph 19) are the most relevant. These data are provided to the test method users, so that they are able to include this information in the test report. Only results produced with QC accepted tissue batches can be accepted for reliable prediction of corrosive classification. An acceptability range (upper and lower limit) for the IC50 or the ET50 is established by the RhE model developer/supplier. The acceptability ranges for the five validated test methods are given in Table 3.

Table 3. **QC batch release criterion**

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM) (18 hours treatment with SDS)(33)	IC_{50} = 1.0 mg/mL	IC_{50} = 3.0 mg/mL
EpiDerm™SCT (EPI-200) (1% Triton X-100)(34)	ET_{50} = 4.0 hours	ET_{50} = 8.7 hours
SkinEthic™ RHE (1% Triton X-100)(35)	ET_{50} = 4.0 hours	ET_{50} = 10.0 hours
epiCS (1% Triton X-100)(36)	ET_{50} = 2.0 hours	ET_{50} = 7.0 hours
LabCyte EPI-MODEL24 SCT (18 hours treatment with SDS) (42)	IC_{50} = 1.4 mg/mL	IC_{50} = 4.0 mg/mL

Application of the Test Chemical and Control Substances

22. At least two tissue replicates should be used for each test chemical and controls for each exposure time. For liquid as well as solid chemicals, sufficient amount of test chemical should be applied to uniformly cover the epidermis surface while avoiding an infinite dose, i.e. a minimum of 70 µL/cm2 or 30 mg/cm2 should be used. Depending on the methods, the epidermis surface should be moistened with deionized or distilled water before application of solid chemicals, to improve contact between the test chemical and the epidermis surface (34) (35) (36) (37) (42). Whenever possible, solids should be tested as a fine powder. The application method should be appropriate for the test chemical (see e.g. references (34-37). At the end of the exposure period, the test chemical should be carefully washed from the epidermis with an aqueous buffer, or 0.9% NaCl. Depending on which of the five validated RhE test methods is used, two or three exposure periods are used per test chemical (for all five valid RhE models: 3 min and 1 hour; for EpiSkin™ an additional exposure time of 4 hours). Depending on the RhE test method used and the exposure period assessed, the incubation temperature during exposure may vary between room temperature and 37ºC.

23. Concurrent negative and positive controls (PC) should be used in each run to demonstrate that viability (with negative controls), barrier function and resulting tissue sensitivity (with the PC) of the tissues are within a defined historical acceptance range. The suggested PC chemicals are glacial acetic acid or 8N KOH depending upon the RhE model used (see Annex 2 and relevant SOP for details). It should be noted that 8N KOH is a direct MTT reducer that might require adapted controls as described in paragraphs 25 and 26. The suggested negative controls are 0.9% (w/v) NaCl or water.

Cell Viability Measurements

24. The MTT assay, which is a quantitative assay, should be used to measure cell viability under this Test Guideline (27). The tissue sample is placed in MTT solution of appropriate concentration (0.3, 0.5 or 1 mg/mL, see Annex 2 and relevant SOP for details) for 3 hours. The precipitated blue formazan product is then extracted from the tissue using a solvent (e.g. isopropanol, acidic isopropanol), and the concentration of formazan is

measured by determining the OD at 570 nm using a filter band pass of maximum ± 30 nm, or by an HPLC/UPLC spectrophotometry procedure (see paragraphs 30 and 31) (38).

25. Test chemicals may interfere with the MTT assay, either by direct reduction of the MTT into blue formazan, and/or by colour interference if the test chemical absorbs, naturally or due to treatment procedures, in the same OD range of formazan (570 ± 30 nm, mainly blue and purple chemicals). Additional controls should be used to detect and correct for a potential interference from these test chemicals such as the non-specific MTT reduction (NSMTT) control and the non-specific colour (NSC) control (see paragraphs 26 to 30). This is especially important when a specific test chemical is not completely removed from the tissue by rinsing or when it penetrates the epidermis, and is therefore present in the tissues when the MTT viability test is performed. Detailed description of how to correct direct MTT reduction and interferences by colouring agents is available in the SOPs for the test methods (34) (35) (36) (37) (42).

