新規試験法提案書

皮膚感作性試験代替法
U937 Cell Line Activation Test (U-SENS™)

平成30年11月

国立医薬品食品衛生研究所

新規試験法提案書

平成 30 年 11 月 5 日

No.　2018-01

皮膚感作性試験代替法　U937 Cell Line Activation Test（U-SENS^{TM}）に関する提案

平成 30 年 10 月 29 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： U-SENS^{TM} において陽性の結果が得られた場合、感作性物質と判定することは可能であるが、感作性強度分類や United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS)のサブカテゴリー分類は困難である。本試験法単独での皮膚感作性の判定は不十分であり、被験物質の特性を十分に理解した上で、Integrated Approaches to Testing and Assessment (IATA) を構成するその他の情報と組み合わせて適切に評価することが必要である。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、Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 442E: *IN VITRO* SKIN SENSITISATION ASSAYS ADDRESSING THE KEY EVENT ON ACTIVATION OF DENDRITIC CELLS ON THE ADVERSE OUTCOME PATHWAY FOR SKIN SENSITISATION, ESAC Opinion No. 2016-03 およびThe U-SENS^{TM} test method Validation Study Reportなどをもとに、皮膚感作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感作性試験代替法 U-SENS^{TM} の使用を提案するものである。

大野泰雄

JaCVAM 評価会議　議長

平林容子

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

大 野 泰 雄 （公益財団法人　木原記念横浜生命科学振興財団）：座長
五十嵐良明 （国立医薬品食品衛生研究所）
石 井 雄 二 （国立医薬品食品衛生研究所　安全性生物試験研究センター）
井 上 智 彰 （日本免疫毒性学会）
今 井 教 安 （日本動物実験代替法学会）
岩瀬裕美子 （日本製薬工業協会）
篠 田 和 俊 （独立行政法人　医薬品医療機器総合機構）
杉山真理子 （日本化粧品工業連合会）
仲 井 俊 司 （日本化学工業協会）
中村るりこ （独立行政法人　製品評価技術基盤機構）
西 川 秋 佳 （国立医薬品食品衛生研究所　安全性生物試験研究センター）
沼 澤　　聡 （日本毒性学会）
野 口 真 希 （独立行政法人　医薬品医療機器総合機構）*
森 田　　健 （日本環境変異原学会）
横 関 博 雄 （日本皮膚免疫アレルギー学会）

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

大 野 泰 雄 （公益財団法人　木原記念横浜生命科学振興財団）：座長
五十嵐良明 （国立医薬品食品衛生研究所）
石 井 雄 二 （国立医薬品食品衛生研究所　安全性生物試験研究センター）
稲 若 邦 文 （日本化学工業協会）
井 上 智 彰 （日本免疫毒性学会）
今 井 教 安 （日本動物実験代替法学会）
岩瀬裕美子 （日本製薬工業協会）
久 保 文 宏 （独立行政法人　医薬品医療機器総合機構）
杉山真理子 （日本化粧品工業連合会）
中村るりこ （独立行政法人　製品評価技術基盤機構）
西 川 秋 佳 （国立医薬品食品衛生研究所　安全性生物試験研究センター/済生会宇都宮病院）
西 村 次 平 （独立行政法人　医薬品医療機器総合機構）
沼 澤　　聡 （日本毒性学会）
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）
増 村 健 一 （日本環境変異原学会）
横 関 博 雄 （日本皮膚免疫アレルギー学会）

任期：平成 30 年 4 月 1 日～平成 32 年 3 月 31 日

JaCVAM 運営委員会

平 林 容 子（国立医薬品食品衛生研究所　安全性生物試験研究センター）：委員長
石 井 孝 司（国立感染症研究所）
大 原　　拓（厚生労働省　医薬・生活衛生局　医薬品審査管理課）
小川久美子（国立医薬品食品衛生研究所　安全性生物試験研究センター　病理部）
奥 田 晴 宏（国立医薬品食品衛生研究所）
諫 田 泰 成（国立医薬品食品衛生研究所　安全性生物試験研究センター　薬理部）
北 嶋　　聡（国立医薬品食品衛生研究所　安全性生物試験研究センター　毒性部）
小池紘一郎（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）
高 木 篤 也（国立医薬品食品衛生研究所　安全性生物試験研究センター　毒性部　動物管理室）
東 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）
蛭 田 浩 一（独立行政法人　医薬品医療機器総合機構）
広 瀬 明 彦（国立医薬品食品衛生研究所　安全性生物試験研究センター　安全性予測評価部）
笛 木　　修（独立行政法人　医薬品医療機器総合機構）
渕 岡　　学（厚生労働省　医薬・生活衛生局　医薬品審査管理課　化学物質安全対策室）
本 間 正 充（国立医薬品食品衛生研究所　安全性生物試験研究センター　変異遺伝部）
小 島　　肇（国立医薬品食品衛生研究所　安全性生物試験研究センター　安全性予測評価部
第二室）：事務局

JaCVAM statement on the U937 Cell Line Activation Test (U-SENS™) Skin Sensitization Test Method

At a meeting held on 29 October 2018 at the National Institute of Health Sciences (NIHS) in Kanagawa, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Proposal: Although it is possible to classify chemicals that yield positive results using the U-SENS™ test method as sensitizers, it is not possible to assess accurately their sensitization strength nor their subcategorization under the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS). The U-SENS™ test method is not suitable for predicting skin sensitization potential on its own; in order to make a suitable assessment, the results of U-SENS™ testing must be used with a thorough understanding of the properties of each test chemical in combination with other information as part of an integrated approach to testing and assessment (IATA). 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) 442E: IN VITRO SKIN SENSITISATION ASSAYS ADDRESSING THE KEY EVENT ON ACTIVATION OF DENDRITIC CELLS ON THE ADVERSE OUTCOME PATHWAY FOR SKIN SENSITISATION, ESAC Opinion No. 2016-03 and EURL ECVAM U-SENS Test Submission Template 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 U-SENS™ skin sensitization test method as a useful means for safety assessment by regulatory agencies.

Yasuo Ohno
Chairperson
JaCVAM Regulatory Acceptance Board

Yoko Hirabayashi
Chairperson
JaCVAM Steering Committee

November 5, 2018

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

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

Mr. Yasuo Ohno (Kihara Memorial Yokohama Foundation for the Advancement of Life Sciences): Chairperson
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Noriyasu Imai (Japanese Society for Alternatives to Animal Experiments)
Mr. Tomoaki Inoue (Japanese Society of Immunotoxicology)
Mr. Yuji Ishii (Biological Safety Research Center: BSRC, NIHS)
Ms. Yumiko Iwase (Japan Pharmaceutical Manufacturers Association)
Mr. Takeshi Morita (Japanese Environmental Mutagen Society)
Mr. Shunji Nakai (Japan Chemical Industry Association)
Ms. Ruriko Nakamura (National Institute of Technology and Evaluation)
Mr. Akiyoshi Nishikawa (BSRC, NIHS)
Mr. Satoshi Numazawa (Japanese Society of Toxicology)
Ms. Maki Noguchi (Pharmaceuticals and Medical Devices Agency) *
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Mr. Hiroo Yokozeki (Japanese Society for Cutaneous Immunology and Allergy)

Term: From 1st April 2016 to 31st March 2018
*: From 1st April 2017 to 31st March 2018

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

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

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

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

皮膚感作性試験代替法　U937 Cell Line Activation Test（U-SENS^{TM}）

目　　次

評価会議報告書

皮膚感作性試験：U937 Cell Line Activation Test（U-SENS^{TM}）

JaCVAM 評価会議

平成 30 年（2018 年）8 月 30 日

JaCVAM 評価会議

大 野 泰 雄 （公益財団法人　木原記念横浜生命科学振興財団）：座長
五十嵐良明 （国立医薬品食品衛生研究所）
石 井 雄 二 （国立医薬品食品衛生研究所　安全性生物試験研究センター）
井 上 智 彰 （日本免疫毒性学会）
今 井 教 安 （日本動物実験代替法学会）
岩瀬裕美子 （日本製薬工業協会）
篠 田 和 俊 （独立行政法人　医薬品医療機器総合機構）
杉山真理子 （日本化粧品工業連合会）
仲 井 俊 司 （日本化学工業協会）
中村るりこ （独立行政法人　製品評価技術基盤機構）
西 川 秋 佳 （国立医薬品食品衛生研究所　安全性生物試験研究センター）
沼 澤　　聡 （日本毒性学会）
野 口 真 希 （独立行政法人　医薬品医療機器総合機構）*
森 田　　健 （日本環境変異原学会）
横 関 博 雄 （日本皮膚免疫アレルギー学会）

任期：平成 28 年 4 月 1 日～平成 30 年 3 月 31 日

*：平成 29 年 4 月 1 日～平成 30 年 3 月 31 日

大 野 泰 雄 （公益財団法人　木原記念横浜生命科学振興財団）：座長
五十嵐良明 （国立医薬品食品衛生研究所）
石 井 雄 二 （国立医薬品食品衛生研究所　安全性生物試験研究センター）
稲 若 邦 文 （日本化学工業協会）
井 上 智 彰 （日本免疫毒性学会）
今 井 教 安 （日本動物実験代替法学会）
岩瀬裕美子 （日本製薬工業協会）
久 保 文 宏 （独立行政法人　医薬品医療機器総合機構）
杉山真理子 （日本化粧品工業連合会）
中村るりこ （独立行政法人　製品評価技術基盤機構）
西 川 秋 佳 （国立医薬品食品衛生研究所　安全性生物試験研究センター/済生会宇都宮病院）
西 村 次 平 （独立行政法人　医薬品医療機器総合機構）
沼 澤　　聡 （日本毒性学会）
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）
増 村 健 一 （日本環境変異原学会）
横 関 博 雄 （日本皮膚免疫アレルギー学会）

任期：平成 30 年 4 月 1 日～平成 32 年 3 月 31 日

U937 Cell Line Activation Test（U-SENS^{TM}）は、多くの皮膚感作性物質が樹状細胞を活性化することを利用し、ヒト組織球系リンパ腫細胞株である U937 細胞に被験物質を曝露したときの細胞表面分子 CD86 の発現変化を測定することにより、皮膚感作性の有無を判定する試験法である[1)]。

U-SENS^{TM} については、European Union Reference Laboratory for Alternatives to Animal Testing（EURL ECVAM）によるバリデーション研究が実施され[2)]、EURL ECVAM Scientific Advisory Committee（ESAC）による評価（ピアレビュー）が完了し[3)]、OECD 専門家会議における議論でより簡便な予測モデルに変更された後 2017 年 10 月に、OECD 試験法ガイドラインリストに追記された（TG442E Annex II）[4)]。JaCVAM 評価会議は、皮膚感作性試験資料編纂委員会により作成された「皮膚感作性試験評価報告書 U937 Cell Line Activation Test（U-SENS^{TM}）」[5)]を用いて、本試験法の妥当性について検討した。

１．試験法の定義

名称：　U937 Cell Line Activation Test（U-SENS^{TM}）

代替する対象毒性試験：　モルモットを用いる皮膚感作性試験（OECD TG406）およびマウスを用いる局所リンパ節試験［Local Lymph Node Assay : LLNA（OECD TG429）、LLNA : DA（OECD TG442A）、LLNA：BrdU-ELISA（OECD TG442B）］

試験法の概略：　本試験法では、樹状細胞のモデルとしてヒト組織球系リンパ腫細胞株である U937 細胞を用い、被験物質が U937 細胞を活性化する能力を評価する。U937 細胞に被験物質を添加して約 45 時間培養した後、Fluorescein isothiocyanate（FITC）で蛍光標識した CD86 に対する特異抗体、および Propidium iodide（PI）を用いて細胞を染色し、フローサイトメーターにより細胞表面分子 CD86 の陽性細胞率、並びに細胞生存率を測定する。被験物質で処理したときの CD86 の陽性細胞率を、媒体のみを添加したコントロールの陽性細胞率と比較し、Stimulation Index（S.I.）を算出する。

２．評価に用いた資料および評価内容の科学的妥当性

本試験法は、皮膚感作性発現機序における重要なステップである抗原提示細胞の活性化反応を指標にしたもので、活性化の際に認められる細胞表面分子の発現上昇を測定するという点で原理的に妥当である。本試験法は、EURL ECVAM によるバリデーション研究[2)]とそれに続く ESAC によるピアレビューにより、実験動物を用いた皮膚感作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おり[3)]、より簡便な予測モデルに変更されて、2017 年 10 月に OECD 試験法ガイドラインに追記されている[4)]。JaCVAM 皮膚感作性試験資料編纂委員会では、現在までに公開されている情報を基に本試験法の皮膚感作性試験代替法としての科学的妥当性について評価した。JaCVAM 評価会議では、ESAC ピアレビューと OECD 試験法ガイドラインを精査し、その評価が妥当であると判断した。

３．本試験法の有用性と適用限界

本試験法は、ヒト組織球性リンパ腫患者から単離された細胞株を用いる *in vitro* 試験法であり、3Rs の精神に合致している。また、１試験あたりの費用は、マウスを用いる LLNA の 1/5 程度であり、試験期間も LLNA に比べて短期間であることから、本試験法は経済性および迅速性の面から有用といえる。なお、陽性対照、陰性対照および媒体対照の測定を継続的に実施し、背景データを作成することが求められている。