26. To identify direct MTT reducers, each test chemical should be added to freshly prepared MTT medium (34) (35) (36) (37) (42). If the MTT mixture containing the test chemical turns blue/purple, the test chemical is presumed to directly reduce the MTT, and further functional check on non-viable epidermis 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 the test chemical in similar amount as viable tissues. Each MTT reducing chemical is applied on at least two killed tissue replicates per exposure time, which undergo the whole skin corrosion test. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the MTT reducer minus the percent non-specific MTT reduction obtained with the killed tissues exposed to the same MTT reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT).

27. To identify potential interference by coloured test chemicals or test chemicals that become coloured when in contact with water or isopropanol and decide on the need for additional controls, spectral analysis of the test chemical in water (environment during exposure) and/or isopropanol (extracting solution) should be performed. If the test chemical in water and/or isopropanol absorbs light in the range of 570 ± 30 nm, further colorant controls should be performed or, alternatively, an HPLC/UPLC-spectrophotometry procedure should be used in which case these controls are not required (see paragraphs 30 and 31). When performing the standard absorbance (OD) measurement, each interfering coloured test chemical is applied on at least two viable tissue replicates per exposure time, which undergo the entire skin corrosion test but are incubated with medium instead of MTT solution during the MTT incubation step to generate a non-specific colour (NSCliving) control. The NSCliving control needs to be performed concurrently per exposure time per coloured test chemical (in each run) due to the inherent biological variability of living tissues. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT, run concurrently to the test being corrected (%NSCliving).

28. Test chemicals that are identified as producing both direct MTT reduction (see paragraph 26) and colour interference (see paragraph 27) will also require a third set of controls, apart from the NSMTT and NSCliving controls described in the previous

paragraphs, when performing the standard absorbance (OD) measurement. This is usually the case with darkly coloured test chemicals interfering with the MTT assay (e.g., blue, purple, black) because their intrinsic colour impedes the assessment of their capacity to directly reduce MTT as described in paragraph 26. These test chemicals may bind to both living and killed tissues and therefore the NSMTT control may not only correct for potential direct MTT reduction by the test chemical, but also for colour interference arising from the binding of the test chemical to killed tissues. This could lead to a double correction for colour interference since the NSCliving control already corrects for colour interference arising from the binding of the test chemical to living tissues. To avoid a possible double correction for colour interference, a third control for non-specific colour in killed tissues (NSCkilled) needs to be performed. In this additional control, the test chemical is applied on at least two killed tissue replicates per exposure time, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step. A single NSCkilled control is sufficient per test chemical regardless of the number of independent tests/runs performed, but should be performed concurrently to the NSMTT control and, where possible, with the same tissue batch. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the test chemical minus %NSMTT minus %NSCliving plus the percent non-specific colour obtained with killed tissues exposed to the interfering test chemical and incubated with medium without MTT, calculated relative to the negative control run concurrently to the test being corrected (%NSCkilled).

29. It is important to note that non-specific MTT reduction and non-specific colour interferences may increase the readouts of the tissue extract above the linearity range of the spectrophotometer. On this basis, each laboratory should determine the linearity range of their spectrophotometer with MTT formazan (CAS # 57360-69-7) from a commercial source before initiating the testing of test chemicals for regulatory purposes. In particular, the standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess direct MTT-reducers and colour interfering test chemicals when the ODs of the tissue extracts obtained with the test chemical without any correction for direct MTT reduction and/or colour interference are within the linear range of the spectrophotometer or when the uncorrected percent viability obtained with the test chemical already defined it as a corrosive (see paragraphs 35 and 36). Nevertheless, results for test chemicals producing %NSMTT and/or %NSCliving ≥ 50% of the negative control should be taken with caution.