4 社で行われたバリデーション研究 [6]において、各社 15 物質を 3 回試験し、施設内再現性の平均は 90％であった。3 社は全ての試験で一致した結果（100%）を示したが、1 社は 9 物質のみ一致した（60%）。4 社による 38 物質に対する評価は 32 物質で一致し、施設間再現性は 84％であった。なお本法の開発者が当初提案した予測モデルは、OECD 専門家会議においてより簡便な予測モデルに変更され、上記の数字はその変更を反映したものである。

OECD 試験法ガイドラインでは、LLNA のデータがある 166 物質についての正確度（感度および特異度）の検討で、LLNA の結果に対して、正確度 86％、感度 91％、特異度 65％であり [7]、様々な化学物質に適用可能であることが示された。しかし、United Nation Globally Harmonized System of Classification and Labelling of Chemicals（UN GHS）分類における軽度～中等度の感作性区分に入る物質は偽陰性の判定が生じやすい傾向がある。また、媒体に溶解または安定的に分散できる物質には適応できるが、界面活性剤等の細胞膜の構造変化を引き起こす物質、揮発性物質、FITC や PI と同じ波長域に強い蛍光を有する物質、プロハプテンおよびプレハプテンのような感作性発現に酸化や細胞による代謝が必要な化学物質は正しく評価されない可能性があり、試験結果の解釈には注意が必要である。

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

社会的受け入れ性：

本試験法は、細胞培養とフローサイトメトリーに習熟した施設であれば実施できる試験法であり、蛍光標識された特異抗体も市販されている。また U937 細胞の入手は容易であり、短期間で安価に実施できる。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、感作性物質と判定することは可能であるが、感作性の強度を分類することは困難である。本試験法単独での皮膚感作性の判定は不十分であり、被験物質の特性を十分に理解した上で、IATA（Integrated Approaches to Testing and Assessment）を構成するその他の情報と組み合わせて適切に評価することが必要である。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) Piroird C, Ovigne JM, Rousset F, Martinozzi-Teissier S, Gomes C, Cotovio J, Alépée N (2015) The Myeloid U937 Skin Sensitization Test (U-SENS) addresses the activation of dendritic cell event in the adverse outcome pathway for skin sensitization. Toxicol In Vitro 29(5):901-16.
2) EURL ECVAM (2017) The U-SENS™ test method Validation Study Report. Accessible at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations
3) EURL ECVAM (2016) ESAC Opinion No. 2016-03 on the L'Oréal-coordinated study on the transferability and reliability of the U-SENS™ test method for skin sensitisation testing. EUR 28178 14 EN; doi 10.2787/815737. Available at: http://publications.jrc.ec.europa.eu/repository/handle/JRC103705
4) OECD TG442E (2017) IN VITRO SKIN SENSITISATION ASSAYS ADDRESSING THE KEY EVENT ON ACTIVATION OF DENDRITIC CELLS ON THE ADVERSE OUTCOME PATHWAY FOR SKIN SENSITISATION. Accessible at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788
5) JaCVAM 皮膚感作性資料編纂委員会：皮膚感作性試験報告書 U937 Cell Line Activation Test（U-SENS™）（2018 年 6 月 6 日）
6) Alépée N, Piroird C, Aujoulat M, Dreyfuss S, Hoffmann S, Hohenstein A, Meloni M, Nardelli L, Gerbeix C, Cotovio J (2015) Prospective multicentre study of the U-SENS test method for skin sensitization testing. Toxicol In Vitro 30(1 Pt B):373-82.
7) EURL ECVAM (2016) Test Submission Template. U-SENS Test Submission Template. European Commission

評価報告書

皮膚感作性試験：U937 Cell Line Activation Test（U-SENS^{TM}）

皮膚感作性試験資料編纂委員会

平成 30 年（2018 年）6 月 6 日

皮膚感作性試験資料編纂委員会

委員長　小 島 幸 一（一般財団法人　食品薬品安全センター）
委　員　足利太可雄（国立医薬品食品衛生研究所）
　　安 達 玲 子（国立医薬品食品衛生研究所）
　　佐 藤 一 博（国立大学法人　福井大学）
　　武 吉 正 博（一般財団法人　化学物質評価研究機構）
　　福 山 朋 季（一般財団法人　残留農薬研究所）

要旨

皮膚感作性は化学物質の安全性評価において重要な評価項目であり、従来、モルモットやマウスを用いた動物実験によって評価されてきた。近年 EU における欧州化学品規制では、安全性評価はコンピューターを用いた定量的構造活性相関（QSAR）モデルや *in vitro* 試験の代替法が推奨されており、動物実験によって安全性が評価された成分を含む化粧品の輸入販売が禁止（2013 年 3 月全面施行）されたことから、動物を用いない *in vitro* 試験法の開発が強く望まれている。

U937 Cell Line Activation Test (U-SENS™)は、多くの皮膚感作性物質が樹状細胞を活性化することを利用し、ヒト組織球性リンパ腫細胞株である U937 細胞を用い、活性化に伴い細胞表面での発現量が変化する CD86 を測定することによって皮膚感作性の有無を判定する試験法である。本報告書は、この U-SENS™ について、TG 442E (2017)、EURL ECVAM Scientific Advisory Committee（ESAC）によって実施されたバリデーション報告書、評価（ピアレビュー）報告書及び試験法開発者の投稿論文などを基に試験手順をまとめ、有用性と限界を評価したものである。

U-SENS™ は、感作性発現機序における第三段階のイベントである樹状細胞が活性化する際の細胞表面分子の発現亢進を利用した *in vitro* 試験法であり、化学物質の感作性を判断する上で重要な情報を与えてくれる。本法は、既に OECD TG 442E としてガイドライン化された h-CLAT と類似の方法である。

U-SENS™ では、マウスを用いる LLNA（Local Lymph Node Assay）の 1/5 程度の消耗品費で被験物質の皮膚感作性を判定することが可能と試算され、試験期間も LLNA に比べ短期であるため、経済性・迅速性の観点で有用性は高いと思われる。なお、細胞の反応性の確認と陽性対照および媒体対照のデータを集積し、ヒストリカルなデータベースを作成・維持し、試験系の再現性を保証する必要がある。

本試験法の EURL ECVAM によるバリデーション試験において、15 物質を用いて 4 施設で実施された施設内再現性の平均は 90%であった。また、38 物質を用いて 4 施設で行われた施設間再現性の平均は 84%であった。

OECD 試験法ガイドラインでは、ヒトに対する感作性の有無の分類で、Basketter らの報告に基づくヒトのクラス 5 と 6 を感作性無しとした場合、感度 100%、特異度 47%、正確度 77%、であった（クラス 6 のみを感作性無しとした場合は、感度 89%、特異度 65%、正確度 85%）（101 物質対象）。LLNA のデータがある 166 物質についての検討では、LLNA の結果に対して、感度 91%、特異度 65%、正確度 86%であった。この 166 物質を見る限り、本法では様々な化学物質の皮膚感作性の予測が可能であることが示されている。

なお、本法の開発者が当初提案した感作性判定予測モデル（OPM: Original Prediction Model）は、OECD 専門家会議においてより簡便な予測モデル（APM: Actual simplified Prediction Model）に変更されたが、上記の施設内および施設間再現性と、*in vivo* との対応性の値は APM を反映したものである。

U-SENS™ で評価した 175 物質と h-CLAT で評価した 142 物質のうち、LLNA のデータがある共通物質は 104 物質であった。この 104 物質について、感度、特異度、正確度を比較したところ、両者での予測性は概ね同様と判断された。

U-SENS™ の試験法は、液相での反応を必要とする試験系であるため、培地あるいは DMSO に 50 mg/mL の濃度で溶解あるいは安定的な分散液であれば試験が可能である。

本試験法は、強度感作性物質{United Nations Globally Harmonized System of Classification and Labelling of Chemicals（UN GHS）1A 分類}に比べて、軽度から中等度感作性物質（UN GHS 1B 分類）で偽陰性の判定が生じやすい傾向にあるため、本法単独での感作性強度分類や UN GHS のサブカテゴリー分類への利用には適さない。

界面活性剤等の細胞膜の構造変化を引き起こすような物質、測定に使用する FITC（Fluorescein isothiocyanate）や PI（Propidium iodide）と同一の波長に強い蛍光を有する物質、揮発性を有する物質などでは、適切に評価されない可能性がある。プレハプテンやプロハプテンに関しては、本法開発の段階では正しく判定されたとしているが、ウェル中での化学的な酸化の進行については未知であり、また、本試験法に用いる細胞の薬物代謝能は限定的であるため、活性化に酸化や代謝系を必要とする化学物質では、正しくその感作性が検出されない可能性がある。

本委員会は、上記の本試験法の様々な限界を勘案すると、本試験法単独では皮膚感作性の判定は不十分で有り、証拠の重み付けや他の試験法との組合せで用いることを推奨する。

1. 緒言

皮膚感作性を評価することは化学物質の安全性評価において重要である。化学物質の皮膚での接触感作性のリスクを動物で予測する試験法としてモルモットを用いる皮膚感作性試験（OECD TG 406）やマウスを用いる局所リンパ節試験（LLNA, OECD TG 429）がある。LLNA には、[^{3}H-Methyl]-thymidine 取込量を測定する方法以外に、放射性同位元素（RI）を使用せずに ATP 量を測定する LLNA : DA（OECD TG 442A）や Bromodeoxyuridine 取込量を測定する LLNA : BrdU-ELISA（OECD TG 442B）がある [1]。

EU において、欧州化学品規制（REACH: Registration, Evaluation, Authorization and Restriction of Chemicals）では、安全性評価においてはコンピューターを用いた定量的構造活性相関（QSAR）モデルや *in vitro* 等による代替法の活用が推奨されており [2]、EU 化粧品規則（EC No 1223/2009）では、動物実験により安全性が評価された成分を使用した化粧品の販売が禁止された [3]（2013 年 3 月全面施行）。そのため、化学物質の皮膚感作性を判定する代替法の開発が強く求められている。

In chemico 試験法としてペプチド結合反応を利用した Direct Peptide Reactivity Assay（DPRA : OECD TG 442C）、*in vitro* 試験法としてケラチノサイト細胞系の標的遺伝子を用いた ARE-Nrf2 Luciferase Test Method（OECD TG 442D）および単球系細胞の活性化を利用した human Cell Line Activation Test（h-CLAT：OECD TG 442E ANNEX I）が OECD から試験法ガイドラインとして公表されている [1]。また、これら以外に h-CLAT 同様、単球系細胞の活性化を利用した U-SENS™ 及び IL-8 Luc assay などの皮膚感作性試験の *in vitro* 法が提案されており、ECVAM 等においてバリデーション研究が行われてきた。

U-SENS™ は、多くの皮膚感作性物質が樹状細胞を活性化することを利用し、ヒト組織球性リンパ腫細胞株である U937 細胞を用い、活性化に伴い細胞表面での発現量が変化する CD86 を測定する試験法である。U-SENS™ のバリデーション研究の結果については、ESAC

による評価（ピアレビュー）が完了し[4]、OECD 専門家会議における議論により予測モデルが変更された後、2017 年 10 月に OECD の試験法ガイドラインリストに追記された（OECD TG 442E ANNEX II）[1]。以下本文中では、本法の開発者が当初提案した感作性判定予測モデル（OPM）による結果と、OECD 専門家会議において変更されたより簡便な予測モデル（APM）による結果の両方を記載した。

JaCVAM 皮膚感作性試験資料編纂委員会（以下、委員会）は、U-SENS™ の皮膚感作性試験代替法としての科学的妥当性について、現在までに公開されている情報をもとに評価したので、その結果を報告する。

2. 試験法の原理

皮膚感作性は、ヒトでは接触皮膚炎、動物（齧歯類）では接触過敏症として知られる化学物質の毒性の一つである。OECD がまとめた Adverse Outcome Pathway (AOP)[5]では、化学物質による皮膚感作は次の 4 つの Key event から成るとされている。

1) 化学物質とタンパク質のシステイン残基あるいはリジン残基との共有結合
2) ケラチノサイトにおける炎症性応答及び Antioxidant/electrophile response element（ARE）-dependent pathway による遺伝子発現
3) 樹状細胞の活性化（特異的細胞表面マーカーの発現、ケモカインやサイトカインの産生）
4) リンパ節における T 細胞の増殖

U-SENS™は上記の第 3 の Key event に対応する試験法である。その基本的原理は単球及び樹状細胞の活性化マーカーである CD86 の発現量の変化を定量化することによって、感作性物質と非感作性物質の判別を行う手法であり、既に OECD TG 442E としてガイドライン化された h-CLAT と類似の方法である。CD86 は抗原提示細胞が T 細胞に抗原提示する際に T 細胞表面の CD28 と相互作用する補助刺激分子であり、樹状細胞の抗原刺激後の分化成熟に伴い発現量が増加することが知られている。抗原提示による T 細胞の活性化に際し、T 細胞受容体（TcR）を介するシグナルと共に CD86/CD28 を介する刺激が極めて重要な役割を担っていることから、感作性物質の判別に有用と考えられる。

本法に用いる U937 細胞はヒト組織球性リンパ腫患者から単離された細胞株[6]であり、h-CLAT で使用する単球性白血病患者由来の THP-1 細胞[7]と同様に骨髄幹細胞由来の細胞である。いずれの細胞もサイトカイン等の刺激によって樹状細胞様の細胞に分化可能であることが報告されており[8]、感作性物質の刺激による CD86 の発現上昇が確認されていることから、感作性物質のスクリーニングに利用可能と考えられる。