30. For coloured test chemicals which are not compatible with the standard absorbance (OD) measurement due to too strong interference with the MTT assay, the alternative HPLC/UPLC-spectrophotometry procedure to measure MTT formazan may be employed (see paragraph 31) (37). The HPLC/UPLC-spectrophotometry system allows for the separation of the MTT formazan from the test chemical before its quantification (38). For this reason, NSCliving or NSCkilled controls are never required when using HPLC/UPLC-spectrophotometry, independently of the chemical being tested. NSMTT controls should nevertheless be used if the test chemical is suspected to directly reduce MTT or has a colour that impedes the assessment of the capacity to directly reduce MTT (as described in paragraph 26). When using HPLC/UPLC-spectrophotometry to measure MTT formazan, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. For test chemicals able to directly reduce MTT, true tissue viability is calculated as the percent tissue viability obtained with living tissues exposed to the test chemical minus %NSMTT. Finally, it should be noted that direct MTT-reducers

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

31. HPLC/UPLC-spectrophotometry may be used also with all types of test chemicals (coloured, non-coloured, MTT-reducers and non-MTT reducers) for measurement of MTT formazan (38). Due to the diversity of HPLC/UPLC-spectrophotometry systems, qualification of the HPLC/UPLC-spectrophotometry system should be demonstrated before its use to quantify MTT formazan from tissue extracts by meeting the acceptance criteria for a set of standard qualification parameters based on those described in the U.S. Food and Drug Administration guidance for industry on bio-analytical method validation (38) (39). These key parameters and their acceptance criteria are shown in Annex 4. Once the acceptance criteria defined in Annex 4 have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure MTT formazan under the experimental conditions described in this Test Guideline.

Acceptance Criteria

32. For each test method using valid RhE models, tissues treated with the negative control should exhibit OD reflecting the quality of the tissues as described in table 2 and should not be below historically established boundaries. Tissues treated with the PC, i.e. glacial acetic acid or 8N KOH, should reflect the ability of the tissues to respond to a corrosive chemical under the conditions of the test method (see Annex 2 and relevant SOP for details). The variability between tissue replicates of test chemical and/or control substances should fall within the accepted limits for each valid RhE model requirements (see Annex 2 and relevant SOP for details) (e.g. the difference of viability between the two tissue replicates should not exceed 30%). If either the negative control or PC included in a run fall out of the accepted ranges, the run is considered as not qualified and should be repeated. If the variability of test chemicals falls outside of the defined range, its testing should be repeated.

Interpretation of Results and Prediction Model

33. The OD values obtained for each test chemical should be used to calculate percentage of viability relative to the negative control, which is set at 100%. In case HPLC/UPLC-spectrophotometry is used, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. The cut-off percentage cell viability values distinguishing corrosive from non-corrosive test chemical (or discriminating between different corrosive sub-categories) are defined below in paragraphs 35 and 36 for each of the test methods covered by this Test Guideline and should be used for interpreting the results.

34. A single testing run composed of at least two tissue replicates should be sufficient for a test chemical when the resulting classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements, a second run may be considered, as well as a third one in case of discordant results between the first two runs.

35. The prediction model for the EpiSkin™ skin corrosion test method (9) (34) (22), associated with the UN GHS (1) classification system, is shown in Table 4:

Table 4. EpiSkin™ prediction model

Viability measured after exposure time points (t='3,' 60 and 240 minutes)	Prediction to consider
< 35% after 3 min exposure	**Corrosive:** Optional Sub-category 1A *
≥ 35% after 3 min exposure **AND** < 35% after 60 min exposure **OR** ≥ 35% after 60 min exposure **AND** < 35% after 240 min exposure	**Corrosive:** A combination of optional Sub-categories 1B-and-1C
≥ 35% after 240 min exposure	**Non-corrosive**

*) According to the data generated in view of assessing the usefulness of the RhE test methods for supporting sub-categorisation, it was shown that around 22 % of the Sub-category 1A results of the EpiSkin™ test method may actually constitute Sub-category 1B or Sub-category 1C substances/mixtures (i.e. over classifications) (see Annex 3).

36. The prediction models for the EpiDerm™ SCT (10) (23) (35), the SkinEthic™ RHE (17) (18) (23) (36), the epiCS® (16) (23) (37) and LabCyte EPI-MODEL24 (41) (42) skin corrosion test methods, associated with the UN GHS (1) classification system, are shown in Table 5:

Table 5. EpiDerm™ SCT, SkinEthic™ RHE epiCS® and LabCyte EPI-MODEL24 SCT

Viability measured after exposure time points (t=3 and 60 minutes)	Prediction to be considered
STEP 1 for EpiDerm™ SCT, SkinEthic™ RHE, epiCS® and LabCyte EPI-MODEL24 SCT	
< 50% after 3 min exposure	Corrosive
≥ 50% after 3 min exposure AND < 15% after 60 min exposure	Corrosive
≥ 50% after 3 min exposure AND ≥ 15% after 60 min exposure	Non-corrosive
STEP 2 for EpiDerm™ SCT - for substances/mixtures identified as Corrosive in step 1	
< 25% after 3 min exposure	Optional Sub-category 1A *
≥ 25% after 3 min exposure	A combination of optional Sub-categories 1B-and-1C
STEP 2 for SkinEthic™ RHE - for substances/mixtures identified as Corrosive in step 1	
< 18% after 3 min exposure	Optional Sub-category 1A *
≥ 18% after 3 min exposure	A combination of optional Sub-categories 1B-and-1C
STEP 2 for epiCS® - for substances/mixtures identified as Corrosive in step 1	
< 15% after 3 min exposure	Optional Sub-category 1A *
≥ 15% after 3 min exposure	A combination of optional Sub-categories 1B-and-1C
STEP 2 for LabCyte EPI-MODEL24 SCT - for substances/mixtures identified as Corrosive in step 1	
< 15% after 3 min exposure	Optional Sub-category 1A *
≥ 15% after 3 min exposure	A combination of optional Sub-categories 1B-and-1C

* According to the data generated in view of assessing the usefulness of the RhE test methods for supporting sub-categorisation, it was shown that around 29%, 31%, 33% and 30% of the Sub-category 1A results of the EpiDerm™ SCT, SkinEthic™ RHE epiCS® and LabCyte EPI-MODEL24 SCT, respectively, may actually constitute Sub-category 1B or Sub-category 1C substances/mixtures (i.e. over-classifications) (see Annex 3).

DATA AND REPORTING

Data

37. For each test, data from individual tissue replicates (e.g. OD values and calculated percentage cell viability for each test chemical, including classification) should be reported in tabular form, including data from repeat experiments as appropriate. In addition, means and ranges of viability and CVs between tissue replicates for each test should be reported. Observed interactions with MTT reagent by direct MTT reducers or coloured test chemicals should be reported for each tested chemical.

Test Report

38. The test report should include the following information:

Test Chemical and Control Substances:

- Mono-constituent substance: chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Multi-constituent substance, UVCB and mixture: characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents;
- Physical appearance, water solubility, and any additional relevant physicochemical properties;
- Source, lot number if available;
- Treatment of the test chemical/control substance prior to testing, if applicable (e.g. warming, grinding);
- Stability of the test chemical, limit date for use, or date for re-analysis if known;
- Storage conditions.

RhE model and protocol used and rationale for it (if applicable)

Test Conditions:

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

Test Procedure:

- Details of the test procedure used (including washing procedures used after exposure period);
- Doses of test chemical and control substances used;
- Duration of exposure period(s) and temperature(s) of exposure;
- Indication of controls used for direct MTT-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (PC, negative control, and NSMTT, NSCliving and NSCkilled, if applicable), per exposure time;
- Description of decision criteria/prediction model applied based on the RhE model used;
- Description of any modifications of the test procedure (including washing procedures).
- Run and Test Acceptance Criteria:
- Positive and negative control mean values and acceptance ranges based on historical data;
- Acceptable variability between tissue replicates for positive and negative controls;
- Acceptable variability between tissue replicates for test chemical.

Results:

- Tabulation of data for individual test chemicals and controls, for each exposure period, each run and each replicate measurement including OD or MTT formazan peak area, percent tissue viability, mean percent tissue viability, differences between replicates, SDs and/or CVs if applicable;
- If applicable, results of controls used for direct MTT-reducers and/or colouring test chemicals including OD or MTT formazan peak area, %NSMTT, %NSCliving, %NSCkilled, differences between tissue replicates, SDs and/or CVs (if applicable), and final correct percent tissue viability;
- Results obtained with the test chemical(s) and control substances in relation to the defined run and test acceptance criteria;
- Description of other effects observed;
- The derived classification with reference to the prediction model/decision criteria used.