U-SENS™では U937 細胞に検体である化学物質を添加して約 45 時間培養した後に CD86 の発現量を蛍光標識した特異的抗体を用いてフローサイトメトリーにより相対蛍光強度を測定し、溶媒のみを作用させた媒体対照の相対蛍光強度との比率（Stimulation Index、S.I.）を基に判定を行う。

3. 試験手順／判定

U-SENS™の試験手順および判定は、被験物質を曝露した U937 細胞における CD86 陽性細胞率と細胞毒性検査によって構成される。

3-1. 細胞調製、試薬および反応性確認試験

U-SENS™ではヒト組織球性リンパ腫細胞株である U937 細胞を使用する。細胞は American Type Culture Collection (ATCC) のような信頼のおける細胞供給元から入手することが望ましい (Clone CRL1593.2)。

U937 細胞は、培養用培地 (RPMI-1640 の基礎培地に 10% Fetal Calf Serum (FCS)、2 mM L-Glutamine、100 units/mL Penicillin および 100 μg/mL Streptomycin を加えたもの) にて、1.5 もしくは 3×10^5 cells/mL の細胞濃度で播種し、2 もしくは 3 日ごとに継代を行い維持する。細胞濃度は 2×10^6 cells/mL を超えないように留意し、Trypan blue で細胞生存率が 90%以上であることを確認する。使用する細胞、FCS、抗体は各ロットについて、試験実施前に反応性の確認を行う。反応性の確認は細胞の起眠から少なくとも 1 週間培養した後に行い、陽性対照物質として Picrylsulfonic acid (TNBS, CAS NO. 2508-19-2, 純度 99%以上) および陰性対照物質として Lactic acid (LA, CAS NO. 50-21-5, 純度 85%以上) を用いる。反応性は TNBS で 1, 12.5, 25, 50, 75, 100 μg/mL、LA で 1, 10, 20, 50, 100, 200 μg/mL の各 6 濃度で確認する。TNBS では CD86 発現率に濃度依存性の陽性反応が得られ、LA では CD86 発現率に陰性反応が得られなければならない。反応性確認を 2 回実施し、いずれも合格した細胞バッチのみを本実験に使用することができる。U937 細胞は融解後 7 週間まで継代させることができ、継代数が 21 を超えないように留意する。

試験実施前に、U937 細胞を 3 もしくは 6×10^5 cells/mL の細胞濃度で培養フラスコに播種し、それぞれ 2 日もしくは 1 日間培養する。実験日に、5×10^5 cells/mL の濃度で新しい培養用培地に再懸濁した U937 細胞を 96 穴平底プレートに 100 μL ずつ加える (各 well あたりの最終細胞数は 0.5×10^5 cells/well)。

3-2. 被験物質および対照物質の調製

試験実施前に被験物質の溶解性を確認する。被験物質が培養用培地で、50 mg/mL の溶解もしくは安定的な懸濁状態が得られる場合は、培養用培地を媒体の第一候補とする。被験物質が培養用培地で実験に適した状態を得られない場合、Dimethyl sulfoxide (DMSO, 純度 99%以上) を媒体の第 2 候補とする。被験物質が培養用培地に溶解する場合、0.4 mg/mL を終濃度として調製し、被験物質が DMSO にしか溶解しない場合は、50 mg/mL を保存溶液として調製する。

被験物質および対照物質は、実験当日に調製する。U-SENS™では用量設定試験を行わないため、初回実験では終濃度として 6 濃度 (1, 10, 20, 50, 100 および 200 μg/mL) となるように培養用培地もしくは、DMSO を 0.4%含んだ培養用培地で調製し、実験を行うべきである。2 回目以降の実験では、1 回目の実験結果を踏まえて少なくとも 4 濃度を用いて実験を行う。培養用培地もしくは 0.4% DMSO で調製した使用液は、等量の U937 細胞懸濁液と混合し、目標濃度を得る。よって使用液は目標濃度の 2 倍となるように調製する必要があ

る (200 μg/mL の終濃度を得る場合は倍の 400 μg/mL を調製する)。目標濃度は、それまでの実験結果をもとに 1, 2, 3, 4, 5, 7.5, 10, 12.5, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180 および 200 μg/mL から 4 濃度以上を選択し、実験に供する。

U-SENS™で用いる媒体対照は、培養用培地もしくは DMSO を 0.4%含有した細胞培養培地とする。

U-SENS™で用いる陽性対照物質は、培養用培地で調製した TNBS とする。TNBS は CD86 発現測定の陽性対照として、プレート中の終濃度が 50 μg/mL となるような 1 濃度を使用し、70%以上の細胞生存率であることを確認する。TNBS がプレート中に 50 μg/mL となるようにするには、1M の保存溶液 (293 mg/mL) を細胞継代培地で調製し、培養用培地で 2,930 倍希釈することで 100 μg/mL の使用液を得る。陰性対照物質としては、LA のプレート中終濃度が 200 μg/mL となるように培養用培地で調製し用いる。各実験のプレート毎に、無処置区、媒体対照区、陰性対照区および陽性対照区を 3 well ずつ設定する。

3-3. 被験物質および対照物質曝露

3-2 で調製した被験物質および対照物質の使用液や媒体対照液と細胞懸濁液とを 96 穴平底プレート内にて 1:1 で混合し、37°C、5% CO_2 in Air 条件下にて 45±3 時間培養する。揮発性の高い被験物質の場合は、ウェル間での被験物質の交差汚染が生じる可能性があるので、プレートをシールするなどの処置を施す。

3-4. 細胞の染色

曝露開始 45±3 時間後に、細胞懸濁液を V 底 96 穴プレートに移し、遠心分離により細胞を沈降させる。上清を捨て、100 μL の氷冷 5%FCS 含有リン酸緩衝生理食塩水 (FCS 含有 PBS) で 1 回洗浄する。遠心分離後、回収した細胞を 100 μL の FCS 含有 PBS で再懸濁し、5 μL (0.25 μg) の FITC 標識抗 CD86 抗体 (BD-Pharmingen, #555657, Clone: Fun-1 もしくは Caltag/Invitrogen, #MHCD8601, Clone: BU63) またはマウス IgG1 アイソタイプ抗体 (BD-Pharmingen, #555748 もしくは Caltag/Invitrogen, #GM4992) を添加し、4°C、暗所で 30 分間静置する。抗体染色後の細胞は、100 μL の FCS 含有 PBS で 2 回、100 μL の氷冷リン酸緩衝液で 1 回洗浄した後、氷冷リン酸緩衝液(サンプルチューブでマニュアル測定する場合は 125 μL、プレートを用いて自動測定する場合は 50 μL)で再懸濁し、測定直前に PI 液 (終濃度が 3 μg/mL)を添加する。

3-5. フローサイトメトリー解析

CD86 発現率と細胞生存率はフローサイトメトリーで解析する。細胞は大きさ (FSC: Forward Scatter Chanel) と顆粒含有量 (SSC: Side Scatter Chanel) を基に計数し、結果を対数で表示し、デブリスを除去した細胞集団 (R1) を選択する。この R1 内の細胞が 10,000 個となるように各 well/チューブごとに細胞のカウントを行う。R1 の細胞を、FL3 (または FL4) と SSC でさらに展開し、PI 陰性 (FL3 が陰性) の生細胞集団を R2 として選択する。細胞生存率は以下の式にて算出する。細胞生存率が低い場合は、死細胞を含む 20,000 個までの条件、もしくはカウント開始後 1 分間のデータを解析することもできる。

$$\text{Cell Viability} = \frac{\text{Number of living cells}}{\text{Total number of acquired cells}} \times 100$$

FL1 陽性細胞 (CD86 陽性) の解析は、生細胞集団 (R2) を FL1 と SSC で展開することで行う。S.I.は媒体対照区と被験物質曝露区の CD86 陽性細胞率を基に下記の計算式より求める。

$$\text{S.I.} = \frac{\%\ \text{of CD86}^+\ \text{treated cells} - \%\ \text{of IgG1}^+\ \text{treated cells}}{\%\ \text{of CD86}^+\ \text{control cells} - \%\ \text{of IgG1}^+\ \text{control cells}} \times 100$$

3-6.　CV70 値と EC150 値の計算法

3.5.で得られた結果を基に、CV70 値（U937 の細胞生存率が 70%を示す被験物質濃度）および EC150 値（CD86 の S.I.値が 150%を示す被験物質濃度）を下記の計算式より求める。

CV70 = C1+[(V1-70)/(V1-V2)*(C2-C1)]

V1: 細胞生存率が 70%以上の、最低の細胞生存率

V2: 細胞生存率が 70%未満の、最高の細胞生存率

C1 と C2: V1 および V2 を示す被験物質濃度

EC150 = C1+[(150-S.I.1)/(S.I.2-S.I.1)*(C2-C1)]

C1: CD86 S.I.値が 150%未満の、最大被験物質濃度

C2: CD86 S.I.値が 150%以上の、最小被験物質濃度

S.I. 1: 濃度 C1 のときの S.I.

S.I. 2: 濃度 C2 のときの S.I.

EC150 および CV70 は測定毎に算出し、CD86 陽性率の被験物質濃度依存性を確認するために使用される。また、被験物質曝露区ないし対照区の平均値を用いて算出する。

3-7. 予測方法

CD86 発現測定では、それぞれの被験物質の予測（陰性もしくは陽性）を行うために、各測定は少なくとも 4 濃度、さらに少なくとも 2 回の独立した測定（別日に実施）を行う。

CD86 の S.I.値が、検査に供した細胞毒性のない（細胞生存率が 70%以上）全ての濃度で 150%未満であり、しかも被験物質の溶解性や細胞毒性のように結果に影響を及ぼすような要因がない場合、その測定における結果は陰性と判断する。一方で陰性以外の全ての場合（S.I.値が 150%以上、結果に影響を及ぼすような要因が存在等）は、その測定における結果を陽性と判断する。

- 少なくとも 2 回の独立した測定において陰性であれば U-SENS™における予測は陰性とする。また、最初の 2 回の測定において陰性であれば 3 回目の測定は行う必要がない。
- 少なくとも 2 回の独立した測定において陽性であれば U-SENS™における予測は陽性とする。また、最初の 2 回の測定において陽性であれば 3 回目の測定は行う必要がない。
- U-SENS™では濃度設定試験を行わないことから、最初の測定において細胞毒性の見られない最も高い濃度でのみ CD86 発現の S.I.値が 150%以上であった場合は、その結果を不十分 (NC: Not Conclusive) と判断し、濃度を追加した測定を少なくとも別途 2 回実施する。3 回目までの結果で判断ができない場合（2 回目と 3 回目の結果が分かれた場合）は 4 回目の測定を行い、その測定結果を U-SENS™における予測とする。2 回目以降の測定では、細胞毒性のない最も高い濃度でのみ S.I.値が 150%以上であったとしても、陽性と判断する。

U-SENS™における予測のフローチャートを図 1 に示す。

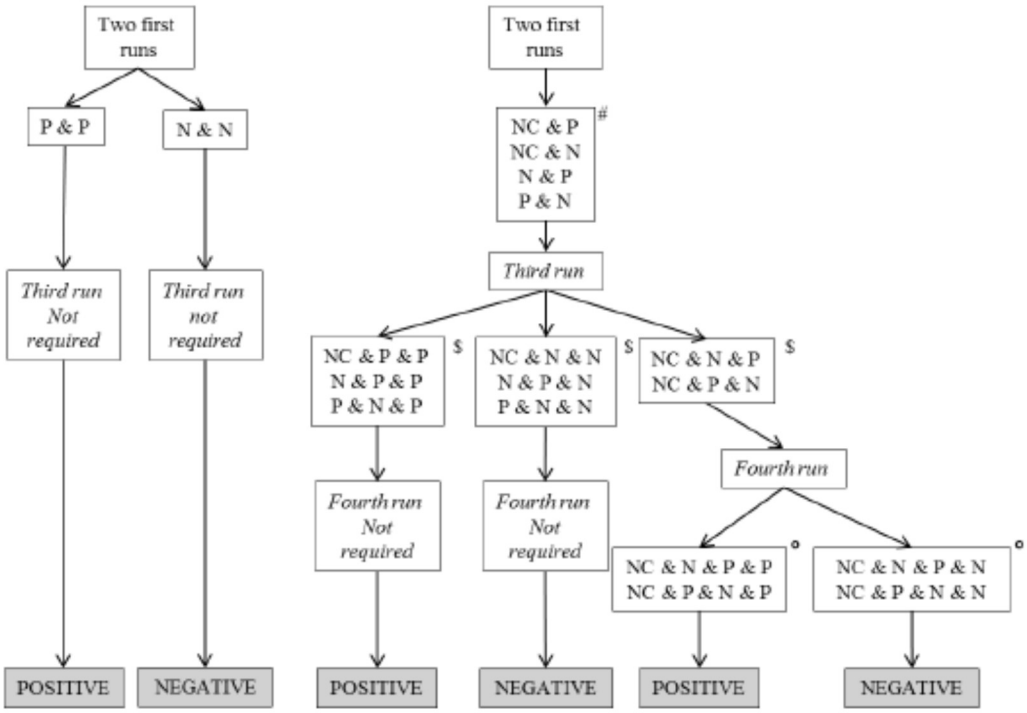


図 1 U-SENS™における予測フローチャート

N: CD86 が陰性かつ結果に影響を及ぼすような事象が認められない場合

P: CD86 が陽性または結果に影響を及ぼすような事象が認められる場合

NC: 最初の測定で、細胞毒性のない最高濃度でのみ CD86 の陽性反応が認められた場合

#: 最初の測定で NC が認められた場合は自動的に 3 回目までの測定が必要となり、3 回目までに予測ができない場合は 4 回目の測定を行う

$: 最初の 2 回の測定結果との組み合わせ

°: 最初の 3 回の測定結果に基づいて実施された 4 回目までの測定結果の組み合わせ

3-8. 試験成立の条件

U-SENS™においては、以下の条件が成立する必要がある。

- 45±3 時間の被験物質曝露終了時に、3 well 設定した無処置 U937 の細胞生存率の平均が 90%より高く、かつ、その CD86 発現率が 2%以上 25%以下である。
- DMSO を媒体とした場合、DMSO 媒体対照区 3 well における細胞生存率の平均が、90%より高くなければならない。さらに DMSO 媒体対照区 3 well の CD86 S.I.の平均は、無処置区平均の 250%未満である。
- 3 回測定のうち少なくとも 2 回で、無処置区における IgG1 アイソタイプコントロールに対する CD86 発現率が 0.6%以上 1.5%未満である。
- 陰性対照区 (LA) の CD86 S.I.が 3 well 中 2 well で陰性 (CD86 S.I.が 150%未満) でかつ細胞生存率が 70%以上でなければならない。
- 陽性対照区 (TNBS) の CD86 S.I.が 3 well 中 2 well で陽性 (CD86 S.I.が 150%以上) でかつ細胞生存率が 70%以上でなければならない。

3-9. 試験手順および判定方法に関する h-CLAT との比較

本試験法は樹状細胞の活性化を指標にした検査法であることから、同様に樹状細胞の活性化に着目した h-CLAT 法との共通点が多い。以下に U-SENS™および h-CLAT の試験手順および判定方法の比較を示す。

	U-SENS	h-CLAT
使用細胞株	U937 (ヒト組織球性リンパ腫細胞株)	THP-1 (ヒト単球性白血病細胞株)
培養用培地	RPMI-1640に10% FCS、L-Glutamine、抗生物質を添加	RPMI-1640に10% FCS、メルカプトエタノール、抗生物質を添加
継代頻度	2~3日おき	2~3日おき
用量設定試験	なし	CV75を決定するために必要
適用可能被験物質濃度	200 μg/mL	5000 μg/mL (生理食塩水及び培地) 1000 μg/mL (DMSO)
被験物質の曝露時間	45±3時間	24±0.5時間
評価方法	細胞生存率とCD86発現率	細胞生存率、CD54およびCD86の相対蛍光強度 (RFI)
予測方法	CD86 S.I.値の150%を基準に2～4回の独立した測定を行い予測する	CD54 RFI値200%、CD86 RFI値150%を基準に2～3回の独立した測定を行い予測する

両試験法は、使用する細胞株が異なるが、培養用の培地、継代方法および継代頻度等の細胞の準備について大きな差異は認められなかった。基本的な被験物質の曝露方法は両試験で共通であったが、試験に使用できる最高被験物質濃度が h-CLAT で 5000 μg/mL (生理食塩水及び培養用培地の場合) ないし 1000 μg/mL (DMSO の場合) なのに対し、U-SENS™では 200 μg/mL であった。被験物質の曝露時間は h-CLAT の 24 時間に対し、U-SENS™では 45 時間が必要であり、細胞の反応性や評価方法の差異が要因と考えられた。試験評価は、h-CLAT が CD54 と CD86 という 2 種類の樹状細胞関連因子の相対蛍光強度を使用して

いるのに対し、U-SENS™は CD86 の発現率を使用している。また、先行の h-CLAT に対して U-SENS™では蛍光強度ではなく発現率を使用している。試験成立条件は、方法の違いに応じて若干の相違があるものの、基本的には同様と考えられた。予測方法は、先行の h-CLAT を参考に U-SENS™の予測フローチャートが作成されていることから、基本的な方針は同様であったが、U-SENS™では用量依存性を指標に入れているため 4 回目測定の可能性が考慮されていた。

4. 精度

本法の技術移転性、施設内再現性、施設間再現性は EURL ECVAM によって評価されている [9]。

4-1. 技術移転性

4 つの感作性物質 TNBS, 1,4-Phenylenediamine (PPD), Abietic acid (AA), 4,4,4-Trinitro-1-Phenylbutane-1,3-dione)と 1 つの非感作性物質 LA の 5 物質を用いて、技術移転性を評価している [9]。まず、Bioassay 社、WIL Research 社、CiToxLAB 社の担当者が開発者である L'Oreal 社で試験法を習得後、それぞれの実験室で週 2 回試験を行い、90%以上の正解があれば成功で、6 週以内に 3 週連続（もしくは 4 週のうち 1 回は失敗）成功が続けば技術移転したとする。

Bioassay 社と WIL Research 社は 2011 年に、CiToxLAB 社は 2013 年に技術移転は問題なく行われた。

4-2. 施設内再現性

2013 年 L'Oreal 社で行われた 3 回施行する Ring trial 2013[10]では、21 物質が施設内再現性で評価された。そのうち 14 物質は、施設間再現性でも評価された。11 物質が感作性物質、10 物質が非感作性物質の計 21 物質である。結果は、Chlorobenzene 以外の 20 物質で感作性(S)と非感作性(NS)が一致し、施設内再現性は 95%（APM）であった（表 1）。OPM での施設内再現性も 95%で差はなかった。

表 1　施設内再現性の評価成績

ID	Chemical name	CAS No	GHS potency category	LLNA potency category	LLNA	U-SENS™ Classification		
						Exp1	Exp2	Exp3
11	Lauryl gallate	1166-52-5	1A	Strong	S	S	S	S
17	Methyldibromoglutaronitlirile	35691-65-7	1A	Strong	S	S	S	S
21	Diethyl maleate	141-05-9	1B	Moderate	S	S	S	S
35	Phenyl benzoate	93-99-2	1B	Moderate	S	S	S	S
36	tetramethylthiuram disulfide (TMTD)	137-26-8	1B	Moderate	S	S	S	S
63	Benzyl benzoate	120-51-4	1B	Weak	S	S	S	S
89	Salicylic acid	69-72-7	NC	NS	NS	NS	NS	NS
109	4-Nitrobenzyl bromide	100-11-8	1A	Extreme	S	S	S	S
116	m-Phenylenediamine	108-45-2	1A	Strong	S	S	S	S
127	m-Aminophenol	591-27-5	1B	Moderate	S	S	S	S
143	12-Bromo-1-dodecanol	3344-77-2	1B	Moderate	S	S	S	S
152	4-Allylanisole	140-67-0	1B	Weak	S	S	S	S
160	Chlorobenzene	108-90-7	NC	NS	NS	NS	NS	S
165	6-Methylcoumarine	92-48-8	NC	NS	NS	S	S	S
71	Methyl salicylate	119-36-8	NC	NS	NS	NS	NS	NS
72	Vaniline	121-33-5	NC	NS	NS	S	S	S
94	Glycerol	56-81-5	NC	NS	NS	NS	NS	NS
161	Sulfanilic acid	121-57-3	NC	NS	NS	NS	NS	NS
162	4-Hydroxybenzoic acid	99-96-7	NC	NS	NS	NS	NS	NS
164	1-Bromobutane	109-65-9	NC	NS	NS	NS	NS	NS
173	Citric acid	77-92-9	NC	NS	NS	NS	NS	NS

grey: substances with discordant classification as compared to the other validation laboratories.

2014 年に L'Oreal 社、WIL Research 社、Bioassay 社、CiToxLAB 社の 4 社で行われた Validation study 2014[10]では 6 つの感作性、9 つの非感作性の計 15 物質を用いて各社 3 回行い施設内再現性が評価された。L'Oreal 社、Bioassay 社、WIL Research 社は全て一致（施設内再現性：WLR=100%）し、CiToxLAB 社は 9 物質の一致に留まり、WLR は 60%であった。4 社の平均は 90%（APM）であった（表 2）。OPM での 4 社の平均値は 92%であった。

表 2　施設内再現性の評価成績 2

ID	Chemical name	CAS No	GHS potency category	L'Oreal			Bioassay			CiToxLAB			WIL Research		
168	Benzoic acid	65-85-0	NC	NS	NS	NS	NS	NS	NS	S	NS	NS	NS	NS	NS
175	Benzyl alcohol	100-51-6	NC	NS	NS	NS	NS	NS	NS	NS	S	S	NS	NS	NS
94	Glycerol	56-81-5	NC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
91	Hexane	110-54-3	NC	NS	NS	NS	NS	NS	NS	NS	NS	S	NS	NS	NS
93	Lactic acid	50-21-5	NC	NS	NS	NS	NS	NS	SN	NS	NS	S	NS	NS	NS
174	Polyethylene glycol	25322-68-2	NC	NS	NS	NS	NS	NS	NS	NS	S	S	NS	NS	NS
163	Saccharin	81-07−2	NC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
172	Streptomycin sulfate	3810-74-0	NC	NS	NS	NS	NS	NS	NS	S	S	NS	NS	NS	NS
167	Vinylidene dichloride	75-35-4	NC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
22	3-Dimethylamino-propylamine	109-55-7	1B	S	S	S	S	S	S	S	S	S	S	S	S
33	Ethylene diamine	107-15-3	1B	S	S	S	S	S	S	S	S	S	S	S	S
7	Methylisothiazolinone	2682-20-4	1A	S	S	S	S	S	S	S	S	S	S	S	S
58	Methylmethacrylate	80-62-6	1B	S	S	S	S	S	S	S	S	S	S	S	S
48	Resorcinol	106-46-3	1B	S	S	S	S	S	S	S	S	S	S	S	S
9	Toluene diamine sulphate	615-50-9	1A	S	S	S	S	S	S	S	S	S	S	S	S

grey: substances with discordant classification as compared to the other validation laboratories.

4-3. 施設間再現性

Ring trial 2013 の 14 物質（11 種の感作性物質と 3 種の非感作性物質）に加えて、Validation study 2014 で 24 物質（8 種の感作性物質と 16 種の非感作性物質）を加えた計 38 物質を Bioassay 社、CiToxLAB 社、L'Oreal 社、WIL Research 社の 4 社で判定し、施設間再現性を評価した。

Ring trial 2013 の 14 物質のうち 12 物質の感作性（S）と非感作性（NS）が 4 社で一致し、施設間再現性は 86%であった。Validation study 2014 の 24 物質のうち 20 物質が 4 社で一致し、施設間再現性は 83%であった。併せて 38 物質中 32 物質で一致し、施設間再現性は 84%（APM）であった（表 3）。OPM でも 84%であった。

表 3　施設間再現性の評価成績

Study	ID	Substance	CAS	LLNA	L'Oreal	Bioassay	CiToxLAB	WIL Research
	164	1-Bromobutane	109-65-9	NS	NS	NS	NS	NS
V	162	4-Hydroxybenzoic acid	99-96-7	NS	NS	NS	NS	NS
a	168	Benzoic acid	65-85-0	NS	NS	NS	NS**	NS
l	175	Benzyl alcohol	100-51-6	NS	NS	NS	S*	NS
i	173	Citric acid	77-92-9	NS	NS	NS	NS	NS
d	94	Glycerol	56-81-5	NS	NS	NS	NS	NS
a	91	Hexane	110-54-3	NS	NS	NS	NS**	NS
t	93	Lactic acid	50-21-5	NS	NS	NS	NS**	NS
i	71	Methyl salicylate	119-36-8	NS	NS	NS	NS	NS
o	174	Polyethylene glycol	100-51-6	NS	NS	NS	S*	NS
n	163	Saccharin	81―07-2	NS	NS	NS	NS	NS
	172	Streptmycin sulfate	3810-74-0	NS	NS	NS	S*	NS
s	161	Sulfanilic acid	121-57-3	NS	NS	NS	NS	NS
t	72	Vanillin	121-33-5	NS	S	NS	S	NS
u	167	Vinylidene dichloride	75-35-4	NS	NS	NS	NS	NS
d	88	Xylene	1330-20-7	NS	NS	NS	NS	NS
y	5	1,4-Phenylenediamine	106-50-3	S	S	S	S	S
2	22	3-Dimethylaminopropylamine	109-55-7	S	S	S	S	S
0	40	Cinnamic alcohol	104-54-1	S	S	S	S	S
1	33	Ethylene diamine	107-15-3	S	S	S	S	S
4	7	Methylisothiazolinone	2682-20-4	S	S	S	S	S
	58	Methylmethacrylate	80-62-6	S	S	S	S	S
	48	Resorciol	108-46-3	S	S	S	S	S
	9	Toluene diamine sulphate	615-50-9	S	S	S	S	S
R	165	6-Methyl coumarine	92-48-8	NS	S	NS	S	S*
i	160	Chlorobenzene	109-90-7	NS	NS**	NS	S	NS
n	89	Salicylic acid	69-72-7	NS	NS	NS	NS	NS
g	152	4-Allylanisole	140-67-0	S	S	S	S	S
	109	4-Nitrobenzyl bromide	100-11-8	S	S	S	S	S
s	143	12-Bromo-1-dodecanol	3344-77-2	S	S	S	S	S
t	63	Benzyl benzoate	12-51-4	S	S	S	S	S
u	21	Diethyl maleate	141-05-9	S	S	S	S	S
d	11	Lauryl galate	1166-52-5	S	S	S	S	S
y	127	m-Aminophenol	591-27-5	S	S	S	S	S
2	116	m-Phenylenedimine	108-45-2	S	S	S	S	S
0	17	Methyldibromo glutaronitrile	35691-65-7	S	S	S	S	S
1	35	Phenyl benzoate	93-99-2	S	S	S*	S	S
3	36	Tetramethylyhiuram disulphate	137-26-8	S	S	S	S	S

grey: substances with discordant classification as compared to the other validation laboratories.
*two 'S' classification and one 'NS'; **two 'NS' classification and one 'S'.