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 (25).

Cell viability: Parameter measuring total activity of a cell population e.g. as ability of cellular mitochondrial dehydrogenases to reduce the vital dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, Thiazolyl blue), which depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

Chemical: means a substance or a mixture.

Concordance: This is a measure of test method performance for test methods that give a categorical result, and is one aspect of relevance. The term is sometimes used interchangeably with accuracy, and is defined as the proportion of all chemicals tested that are correctly classified as positive or negative. Concordance is highly dependent on the prevalence of positives in the types of test chemical being examined (25).

ET50: Can be estimated by determination of the exposure time required to reduce cell viability by 50% upon application of the benchmark chemical at a specified, fixed concentration, see also IC50.

GHS (Globally Harmonized System of Classification and Labelling of Chemicals): 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).

HPLC: High Performance Liquid Chromatography.

IATA: Integrated Approach on Testing and Assessment.

IC50: Can be estimated by determination of the concentration at which a benchmark chemical reduces the viability of the tissues by 50% (IC50) after a fixed exposure time, see also ET50.

ET50. Infinite dose: Amount of test chemical applied to the epidermis exceeding the amount required to completely and uniformly cover the epidermis surface.

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

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

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

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.

NC: Non corrosive.

NSCkilled control: Non-Specific Colour control in killed tissues.

NSCliving control : Non-Specific Colour control in living tissues.

NSMTT: Non-Specific MTT reduction.

OD: Optical Density

PC: Positive Control, a replicate 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.

Performance standards (PS): 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 similar levels of reliability and accuracy, 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 (25).

Relevance: Description of relationship of the test method to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test method correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (25).

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 (25).

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

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test method. 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 (25).

Skin corrosion in vivo: The production of irreversible damage of the skin; namely, visible necrosis through the epidermis and into the dermis, following the application of a test chemical for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discoloration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test method. 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 (25).

Substance: means 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.

Test chemical: means what is being tested.

UPLC: Ultra-High Performance Liquid Chromatography.

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

ANNEX 2 - MAIN TEST METHOD COMPONENTS OF THE RhE TEST METHODS VALIDATED FOR SKIN CORROSION TESTING

Nr.	1	2	3	4	5
Test Method Component	**EpiSkin™**	**EpiDerm™ SCT**	**SkinEthic™ RHE**	**epiCS®**	**LabCyte EPI-MODEL24 SCT**
Model surface	0.38 cm^2	0.63 cm^2	0.5 cm^2	0.6 cm^2	0.3 cm^2
Number of tissue replicates	At least 2 per exposure time	2-3 per exposure time	At least 2 per exposure time	At least 2 per exposure time	At least 2 per exposure time
Treatment doses and application	Liquids and viscous: 50 ± 3 µL (131.6 µL/cm^2) Solids: 20± 2 mg (52.6 mg/cm^2) +100 µL±5µL NaCl solution (9 g/L) Waxy/sticky: 50 ± 2 mg (131.6 mg/cm^2) with a nylon mesh	Liquids: 50 µL (79.4 µL/cm^2) with or without a nylon mesh *Pre-test compatibility of test chemical with nylon mesh* Semi solids: 50 µL (79.4 µL/cm^2) Solids: 25 µL H_2O (or necessary) + 25 mg (39.7 mg/cm^2) Waxes: flat "disc like" piece of ca. 8 mm diameter placed atop the tissue wetted with 15µL H_2O.	Liquids and viscous:40 ± 3 µL (80µL/cm^2) using nylonmesh *Pre-test compatibility of test chemical with nylon mesh* Solids: 20 µL ± 2µl H_2O + 20± 3 mg (40 mg/cm2) Waxy/sticky: 20 ± 3 mg (40 mg/cm^2) with a nylon mesh	Liquids and viscous:50 µL (83.3µL/cm^2) using nylonmesh *Pre-test compatibility of test chemical with nylon mesh* Semi solids: 50 µL (83.3 µL/cm^2) Solids: 25 mg (41.7 mg/cm^2) + 25 µL H_2O (or more if necessary) Waxy/sticky: flat "cookie like" piece of ca. 8 mm diameter placed atop the tissue wetted with 15µL H_2O	Liquids and viscous:50 µL (166.7µL/cm^2) Solids: 50± 2 mg (166.7 mg/cm^2) + 50 µL H_2O Waxy: Use a positive displacement pipette and tip as liquid and viscous substance.