5. 正確度（および感度と特異度）[1*]

Validation study 2014 の 24 物質の結果を LLNA の結果（Xylene は偽陽性となったが、ヒトでの評価は陰性のため最終的に陰性とした）を元に正確度を出した。8 つの感作性物質は 4 社とも陽性となり感度は 100%であった [10]。特異度は CiToxLAB 社が 81%で最も低く WIL Research 社と Bioassay 社が 100%であった。正確度は CiToxLAB 社で 88%、L'Oreal 社で 96%、WIL Research 社と Bioassay 社で 100%だった。4 社平均では、感度 100%、特異度 94%、正確度 96%だった。

施設間再現性で用いた Ring trial 2013 と Validaton study 2014 を合わせた 38 物質の結果を LLNA データを参考に分析して正確度を出している（APM）。CiToxLAB 社と L'Oreal 社は 19 の感作性物質は全て陽性と判定され、感度は 100%であった。特異度は CiToxLAB 社が最も低く 68%、Bioassay 社が最も高く 100%であった。正確度は CiToxLAB 社で 87%、L'Oreal 社は 95%、WIL Research 社と Bioassay 社は 97%であった。4 社平均では、感度 99%、特異度 88%、正確度 93%であった [10]。4 社平均を OPM で評価した場合は、感度 98%、特異度 90%、正確度 93%であった。

本法の感度及び特異度の正確度を分析するため、175 物質を L'Oreal 社が評価している [11]。以下全て APM による評価結果を示す。175 物質はヒトまたはモルモットの LLNA のデータが有る物質で、*in vitro* 試験でも評価可能な物質である。70%は化粧品関連で、そのうち香料が 29%、保存剤が 15%、染料が 6%であった。175 物質中 65%が Aptula and Roberts (2006)の 5 つのタンパク結合様式（Michael acceptor, Shiff base formation, acyl transfer agent, SN2 (substitution nucleophilic bi-molecular)または SNAr (nucleophilic aromatic substitution)）の少なくとも一つに属していた [12]。

理想的にはヒトのデータを参考とすべきであるが、LLNA の EC3 とヒトの感作性との間に良い相関が見られるため、LLNA が *in vitro* 皮膚感作性試験の評価のための基礎となっている。175 物質中 166 物質に LLNA のデータがあり、101 物質はヒトのデータが、そして 92 物質は LLNA とヒトの両方のデータがあった（図 2）。LLNA のデータがある 166 物質についての検討では、LLNA の結果に対して、感度 91%、特異度 65%、正確度 86%であった。

ヒトのカテゴリー1 から 4（Basketter ら（2014）[13]）の明らかな感作性物質の U-SENS™ の感度は 100%であった [9]。101 物質のヒトのデータに対しては、U-SENS™ は感度 100%、特異度 47%、正確度 77%であった。LLNA しかデータが無い 74 物質に対しては、感度 91%、特異度 76%、正確度 88%であった。

ヒトのデータがある場合は、まずヒトのデータから、Basketter ら（2014）[13]のカテゴリー1 から 4 までを感作性物質とし、クラス 5 と 6 を非感作性物質として判定した。ヒトのデータが無い場合は LLNA から判定した。結果は、感度 96%、特異度 55%、正確度 82%であった [9]。

U-SENS™ と同じく第三段階のイベントである、樹状細胞が活性化する際の細胞表面分子

[1*]値は、文献 9)は最新の予測モデル（APM）を用いているが、10)は旧予測モデル（OPM）を用いている。

の発現亢進を利用した *in vitro* 試験法である h-CLAT との比較を行った。対象物質は、L'Oreal 社が評価した 175 物質[9]のうち LLNA のデータがある 166 物質（APM による評価）と、h-CLAT の評価化合物 142 物質（すべて LLNA データあり）[14]の共通の化合物（104 物質）とした。この 104 物質について、LLNA に対する U-SENS™ の予測性は、感度 93%、特異度 61%、正確度 86%であり、h-CLAT の予測性は、感度 90%、特異度 68%、正確度 85%であった。したがってこの２つの試験法の LLNA に対する予測性は概ね同様と判断された。

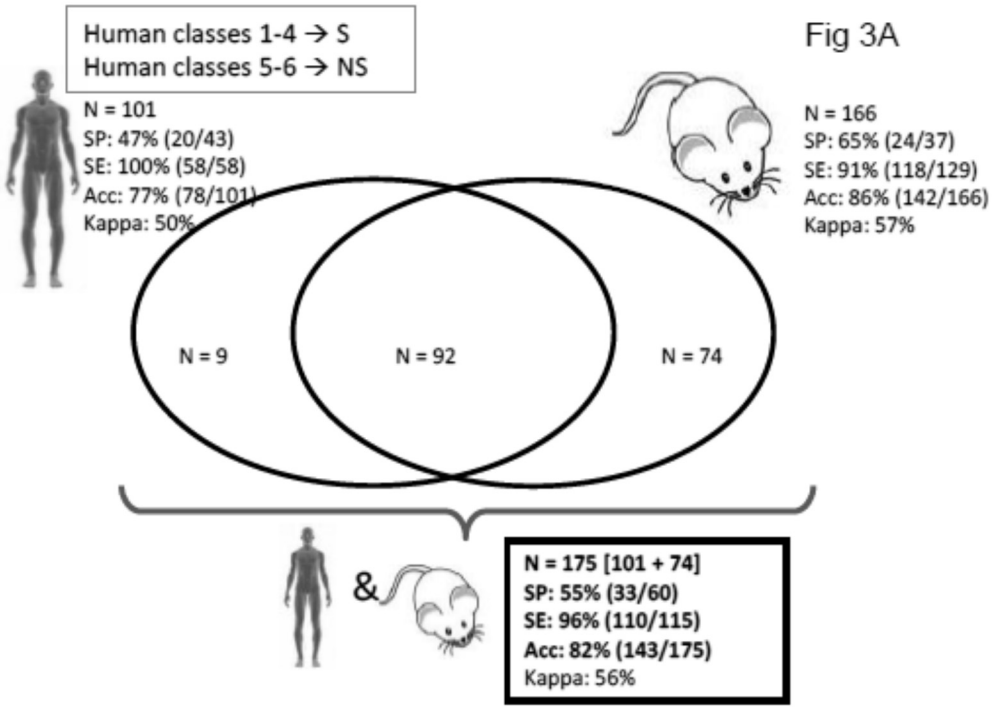


図 2　U-SENS™ の正確度のために用いた 175 物質のヒトと LLNA によるデータ（引用文献 9）より）

6. 評価可能な物質の範囲

L'Oreal 社が評価した 175 物質では、表 4 に示す通り、様々な化学物質の皮膚感作性の予測が可能であることが示されている[9]。なお、Ethyl vanillin (No. 169)、Polyethylene glycol (No. 174)、Benzyl alcohol (No. 175)の 3 物質は、OPM では陰性と判定されていたが[11]、表 4 に示すように APM では陽性と判定された。

表 4 Database of 175 chemicals used in the evaluation of the predictive performance ot the U-SENS™ assay

N°	Substance	CAS number	Human potency cat.[a]	LLNA[b]			U-SENS				Human versus LLNA / U-SENS[d]
				EC3 (%)	Potency cat.[c]	CLP cat.	Vehicle	Class	EC150 (µg/mL)	CV70 (µg/mL)	
1	Tetrachlorosalicylanilide	1154-59-2	1	0.04	Extreme	Cat. 1A	DMSO	POSITIVE	2	2	✓
2	2,4-Dinitrochlorobenzen DNCB	97-00-7	1	0.05	Extreme	Cat. 1A	DMSO	POSITIVE	1	1	✓
3	Diphenylcyclopropenone	886-38-4	1	0.05	Extreme	Cat. 1A	DMSO	POSITIVE	0.2	1.4	✓
4	Potassium dichromate	7778-50-9	1	0.08	Extreme	Cat. 1A	RPMI	POSITIVE	0.9	0.9	✓
5	1,4-Phenylenediamine	106-50-3	1	0.11	Strong	Cat. 1A	RPMI	POSITIVE	2	21	✓
6	Dimethyl fumarate	624-49-7	1	0,35	Strong	Cat. 1A	RPMI	POSITIVE	1	27	✓
7	Methylisothiazolinone (MI pure)	2682-20-4	1	0.4	Strong	Cat. 1A	RPMI	POSITIVE	1	6	✓
8	Glutaraldehyde (act. 50%)	111-30-8	2	0.1	Strong	Cat. 1A	RPMI	POSITIVE	83	>200	✓
9	Toluene diamine sulphate	615-50-9	2	0.2	Strong	Cat. 1A	RPMI	POSITIVE	2	12	✓
10	Gold chloride	13453-07-1	2	0.3	Strong	Cat. 1A	RPMI	POSITIVE	0.1	0.1	✓
11	Lauryl gallate	1166-52-5	2	0.3	Strong	Cat. 1A	DMSO	POSITIVE	0.7	0.7	✓
12	Propyl gallate	121-79-9	2	0.32	Strong	Cat. 1A	RPMI	POSITIVE	2	33	✓
13	2-Nitro-1,4-phenylenediamine	5307-14-2	2	0.4	Strong	Cat. 1A	DMSO	POSITIVE	5	115	✓
14	2-aminophenol	95-55-6	2	0.4	Strong	Cat. 1A	DMSO	POSITIVE	0.1	8	✓
15	Formaldehyde (act. 37%)	50-00-0	2	0.4	Strong	Cat. 1A	DMSO	POSITIVE	6	6	✓
16	Methyl heptine carbonate	111-12-6	2	0.5	Strong	Cat. 1A	DMSO	POSITIVE	39	70	✓
17	Methyldibromo glutaronitrile	35691-65-7	2	0.9	Strong	Cat. 1A	DMSO	POSITIVE	3	6	✓
18	Isoeugenol	97-54-1	2	1.2	Moderate	Cat. 1A	DMSO	POSITIVE	45	115	✓
19	Glyoxal (act. 40%)	107-22-2	2	1.4	Moderate	Cat. 1A	RPMI	POSITIVE	33	93	✓
20	Cinnamic aldehyde	104-55-2	2	2	Moderate	Cat. 1B	DMSO	POSITIVE	1	8	✓
21	Diethyl maleate	141-05-9	2	2.1	Moderate	Cat. 1B	DMSO	POSITIVE	7	30	✓
22	3-Dimethylaminopropylamine	109-55-7	2	2.2	Moderate	Cat. 1B	RPMI	POSITIVE	103	>200	✓
23	1,2-Benzisothiazolin-3-one	2634-33-5	2	2.3	Moderate	Cat. 1B	DMSO	POSITIVE	0.4	3	✓
24	Thioglycerol	96-27-5	2	3.6	Moderate	Cat. 1B	RPMI	POSITIVE	88	>200	✓
25	Lyral	31906-04-4	2	17.1	Weak	Cat. 1B	DMSO	POSITIVE	6	24	✓
26	Metol	55-55-0	3	0.8	Strong	Cat. 1A	RPMI	POSITIVE	0.3	3	✓
27	1,4-Dihydroquinone	123-31-9	3	0.11	Strong	Cat. 1A	RPMI	POSITIVE	1	6	✓
28	Chlorpromazine	50-53-3	3	0.14	Strong	Cat. 1A	DMSO	POSITIVE	6	6	✓
29	Benzoyl peroxide	94-36-0	3	0.3	Strong	Cat. 1A	DMSO	POSITIVE	27	33	✓
30	Hydroxyethyl acrylate	868-77-9	3	1.4	Moderate	Cat. 1A	RPMI	POSITIVE	40	>200	✓
31	Bisphenol A-diglycidyl ether	1675-54-3	3	1.5	Moderate	Cat. 1A	DMSO	POSITIVE	10	26	✓
32	2-Mercaptobenzothiazole	149-30-4	3	1.7	Moderate	Cat. 1A	DMSO	POSITIVE	40	80	✓
33	Ethylene diamine	107-15-3	3	2.2	Moderate	Cat. 1B	RPMI	POSITIVE	16	58	✓
34	Farnesol	4602-84-0	3	4.8	Moderate	Cat. 1B	DMSO	POSITIVE	8	34	✓
35	Phenyl benzoate	93-99-2	3	5.2	Moderate	Cat. 1B	DMSO	POSITIVE	101	>200	✓
36	Tetramethylthiuramdisulfide	137-26-8	3	6	Moderate	Cat. 1B	DMSO	POSITIVE	0.1	3	✓
37	Citral	5392-40-5	3	13	Weak	Cat. 1B	DMSO	POSITIVE	4	13	✓
38	Eugenol	97-53-0	3	13	Weak	Cat. 1B	DMSO	POSITIVE	29	142	✓
39	Abietic acid	514-10-3	3	15	Weak	Cat. 1B	DMSO	POSITIVE	37	66	✓
40	Cinnamic alcohol	104-54-1	3	21	Weak	Cat. 1B	DMSO	POSITIVE	21	110	✓
41	Imidazolidinyl urea	39236-46-9	3	24	Weak	Cat. 1B	RPMI	POSITIVE	11	28	✓
42	Coumarin	91-64-5	3	30	Weak	Cat. 1B	DMSO	POSITIVE	152	>200	✓
43	Butyl glycidyl ether	2426-08-6	3	31	Weak	Cat. 1B	RPMI	POSITIVE	149	>200	✓
44	Chlorhexidine gluconate	55-56-1	4	na	na	na	DMSO	POSITIVE	4	4	✓
45	Bronopol	52-51-7	4	na	na	na	RPMI	POSITIVE	2	6	✓
46	Hexyl salicylate	6259-76-3	4	0.18	Strong	Cat. 1A	DMSO	POSITIVE	27	27	✓
47	Iodopropynyl butylcarbamate	55406-53-6	4	0.87	Strong	Cat. 1A	DMSO	POSITIVE	2	4	✓
48	Resorcinol	108-46-3	4	5.5	Moderate	Cat. 1B	RPMI	POSITIVE	5	173	✓
49	Amyl cinnamic aldehyde	122-40-7	4	11	Weak	Cat. 1B	DMSO	POSITIVE	13	27	✓
50	Lillial	80-54-6	4	19	Weak	Cat. 1B	DMSO	POSITIVE	9	34	✓
51	Hydroxycitronellal	107-75-5	4	20	Weak	Cat. 1B	DMSO	POSITIVE	6	9	✓
52	Benzocaine	94-09-7	4	22	Weak	Cat. 1B	DMSO	POSITIVE	103	>200	✓
53	Amylcinnamyl alcohol	101-85-9	4	25	Weak	Cat. 1B	DMSO	POSITIVE	15	40	✓
54	Geraniol	106-24-1	4	26	Weak	Cat. 1B	RPMI	POSITIVE	54	134	✓
55	Linalool	78-70-6	4	30	Weak	Cat. 1B	DMSO	POSITIVE	41	>200	✓
56	Ethyleneglycol dimethacrylate	97-90-5	4	35	Weak	Cat. 1B	DMSO	POSITIVE	81	>200	✓
57	Aniline	62-53-3	4	89	Weak	Cat. 1B	RPMI	POSITIVE	63	>200	✓
58	Methylmethacrylate	80-62-6	4	90	Weak	Cat. 1B	DMSO	POSITIVE	157	>200	✓
59	Anethole	104-46-1	5	2.3	Moderate	Cat. 1B	DMSO	POSITIVE	>200	>200	✓
60	Benzyl salicylate	118-58-1	5	2.9	Moderate	Cat. 1B	DMSO	POSITIVE	66	66	✓