Nr.	1	2	3	4	5
Test Method Component	**EpiSkin™**	**EpiDerm™ SCT**	**SkinEthic™ RHE**	**epiCS®**	**LabCyte EPI-MODEL24 SCT**
Pre-check for direct MTT reduction	50 µL (liquid) or 20 mg (solid) + 2 mL MTT 0.3 mg/mL solution for 180±5 min at 37°C, 5% CO_2, 95% RH ➔ if solution turns blue/purple, water-killed adapted controls should be performed	50 µL (liquid) or 25 mg (solid) + 1 mL MTT 1 mg/mL solution for 60 min at 37°C, 5% CO_2, 95% RH ➔ if solution turns blue/purple, freeze-killed adapted controls should be performed	40 µL (liquid) or 20 mg (solid) + 1 mL MTT 1 mg/mL solution for 180±15 min at 37°C, 5% CO_2, 95% RH ➔ if solution turns blue/purple, freeze-killed adapted controls should be performed	50 µL (liquid) or 25 mg (solid) + 1 mL MTT 1 mg/mL solution for 60 min at 37°C, 5% CO_2, 95% RH ➔ if solution turns blue/purple, freeze-killed adapted controls should be performed	50 µL (liquid) or 50 mg (solid) + 500 µL MTT 0.5 mg/mL solution for 60 min at 37°C, 5% CO_2, 95% RH ➔ if solution turns blue/purple, freeze-killed adapted controls should be performed
Pre-check for colour interference	10 µL (liquid) or 10 mg (solid) + 90µL H2O mixed for 15 min at RT ➔ if solution becomes coloured, living adapted controls should be performed	50 µL (liquid) or 25 mg (solid) + 300 µL H2O mixed for 60 min at 37oC, 5% CO2, 95% RH ➔ if solution becomes coloured, living adapted controls should be performed	40 µL (liquid) or 20 mg (solid) + 300 µL H2O mixed for 60 min at RT ➔ if solution becomes coloured, living adapted controls should be performed	50 µL (liquid) or 25 mg (solid) + 300 µL H2O mixed for 60 min at 37oC, 5% CO2, 95% RH ➔ if solution becomes coloured, living adapted controls should be performed	50 µL (liquid) or 50 mg (solid) + 500 µL H2O mixed for 60 min at 37oC, 5% CO2, 95% RH ➔ if solution becomes coloured, living adapted controls should be performed
Exposure time and temperature	3 min, 60 min (±5 min) and 240 min (±10 min) In ventilated cabinet Room Temperature (RT, 18-28oC)	3 min at RT, and 60 min at 37oC, 5% CO2, 95% RH	3 min at RT, and 60 min at 37oC, 5% CO2, 95% RH	3 min at RT, and 60 min at 37oC, 5% CO2, 95% RH	3 min at RT, and 60 min at 37oC, 5% CO2, 95% RH
Rinsing	25 mL 1x PBS (2 mL/throwing)	20 times with a constant soft stream of 1x PBS	20 times with a constant soft stream of 1x PBS	20 times with a constant soft stream of 1x PBS	10 times or more with a constant strong stream of 1x PBS
Negative control	50 µL NaCl solution (9 g/L) Tested with every exposure time	50 µL H2O Tested with every exposure time	40 µL H2O Tested with every exposure time	50 µL H2O Tested with every exposure time	50 µL H2O Tested with every exposure time
Positive control	50 µL Glacial acetic acid Tested only for 4 hours	50 µL 8N KOH Tested with every exposure time	40 µL 8N KOH Tested only for 1 hour	50 µL 8N KOH Tested with every exposure time	50 µL 8N KOH Tested only for 1 hour
MTT solution	2 mL 0.3 mg/mL	300 µL 1 mg/mL	300 µL 1 mg/mL	300 µL 1 mg/mL	500 µL 0.5 mg/mL
MTT incubation	180 min (±15 min) at 37oC, 5%	180 min at 37oC, 5% CO2, 95%	180 min (±15 min) at 37oC, 5%	180 min at 37oC, 5% CO2, 95%	180 min (±5 min) at 37oC, 5%

Nr.	1	2	3	4	5
Test Method Component	**EpiSkin™**	**EpiDerm™ SCT**	**SkinEthic™ RHE**	**epiCS®**	**LabCyte EPI-MODEL24 SCT**
time and temperature	CO2, 95% RH	RH	CO2, 95% RH	RH	CO2, 95% RH
Test Method Component	EpiSkin™ EIT	EpiDerm™ SCT	SkinEthic™ RHE EIT	epiCS®	LabCyte EPI-MODEL24 SCT
Extraction solvent	500 µL acidified isopropanol (0.04 N HCl in isopropanol) (isolated tissue fully immersed)	2 mL isopropanol (extraction from top and bottom of insert)	1.5 mL isopropanol (extraction from top and bottom of insert)	2 mL isopropanol (extraction from top and bottom of insert)	300 µL isopropanol (isolated tissue fully immersed)
Extraction time And temperature	Overnight at RT, protected from light	Overnight without shaking at RT or for 120 min with shaking (~120 rpm) at RT	Overnight without shaking at RT or for 120 min with shaking (~120 rpm) at RT	Overnight without shaking at RT or for 120 min with shaking (~120 rpm) at RT	Overnight at RT, protected from light
OD reading	570 nm (545 - 595 nm) without reference filter	570 nm (or 540 nm) without reference filter	570 nm (540 - 600 nm) without reference filter	540 - 570 nm without reference filter	570 nm with reference filter 650 nm
Tissue Quality Control	18 hours treatment with SDS 1.0mg/mL ≤ IC_{50} ≤ 3.0mg/mL	Treatment with 1% Triton X-100 4.08 hours ≤ ET_{50} ≤ 8.7 hours	Treatment with 1% Triton X-100 4.0 hours ≤ ET_{50} ≤ 10.0 hours	Treatment with 1% Triton X-100 2.0 hours ≤ ET_{50} ≤ 7.0 hours	18 hours treatment with SDS 1.4mg/mL ≤ IC_{50} ≤ 4.0 mg/mL
Acceptability Criteria	1. Mean OD of the tissue replicates treated with the negative control (NaCl) should be ≥ 0.6 and ≤ 1.5 for every exposure time 2. Mean viability of the tissue replicates exposed for 4 hours with the positive control (glacial acetic acid), expressed as % of the negative control, should be ≤ 20% 3. In the range 20-100% viability and for ODs ≥ 0.3, difference of viability between the two tissue replicates should not	1. Mean OD of the tissue replicates treated with the negative control (H_2O) should be ≥ 0.8 and ≤ 2.8 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour with the positive control (8N KOH), expressed as % of the negative control, should be ≤ 15% 3. In the range 20 - 100% viability, the Coefficient of Variation (CV) between tissue replicates should be ≤ 30%	1. Mean OD of the tissue replicates treated with the negative control (H_2O) should be ≥ 0.8 and ≤ 3.0 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour (and 4 hours, if applicable) with the positive control (8N KOH), expressed as % of the negative control, should be ≤ 15% 3. In the range 20-100% viability and for ODs ≥ 0.3, difference of viability between the two	1. Mean OD of the tissue replicates treated with the negative control (H_2O) should be ≥ 0.8 and ≤ 2.8 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour with the positive control (8N KOH), expressed as % of the negative control, should be ≤ 15%. 3. In the range 20-100% viability and for ODs ≥ 0.3, difference of viability between the two tissue replicates should not	1. Mean OD of the tissue replicates treated with the negative control (H_2O) should be ≥ 0.7 and ≤ 2.5 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour with the positive control (8N KOH), expressed as % of the negative control, should be ≤ 15%. 3. In the range 20-100% viability and for ODs ≥ 0.3, difference of viability between the two tissue replicates should not

Nr.	1	2	3	4	5
Test Method Component	EpiSkin™	EpiDerm™ SCT	SkinEthic™ RHE	epiCS®	LabCyte EPI-MODEL24 SCT
	exceed 30%.		tissue replicates should not exceed 30%.	exceed 30%.	exceed 30%.