61	Anisyl alcohol	105-13-5	5	5.9	Moderate	Cat. 1B		DMSO	NEGATIVE	>200	>200	M
62	Hexyl cinnamic aldehyde	101-86-0	5	8	Moderate	Cat. 1B		DMSO	POSITIVE	5	22	✓
63	Benzyl benzoate	120-51-4	5	17	Weak	Cat. 1B		DMSO	POSITIVE	>200	>200	✓
64	Pentachlorophenol	87-86-5	5	20	Weak	Cat. 1B		DMSO	POSITIVE	21	21	✓
65	Benzaldehyde	100-52-7	5	25	Weak	Cat. 1B		DMSO	POSITIVE	85	175	✓
66	Diethanolamine	111-42-2	5	40	Weak	Cat. 1B		RPMI	POSITIVE	27	>200	✓
67	Isopropyl myristate	110-27-0	5	44	Weak	Cat. 1B		DMSO	NEGATIVE	>200	>200	M
68	Citronellol	106-22-9	5	43.5	Weak	Cat. 1B		DMSO	POSITIVE	25	>200	✓
69	Limonene (not oxidised)	138-86-3	5	69	Weak	Cat. 1B		DMSO	POSITIVE	26	43	✓
70	Pyridine	110-86-1	5	72	Weak	Cat. 1B		RPMI	NEGATIVE	>200	>200	M
71	Methyl salicylate	119-36-8	5	NC	NS	no cat.		DMSO	NEGATIVE	>200	>200	L&M
72	Vanillin	121-33-5	5	NC	NS	no cat.		DMSO	POSITIVE	50	>200	L
73	4-Aminobenzoic acid	150-13-0	5	NC	NS	no cat.		DMSO	NEGATIVE	>200	>200	L&M
74	Propyl paraben	94-13-3	5	NC	NS	no cat.		RPMI	POSITIVE	24	122	L
75	Benzalkonium chloride	8001-54-5	5	NC	NS	no cat.		RPMI	POSITIVE	0.1	1	L
76	Hydrocortisone	50-23-7	5	NC	NS	no cat.		DMSO	NEGATIVE	>200	>200	L&M
77	Propylene glycol	57-55-6	5	NC	NS	no cat.		RPMI	NEGATIVE	>200	>200	L&M
78	Isopropanol	67-63-0	5	NC	NS	no cat.		RPMI	NEGATIVE	>200	>200	L&M
79	Phenoxyethanol	122-99-6	5	NC	NS	no cat.		DMSO	POSITIVE	176	>200	L
80	Sorbic acid	110-44-1	5	na	na	na		DMSO	POSITIVE	115	>200	✓
81	Triclosan	3380-34-5	5	na	na	na		DMSO	POSITIVE	4	5	✓
82	Triethanolamine	102-71-6	5	na	na	na		RPMI	POSITIVE	189	>200	✓
83	Butylene glycol	107-88-0	5	na	na	na		RPMI	NEGATIVE	>200	>200	M
84	Cetrimide	57-09-0	5	na	na	na		RPMI	POSITIVE	0.8	0.8	✓
85	Tocopherol	59-02-9	6	7.4	Moderate	Cat. 1B		RPMI	NEGATIVE	>200	>200	L
86	Sodium lauryl sulfate	151-21-3	6	14	Weak	Cat. 1B		RPMI	POSITIVE	55	76	L&M
87	DMSO	67-68-5	6	72	Weak	Cat. 1B		RPMI	NEGATIVE	>200	>200	L
88	Xylene	1330-20-7	6	96	Weak	Cat. 1B		DMSO	NEGATIVE	>200	>200	L
89	Salicylic acid	69-72-7	6	NC	NS	no cat.		DMSO	NEGATIVE	>200	>200	✓
90	Octanoic acid	124-07-2	6	NC	NS	no cat.		RPMI	POSITIVE	70	>200	M
91	Hexane	110-54-3	6	NC	NS	no cat.		RPMI	NEGATIVE	>200	>200	✓
92	Diethyl phthalate	84-66-2	6	NC	NS	no cat.		DMSO	POSITIVE	121	>200	M
93	Lactic acid	50-21-5	6	NC	NS	no cat.		RPMI	NEGATIVE	>200	>200	✓
94	Glycerol/Glycerin	56-81-5	6	NC	NS	no cat.		RPMI	NEGATIVE	>200	>200	✓
95	1-Butanol	71-36-3	6	NC	NS	no cat.		RPMI	NEGATIVE	>200	>200	✓
96	Tween 80	9005-65-6	6	NC	NS	no cat.		RPMI	POSITIVE	9	200	M
97	Phenol	108-95-2	6	NC	NS	no cat.		DMSO	POSITIVE	22	102	M
98	Dextran	3371-50-4	6	NC	NS	no cat.		RPMI	NEGATIVE	>200	>200	✓
99	Diethyl toluamide	94271-03-1	6	NC	NS	no cat.		DMSO	POSITIVE	53	>200	M
100	Sorbitol	50-70-4	6	na	na	na		RPMI	NEGATIVE	>200	>200	✓
101	Glucose	50-99-7	6	na	na	na		RPMI	NEGATIVE	>200	>200	✓
102	2,4-Dinitrofluorobenzene	70-34-8	na	0.032	Extreme	Cat. 1A		RPMI	POSITIVE	1	4	-
103	Oxazolone	15646-46-5	na	0.003	Extreme	Cat. 1A		DMSO	POSITIVE	145	>200	-
104	MCI/MI (act. 1.5%)	55965-84-9	na	0.005	Extreme	Cat. 1A		RPMI	POSITIVE	11	34	-
105	7,12-Dimethylbenz[α]anthracene	57-97-6	na	0.006	Extreme	Cat. 1A		RPMI	POSITIVE	47	>200	-
106	p-Benzoquinone	106-51-4	na	0.01	Extreme	Cat. 1A		RPMI	POSITIVE	2	15	-
107	3-Methylcatechol	488-17-5	na	0.02	Extreme	Cat. 1A		RPMI	POSITIVE	0.3	4	-
108	Bandrowski's base	20048-27-5	na	0.04	Extreme	Cat. 1A		DMSO	POSITIVE	1	3	-
109	4-Nitrobenzyl bromide	100-11-8	na	0.05	Extreme	Cat. 1A		DMSO	POSITIVE	0.4	0.4	-
110	Methylnitrosourea	684-93-5	na	0.05	Extreme	Cat. 1A		RPMI	POSITIVE	13	>200	-
111	Cyanuric chloride	108-77-0	na	0.09	Extreme	Cat. 1A		RPMI	POSITIVE	15	68	-
112	p-aminophenol	123-30-8	na	-	Strong	Cat. 1A		RPMI	POSITIVE	2	6	-
113	Phthalic anhydride	85-44-9	na	0.16	Strong	Cat. 1A		DMSO	NEGATIVE	>200	>200	D
114	Benzyl bromide	100-39-0	na	0.2	Strong	Cat. 1A		DMSO	POSITIVE	8	15	-
115	2,4,6-Trinitrobenzenesulfonic acid	2508-19-2	na	0.36	Strong	Cat. 1A		RPMI	POSITIVE	10	>200	-
116	3-Phenylenediamine	108-45-2	na	0.49	Strong	Cat. 1A		RPMI	POSITIVE	4	137	-
117	CD3	25646-71-3	na	0.6	Strong	Cat. 1A		RPMI	POSITIVE	1	4	-
118	Squaric acid diethyl ester	5231-87-8	na	0.9	Strong	Cat. 1A		DMSO	POSITIVE	32	71	-
119	1-Phenyl-1,2-propanedione	579-07-7	na	1.3	Moderate	Cat. 1A		DMSO	POSITIVE	35	77	-
120	1-Naphthol	90-15-3	na	1.3	Moderate	Cat. 1A		DMSO	POSITIVE	3	31	-
121	2-Hydroxyethyl acrylate	818-61-1	na	1.4	Moderate	Cat. 1A		RPMI	POSITIVE	2	11	-
122	Nonanoyl chloride	764-85-2	na	1.8	Moderate	Cat. 1A		DMSO	POSITIVE	22	46	-
123	N,N-bis(2-hydroxyethyl)-p- phenylenediamine sulfate	54381-16-7	na	1.04	Moderate	Cat. 1A		RPMI	POSITIVE	8	29	-
124	Methyl-2-nonynoate	111-80-8	na	2.5	Moderate	Cat. 1B		DMSO	POSITIVE	18	105	-
125	3,3,5-trimethylhexanoyl chloride	36727-29-4	na	2.7	Moderate	Cat. 1B		DMSO	POSITIVE	17	48	-
126	Phenylacetaldehyde	122-78-1	na	3	Moderate	Cat. 1B		DMSO	POSITIVE	7	17	-
127	3-Aminophenol	591-27-5	na	3.2	Moderate	Cat. 1B		DMSO	POSITIVE	11	128	-
128	diethyl sulfate	64-67-5	na	3.3	Moderate	Cat. 1B		RPMI	POSITIVE	22	>200	-
129	Benzylideneacetone	122-57-6	na	3.7	Moderate	Cat. 1B		RPMI	POSITIVE	3	7	-
130	3-Propylidenephthalide	17369-59-4	na	3.7	Moderate	Cat. 1B		DMSO	POSITIVE	29	>200	-