ANNEX 3 - PERFORMANCE OF TEST METHODS FOR SUB-CATEGORISATION

The table below provides the performances of the five test methods calculated based on a set of 79 or 80 chemicals tested by the five test developers. Calculations of four test methods (EpiSkin™, EpiDerm™ SCT, SkinEthic™ RHE and epiCS®) were performed by the OECD Secretariat, reviewed and agreed by an expert subgroup (21) (23). Calculation of LabCyte EPI-MODEL24 SCT was performed by the test developer, reviewed and agreed by the validation management group and a peer review panel (41) (43).

STATISTICS ON PREDICTIONS OBTAINED ON THE ENTIRE SET OF CHEMICALS (n= 80 chemicals tested over 2 independent runs for epiCS® or 3 independent runs for EpiDerm™ SCT, EpiSkin™ and SkinEthic™RHE *i.e.* respectively 159* or 240 classifications.
n= 79 chemicals tested over 3 independent runs for LabCyte EPI-MODEL24 SCT, *i.e.* 237 classification.)**
*one chemical was tested once in epiCS® because of no availability (23).
** one chemical was not tested in LabCyte EPI-MODEL24 SCT because of no availability.

	EpiSkin	EpiDerm	SkinEthic	epiCS	LabCyte EPI-MODEL24
Overclassifications:					
1B-and-1C overclassified 1A	21.5%	29.0%	31.2%	32.8%	30.0%
NC overclassified 1B-and-1C	20.7%	23.4%	27.0%	28.4%	18.9%
NC overclassified 1A	0.0%	2.7%	0.0%	0.0%	2.7%
Overclassified as Corrosive	20.7%	26.1%	27.0%	28.4%	21.6%
Global overclassification rate (all categories)	17.9%	23.3%	24.5%	25.8%	21.5%
Underclassifications:					
1A underclassified 1B-and-1C	16.7%	16.7%	16.7%	12.5%	13.9%
1A underclassified NC	0.0%	0.0%	0.0%	0.0%	0.0%
1B-and-1C underclassified NC	2.2%	0.0%	7.5%	6.6%	0.0%
Global underclassification rate (all categories)	3.3%	2.5%	5.4%	4.4%	2.1%
Correct Classifications:					
1A correctly classified	83.3%	83.3%	83.3%	87.5%	86.1%
1B-and-/1C correctly classified	76.3%	71.0%	61.3%	60.7%	70.0%
NC correctly classified	79.3%	73.9%	73.0%	71.62%	78.4%
Overall Accuracy	78.8%	74.2%	70.0%	69.8%	76.4%

ANNEX 4 - Key parameters and acceptance criteria for qualification of an HPLC/UPLC-spectrophotometry system for measurement of MTT formazan extracted from RhE tissues

Parameter	Protocol Derived from FDA Guidance (36)(38)	Acceptance Criteria
Selectivity	Analysis of isopropanol, living blank (isopropanol extract from living RhE tissues without any treatment), dead blank (isopropanol extract from killed RhE tissues without any treatment)	$Area_{interference}$ = 20% of $Area_{LLOQ}$[1]
Precision	Quality Controls (i.e., MTT formazan at 1.6 g/mL, 16 g/mL and 160 g/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	$Area_{interference}$ = 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 g/mL); Quality Controls in isopropanol (n=5)	Calibration Curves:%Dev = 15% or = 20% for LLOQ Quality Controls:
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)	%Dev= 15% and CV = 15%
Short Term Stability of MTT Formazan in RhE 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 RhE Tissue Extract, if required	Quality Controls in living blank (n='3)' analysed theday of the preparation and after several days of storageat a specified temperature (e.g., 4ºC, -20ºC, -80ºC)	%Dev = 15%

Note:
[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 i.e., 200 µg/mL.