131	Squaric acid	2892-51-5	na	4.3	Moderate	Cat. 1B	RPMI	POSITIVE	63	>200	-
132	a-Methyl cinnamic aldehyde	101-39-3	na	4.5	Moderate	Cat. 1B	DMSO	POSITIVE	9	>200	-
133	Nickel sulfate	10101-97-0	na	4.8	Moderate	Cat. 1B	RPMI	POSITIVE	16	55	-
134	trans-2-Hexenal	6728-26-3	na	5.5	Moderate	Cat. 1B	RPMI	POSITIVE	4	51	-
135	3,4-Dihydrocoumarin	119-84-6	na	5.6	Moderate	Cat. 1B	DMSO	NEGATIVE	>200	>200	D
136	2-Methoxy-4-methyl-phenol	93-51-6	na	5.8	Moderate	Cat. 1B	DMSO	POSITIVE	13	159	-
137	Diethylenetriamine	111-40-0	na	5.8	Moderate	Cat. 1B	RPMI	NEGATIVE	>200	>200	D
138	1-Bromoeicosane	4276-49-7	na	6.1	Moderate	Cat. 1B	RPMI	NEGATIVE	>200	>200	D
139	2-Phenylpropionaldehyde	93-53-8	na	6.3	Moderate	Cat. 1B	DMSO	POSITIVE	4	12	-
140	4-Chloroaniline	106-47-8	na	6.5	Moderate	Cat. 1B	DMSO	POSITIVE	1	182	-
141	Dihydroeugenol	2785-87-7	na	6.8	Moderate	Cat. 1B	DMSO	POSITIVE	47	122	-
142	Undec-10-enal	112-45-8	na	6.8	Moderate	Cat. 1B	RPMI	POSITIVE	9	29	-
143	12-Bromo-1-dodecanol	3344-77-2	na	6.9	Moderate	Cat. 1B	DMSO	POSITIVE	9	28	-
144	Safranal	116-26-7	na	7.5	Moderate	Cat. 1B	DMSO	POSITIVE	12	39	-
145	Methyl methanesulphonate	66-27-3	na	8.1	Moderate	Cat. 1B	RPMI	POSITIVE	2	33	-
146	Tween 21	9005-64-5	na	-	Weak	Cat. 1B	RPMI	POSITIVE	68	>200	-
147	Farnesal	19317-11-4	na	12	Weak	Cat. 1B	DMSO	POSITIVE	6	6	-
148	1-Bromohexane	111-25-1	na	10	Weak	Cat. 1B	DMSO	POSITIVE	>200	183	-
149	2-Ethylhexyl acrylate	103-11-7	na	10	Weak	Cat. 1B	DMSO	POSITIVE	15	200	-
150	2,3-Butanedion	431-03-8	na	11	Weak	Cat. 1B	RPMI	POSITIVE	11	103	-
151	Oxalic acid	144-62-7	na	15	Weak	Cat. 1B	DMSO	NEGATIVE	>200	>200	D
152	4-Allylanisole	140-67-0	na	18	Weak	Cat. 1B	DMSO	POSITIVE	98	>200	-
153	Benzyl cinnamate	103-41-3	na	18.4	Weak	Cat. 1B	DMSO	POSITIVE	177	>200	-
154	4,4,4-Trifluro-1-phenylbutane-1,3-dione	326-06-7	na	20	Weak	Cat. 1B	DMSO	POSITIVE	18	41	-
155	alpha-iso-Methylionone	127-51-5	na	21.8	Weak	Cat. 1B	DMSO	POSITIVE	22	36	-
156	Cyclamen aldehyde	103-95-7	na	22	Weak	Cat. 1B	DMSO	POSITIVE	30	117	-
157	Undecylenic acid	112-38-9	na	25	Weak	Cat. 1B	DMSO	POSITIVE	18	75	-
158	R(+)-Limonene	5989-27-5	na	69	Weak	Cat. 1B	RPMI	POSITIVE	30	>200	-
159	Tartaric acid	87-69-4	na	NC	NS	no cat.	RPMI	NEGATIVE	>200	>200	-
160	Chlorobenzene	108-90-7	na	NC	NS	no cat.	DMSO	NEGATIVE	>200	>200	-
161	Sulfanilic acid	121-57-3	na	NC	NS	no cat.	RPMI	NEGATIVE	>200	>200	-
162	4-Hydroxybenzoic acid	99-96-7	na	NC	NS	no cat.	DMSO	NEGATIVE	>200	>200	-
163	Saccharin	81-07-2	na	NC	NS	no cat.	RPMI	NEGATIVE	>200	>200	-
164	1-Bromobutane	109-65-9	na	NC	NS	no cat.	DMSO	NEGATIVE	>200	>200	-
165	6-Methyl coumarin	92-48-8	na	NC	NS	no cat.	DMSO	POSITIVE	94	187	D
166	Ethyl benzoylacetate	94-02-0	na	NC	NS	no cat.	DMSO	NEGATIVE	>200	>200	-
167	Vinylidene dichloride	75-35-4	na	NC	NS	no cat.	DMSO	NEGATIVE	>200	>200	-
168	Benzoic acid	65-85-0	na	NC	NS	no cat.	RPMI	NEGATIVE	>200	>200	-
169	Ethyl vanillin	121-32-4	na	NC	NS	no cat.	DMSO	POSITIVE	66	66	D
170	Sulfanilamide	63-74-1	na	NC	NS	no cat.	RPMI	NEGATIVE	>200	>200	-
171	Kanamycin (sulfate)	25389-94-0	na	NC	NS	no cat.	RPMI	NEGATIVE	>200	>200	-
172	Streptomycin sulfate	3810-74-0	na	NC	NS	no cat.	RPMI	NEGATIVE	>200	>200	-
173	Citric acid	77-92-9	na	NC	NS	no cat.	RPMI	NEGATIVE	>200	>200	-
174	Polyethylene glycol	25322-68-3	na	NC	NS	no cat.	RPMI	POSITIVE	73	>200	D
175	Benzyl alcohol	100-51-6	na	NC	NS	no cat.	DMSO	POSITIVE	176	>200	D

[a] Human Skin Sensitizing Potency category (Basketter et al., 2014) ; na: not available

[b] LLNA (TG429 and EU test method B.42 (OECD, 2010a; UN 2011)) ; na: not available

[c] LLNA potency category based on the EC3 value as proposed by Kimber et al. (2003).

[d] Human versus LLNA and/or U-SENS™ S/NS classifications: ✓: concordant hazard classifications between human, LLNA and U-SENS™; L: discordant hazard classifications between LLNA and human; M: discordant hazard classifications between U-SENS™ and human; -: concordant hazard classifications between LLNA and U-SENS™ without any human data available; D: discordant hazard classifications between LLNA and U-SENS™ without any human data available.

7. 有用性と限界

本法は細胞実験とフローサイトメトリーを組み合わせた試験法であり、細胞培養機器及びフローサイトメーターを保有し、その技術に習熟した施設であれば実施可能と思われる。

また、本法は動物を用いない *in vitro* の手法であり、科学的目的のために実施される動物実験に関し、「動物の愛護及び管理に関する法律」及び 3R の精神と合致している。さらに U-SENS™ 及び LLNA 実施の際に必要となる消耗品費は、LLNA では 1 アッセイ当たり約 10 万円であるのに対し、U-SENS™ では約 1.8 万円と試算された。試験期間も LLNA より短期間で実施可能であることから、試験法として迅速性・経済性の面から有用と思われる。

技術的限界として、液相での反応を必要とする試験系であるため、培地に 50 mg/mL、これに所定の濃度で不溶の場合は、DMSO に 50 mg/mL の濃度で溶解あるいは安定的に分散する必要がある（ただし、科学的根拠があれば他の溶媒も使用可能とされている）。

また、本法はフローサイトメーターを用いる手法であり、蛍光を有する物質も評価は可能であるが、FITC や PI と同一波長に強い蛍光を有する物質は測定を妨害する可能性がある。更に、過度の細胞毒性を有する物質は細胞の構造変化を引き起こし正しく評価されない可能性がある。また、他の細胞を用いる試験系と同様に、揮発性物質は飛散による物質のロスや近隣のウェルへのクロスコンタミを起こすため、適切に評価されない可能性がある。

プレハプテン（酸化により活性化される物質）やプロハプテン（P450 等による代謝活性化を必要とする物質）に関しては、これまでの検討では正しく判定された（プレハプテン 4 種及びプロハプテン 7 種、OPM と APM で判定結果は同じ）[11]。しかし、ウェル中での化学的な酸化の進行については未知であり、また、本細胞の薬物代謝能は限定的であるため、未検討のプロハプテンやプレハプテンについては偽陰性を生じる可能性もある [15]。

界面活性剤のような細胞膜に影響を与える物質は、CD86 発現の非特異的増大により偽陽性となり得るため、このような物質が陽性と判定された場合は注意が必要である。

また、混合物に関しては、本法の適用例はこれまで 1 例のみであり（OPM と APM で判定結果は同じ）、適用可能性に関する十分な情報は得られていない [11]。従って、混合物に対する本法の適用については注意が必要である。

本法は、IATA (Integrated Approaches to Testing and Assessment)において他の試験法と組み合わせることにより、感作性物質と非感作性物質との区別に使用することが可能である [16]。しかし、強度感作性物質（UN GHS 1A 分類）よりも軽度～中等度感作性物質（UN GHS 1B 分類）で偽陰性の判定が生じやすい傾向にあるため、本法単独での感作性強度分類や UN GHS のサブカテゴリー分類への応用には適さない。

8. 結論

U-SENS^{TM} は、感作性発現機序における第三段階のイベントである樹状細胞が活性化する際の細胞表面分子の発現亢進を利用した *in vitro* 試験法である。ヒト組織球性リンパ腫細胞株である U-937 細胞を用い、活性化に伴い細胞表面での発現量が増加する CD86 を測定する試験法で、化学物質の感作性を判断する上で重要な情報を与えてくれる。

マウスを用いる LLNA の 1/5 程度の消耗品費で実施可能と試算され、試験期間も LLNA に比べ短期間であるため、試験法として迅速性・経済性の面から有用性は高いと思われる。本法は、細胞実験とフローサイトメトリーを組み合わせた試験法であり、細胞培養機器およびフローサイトメーターを保有し、その技術に習熟した施設であれば実施可能と考えられる。なお、細胞の反応性の確認と陽性対照および媒体対照の測定で発生するデータを基にヒストリカルなデータベースを作成・維持し、試験系の再現性を保証するために用いる必要がある。

本試験法のバリデーション試験において、15 物質を用いて実施された施設内再現性は、60～100％であり、平均は 90％であった。OECD 専門家会議における議論で、感作性予測モデルが一部改訂され(APM)、判定の煩雑さが解消された。APM で評価すると、ヒトに対す

る感作性の有無の分類で、Basketter らの報告に基づくヒトのクラス 5 と 6 を感作性無しとした場合、感度 100%、特異度 47%、正確度 77%であったが、クラス 6 のみを感作性無しとした場合は、感度 89%、特異度 65%、正確度 85%であった（101 物質対象）。LLNA のデータがある 166 物質についての検討では、LLNA の結果に対して、感度 91%、特異度 65%、正確度 86%であった。この 166 物質を見る限り、本法は様々な化学物質の皮膚感作性の予測が可能であることが示されている。

U-SENS^{TM} で評価した 175 物質と h-CLAT で評価した 142 物質のうち、LLNA のデータがある共通物質は 104 物質であった。この 104 物質について、感度、特異度、正確度を比較したところ、両者での予測性は概ね同様と判断された。

U-SENS^{TM} の試験法は、液相での反応を必要とする試験系であるため、培地あるいは DMSO に 50 mg/mL の濃度で溶解あるいは安定的に分散するものであれば試験が可能である。

界面活性剤等の細胞膜の構造変化を引き起こすような物質、測定に使用する FITC や PI と同一波長に強い蛍光を有する物質、揮発性を有する物質などでは、適切に評価されない可能性がある。プレハプテンやプロハプテンに関しては、本法開発の段階では正しく判定されたとしているが、ウェル中での化学的な酸化の進行については未知であり、また、本細胞の薬物代謝能は限定的であるため、未検討のプロハプテンやプレハプテンについては偽陰性を生じる可能性もある。さらに、本法は混合物に関しては適用可能性に関する十分な情報は得られていない、従って、混合物に対する本法の適用については注意が必要である。

本試験法は、強度感作性物質（UN GHS 1A 分類）に比べて、軽度から中等度感作性物質（UN GHS 1B 分類）で偽陰性の判定が生じやすい傾向にあるため、本法単独での感作性強度分類や UN GHS のサブカテゴリー分類への利用には適さない。本試験法に用いる細胞の薬物代謝能は限定的であるため、活性化に代謝系を必要とする化学物質では、正しくその感作性が検出されない可能性がある。

本委員会は、上記の本試験法の様々な限界を勘案すると、本試験法単独では皮膚感作性の判定は不十分で有り、証拠の重み付けや他の試験法との組合せで用いることを推奨する。

引用文献

1) http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788
2) http://www.reach-serv.com/index.php?option=com_content&task=view&id=106&Itemid=115
3) http://data.europa.eu/eli/reg/2009/1223/2016-08-12
4) EC EURL ECVAM. (2016). ESAC Opinion No. 2016-03 on the L'Oréal-coordinated study on the transferability and reliability of the U-SENS™ test method for skin sensitisation testing. EUR 28178 14 EN; doi 10.2787/815737. Available at: http://publications.jrc.ec.europa.eu/repository/handle/JRC103705
5) OECD (2014), The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins, OECD Publishing, Paris, http://dx.doi.org/10.1787/9789264221444-en
6) Sundström C, Nilsson K (1976), Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer. 17(5):565-77.
7) Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. (1980), Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer. 26(2):171-6.
8) W. Chanput, V. Peters, H. Wichers (2015), THP-1 and U937 Cells, In The Impact of Food Bio-Actives on Gut Health, K. Verhoeckx et al. (eds.), Springer Nature
9) EURL ECVAM (2016) Test Submission Template. U-SENS Test Submission Template. European Commission
10) Alépée N, Piroird C, Aujoulat M, Dreyfuss S, Hoffmann S, Hohenstein A, Meloni M, Nardelli L, Gerbeix C, Cotovio J (2015). Prospective multicentre study of the U-SENS test method for skin sensitization testing. Toxicol In Vitro 30(1 Pt B):373-82.
11) Piroird C, Ovigne JM, Rousset F, Martinozzi-Teissier S, Gomes C, Cotovio J, Alépée N (2015). The Myeloid U937 Skin Sensitization Test (U-SENS) addresses the activation of dendritic cell event in the adverse outcome pathway for skin sensitization. Toxicol In Vitro 29(5):901-16.
12) Aptula and Roberts (2006) Mechanistic applicability domains for nonanimal-based predicton of toxicological end points: general principles and application to reactive toxicity. Chem Res Toxicol; 19: 1097-1105.
13) Basketter, DA; Alepee, N; Ashikaga, T; Barroso, J; Gilmour, N; Goebel, C; Hibatallah, J; Hoffmann, S; Kern, P; Martinozzi-Teissier, S; Maxwell, G; Reisinger, K; Sakaguchi, H; Schepky, A; Tailhardat, M; Templier (2014) Categorization of Chemicals According to Their Relative Human Skin Sensitizing Potency. Dermatitis 25:11-21.
14) EC EURL ECVAM (2015), Re-analysis of the within and between laboratory reproducibility of the human Cell Line Activation Test (h-CLAT). Accessible at: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/eurl-ecvam-recommendation-on-the-human-cell-line-activation-test-h-clat-for-skin-sensitisation-testing
15) Fabian E, Vogel D, Blatz V, Ramirez T, Kolle S, Eltze T, van Ravenzwaay B, Oesch F, Landsiedel R (2013) Xenobiotic metabolizing enzyme activities in cells used for testing skin

sensitization in vitro. Arch. Toxicol., 87, 1683-1696.
16) Guidance Document on the Reporting of Defined Approaches and Individual Information Sources to be Used within Integrated Approaches to Testing and Assessment (IATA) for Skin Sensitisation, Annex 1 and Annex 2. http://www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm

OECD/OCDE 442E
Adopted:
25 June 2018

KEY EVENT-BASED TEST GUIDELINE

IN VITRO SKIN SENSITISATION ASSAYS ADDRESSING THE KEY EVENT ON ACTIVATION OF DENDRITIC CELLS ON THE ADVERSE OUTCOME PATHWAY FOR SKIN SENSITISATION

GENERAL INTRODUCTION

Activation of dendritic cells Key Event based Test Guideline

1. A skin sensitiser refers to a substance that will lead to an allergic response following skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). There is general agreement on the key biological events underlying skin sensitisation. The current knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised as an Adverse Outcome Pathway (AOP) (2), starting with the molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. In this instance, the molecular initiating event (i.e. the first key event) is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells (DC), typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell activation and proliferation, which is indirectly assessed in the murine Local Lymph Node Assay (LLNA) (3).

2. This Test Guideline (TG) describes *in vitro* assays that address mechanisms described under the Key Event on activation of dendritic cells of the AOP for skin sensitisation (2). The TG comprises test methods to be used for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (1).

The test methods described in this TG are:

- Human Cell Line Activation test (h-CLAT)
- U937 cell line activation Test (U-SENS™)
- Interleukin-8 Reporter Gene Assay (IL-8 Luc assay)

In accordance with the decision of the Council on a delegation of authority to amend Annex I of the decision of the council on the Mutual Acceptance of Data in the assessment of chemicals [C(2018)49], this Guideline was amended by the OECD's Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology by written procedure on 25 June 2018.

3. The test methods included in this Test Guideline may differ in relation to the procedure used to generate the data and the readouts measured but can be used indiscriminately to address countries' requirements for test results on the Key Event on activation of dendritic cells of the AOP for skin sensitisation while benefiting from the Mutual Acceptance of Data.

Background and principles of the test methods included in the Key Event based Test Guideline

4. The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods that use guinea-pigs, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman, and the Buehler Test (TG 406) (4), assess both the induction and elicitation phases of skin sensitisation. The murine tests, the LLNA (TG 429) (3) and its two non-radioactive modifications, LLNA: DA (TG 442 A) (5) and LLNA: BrdU-ELISA (TG 442 B) (6), all assess the induction response exclusively, and have also gained acceptance, since they provide an advantage over the guinea pig tests in terms of animal welfare together with an objective measurement of the induction phase of skin sensitisation.

5. Recently mechanistically-based *in chemico* and *in vitro* test methods addressing the first key event (OECD TG 442C; Direct Peptide Reactivity Assay (7)), and second key event (OECD TG 442D; ARE-Nrf2 Luciferase Test Method (8)) of the skin sensitisation AOP have been adopted for contributing to the evaluation of the skin sensitisation hazard potential of chemicals.

6. Test methods described in this TG either quantify the change in the expression of cell surface marker(s) associated with the process of activation of monocytes and DC following exposure to sensitisers (e.g. CD54, CD86) or the changes in IL-8 expression, a cytokine associated with the activation of DC. Skin sensitisers have been reported to induce the expression of cell membrane markers such as CD40, CD54, CD80, CD83, and CD86 in addition to induction of proinflammatory cytokines, such as IL-1β and TNF-α, and several chemokines including IL-8 (CXCL8) and CCL3 (9) (10) (11) (12), associated with DC activation (2).

7. However, as DC activation represents only one key event of the skin sensitisation AOP (2) (13), information generated with test methods measuring markers of DC activation alone may not be sufficient to conclude on the presence or absence of skin sensitisation potential of chemicals. Therefore data generated with the test methods described in this Test Guideline are proposed to support the discrimination between skin sensitisers (i.e. UN GHS Category 1) and non-sensitisers when used within Integrated Approaches to Testing and Assessment (IATA), together with other relevant complementary information, e.g. derived from in vitro assays addressing other key events of the skin sensitisation AOP as well as non-testing methods, including read-across from chemical analogues (13). Examples of the use of data generated with these methods within Defined Approaches, i.e. approaches standardised both in relation to the set of information sources used and in the procedure applied to the data to derive predictions, have been published (13) and can be employed as useful elements within IATA.

8. The test methods described in this Test Guideline cannot be used on their own, neither to sub-categorise skin sensitisers into subcategories 1A and 1B as defined by UN GHS (1), for authorities implementing these two optional subcategories, nor to predict potency for safety assessment decisions. However, depending on the regulatory framework, positive results generated with these methods may be used on their own to classify a chemical into UN GHS category 1.

9. The term "test chemical" is used in this Test Guideline to refer to what is being tested[1] and is not related to the applicability of the test methods to the testing of mono-constituent substances, multi-constituent substances and/or mixtures. Limited information is currently available on the applicability of the test methods to multi-constituent substances/mixtures (14) (15). The test methods are nevertheless technically applicable to the testing of multi-constituent substances and 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. Moreover, when testing multi-constituent substances or mixtures, consideration should be given to possible interference of cytotoxic constituents with the observed responses.

LITERATURE

1. United Nations UN (2015), Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Sixth revised edition. New York & Geneva: United Nations Publications. ISBN: 978-92-1-117006-1.

   Available at: [https://www.unece.org/trans/danger/publi/ghs/ghs_rev06/06files_e.html].

2. OECD (2012), The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168. Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2012)10/PART1&docLanguage=En

3. OECD (2010), The Local Lymph Node Assay. OECD Guideline for the Testing of Chemicals No. 429, Organisation for Economic Cooperation and Development, Paris. Available at: http://www.oecd.org/env/testguidelines

4. OECD (1992), Skin Sensitisation. OECD Guideline for the Testing of Chemicals No. 406, Organisation for Economic Cooperation and Development, Paris. Available at: http://www.oecd.org/env/testguidelines

5. OECD (2010), Skin Sensitisation: Local Lymph Node Assay: DA. OECD Guideline for the Testing of Chemicals No. 442A, Organisation for Economic Cooperation and Development, Paris. Available at: http://www.oecd.org/env/testguidelines

6. OECD (2010), Skin sensitisation: Local Lymph Node Assay: BrdU-ELISA. OECD Guideline for the Testing of Chemicals No. 442B, Organisation for Economic Cooperation and Development, Paris. Available at: http://www.oecd.org/env/testguidelines

7. OECD (2015), OECD Guideline for the Testing of Chemicals No. 442C: In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA). Paris, France: Organisation for Economic Cooperation and Development. Available at: http://www.oecd.org/env/testguidelines

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

8. OECD (2015), OECD Guideline for the Testing of Chemicals No. 442D: In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method. Paris, France: Organisation for Economic Cooperation and Development. Available at: http://www.oecd.org/env/testguidelines

9. Steinman RM. 1991. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9:271-96.

10. Caux C, Vanbervliet B, Massacrier C, Azuma M, Okumura K, Lanier LL, and Banchereau J. 1994. B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. J Exp Med 180:1841-7.

11. Aiba S, Terunuma A, Manome H, and Tagami H. 1997. Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. Eur J Immunol 27:3031-8.

12. Aiba S, Manome H, Nakagawa S, Mollah ZU, Mizuashi M, Ohtani T, Yoshino Y, and Tagami. H. 2003. p38 mitogen-activated protein kinase and extracellular signal-regulated kinases play distinct roles in the activation of dendritic cells by two representative haptens, NiCl2 and DNCB. J Invest Dermatol 120:390-8.

13. OECD (2016). Series on Testing & Assessment No. 256: Guidance Document On The Reporting Of Defined Approaches And Individual Information Sources To Be Used Within Integrated Approaches To Testing And Assessment (IATA) For Skin Sensitisation, Annex 1 and Annex 2. ENV/JM/HA(2016)29. Organisation for Economic Cooperation and Development, Paris. Available at: [https://community.oecd.org/community/iatass].

14. Ashikaga T, Sakaguchi H, Sono S, Kosaka N, Ishikawa M, Nukada Y, Miyazawa M, Ito Y, NishiyamaN, Itagaki H. (2010), A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). Altern. Lab. Anim. 38, 275-284.

15. Piroird, C., Ovigne, J.M., Rousset, F., Martinozzi-Teissier, S., Gomes, C., Cotovio, J., Alépée, N. (2015). The Myeloid U937 Skin Sensitization Test (U-SENS) addresses the activation of dendritic cell event in the adverse outcome pathway for skin sensitization. *Toxicol. In Vitro* 29, 901-916.

ANNEX I: *IN VITRO* SKIN SENSITISATION: HUMAN CELL LINE ACTIVATION TEST (H-CLAT)

INITIAL CONSIDERATIONS AND LIMITATIONS

1. The h-CLAT method quantifies changes in the expression of cell surface markers associated with the process of activation of monocytes and dendritic cells (DC) (i.e. CD86 and CD54), in the human monocytic leukaemia cell line THP-1, following exposure to sensitisers (1) (2). The measured expression levels of CD86 and CD54 cell surface markers are then used for supporting the discrimination between skin sensitisers and non-sensitisers.

2. The h-CLAT method has been evaluated in a European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)-coordinated validation study and subsequent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC). Considering all available evidence and input from regulators and stakeholders, the h-CLAT was recommended by EURL ECVAM (3) to be used as part of an IATA to support the discrimination between sensitisers and non-sensitisers for the purpose of hazard classification and labelling. Examples of the use of h-CLAT data in combination with other information are reported in the literature (4) (5) (6) (7) (8) (9) (10) (11).

3. The h-CLAT method proved to be transferable to laboratories experienced in cell culture techniques and flow cytometry analysis. The level of reproducibility in predictions that can be expected from the test method is in the order of 80% within and between laboratories (3) (12). Results generated in the validation study (13) and other published studies (14) overall indicate that, compared with LLNA results, the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers is 85% (N=142) with a sensitivity of 93% (94/101) and a specificity of 66% (27/41) (based on a re-analysis by EURL ECVAM (12) considering all existing data and not considering negative results for chemicals with a Log Kow greater than 3.5 as described in paragraph 4). False negative predictions with the h-CLAT are more likely to concern chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A) (4) (13) (15). Taken together, this information indicates the usefulness of the h-CLAT method to contribute to the identification of skin sensitisation hazards. However, the accuracy values given here for the h-CLAT as a stand-alone test method are only indicative, since the test method should be considered in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General introduction. Furthermore, when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in humans.

4. On the basis of the data currently available, the h-CLAT method was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined in *in vivo* studies) and physicochemical properties (3) (14) (15). The h-CLAT method is applicable to test chemicals soluble or that form a stable dispersion (i.e. a colloid or suspension in which the test chemical does not settle or separate from the solvent/vehicle into different phases) in an appropriate solvent/vehicle (see paragraph 14). Test chemicals with a Log Kow greater than 3.5 tend to produce false negative results (14). Therefore negative results with test chemicals with a Log Kow greater than 3.5 should not be considered. However, positive results obtained with test chemicals with a Log Kow greater than 3.5 could still be used to support the identification of the test chemical as a skin sensitiser. Furthermore, because of the limited metabolic capability of the cell line used (16) and because of the

experimental conditions, pro-haptens (i.e. substances requiring enzymatic activation for example via P450 enzymes) and pre-haptens (i.e. substances activated by oxidation) in particular with a slow oxidation rate may also provide negative results in the h-CLAT (15). Fluorescent test chemicals can be assessed with the h-CLAT (17), nevertheless, strong fluorescent test chemicals emitting at the same wavelength as fluorescein isothiocyanate (FITC) or as propidium iodide (PI), will interfere with the flow cytometric detection and thus cannot be correctly evaluated using FITC-conjugated antibodies or PI. In such a case, other fluorochrome-tagged antibodies or other cytotoxicity markers, respectively, can be used as long as it can be shown they provide similar results as the FITC-tagged antibodies (see paragraph 24) or PI (see paragraph 18) e.g. by testing the proficiency substances in Appendix II. In the light of the above, negative results should be interpreted in the context of the stated limitations and together with other information sources within the framework of IATA. In cases where there is evidence demonstrating the non-applicability of the h-CLAT method to other specific categories of test chemicals, it should not be used for those specific categories.

5. As described above, the h-CLAT method supports the discrimination between skin sensitisers from non-sensitisers. However, it may also potentially contribute to the assessment of sensitising potency (4) (5) (9) when used in integrated approaches such as IATA. Nevertheless, further work, preferably based on human data, is required to determine how h-CLAT results may possibly inform potency assessment.

6. Definitions are provided in Appendix I.

PRINCIPLE OF THE TEST

7. The h-CLAT method is an *in vitro* assay that quantifies changes of cell surface marker expression (i.e. CD86 and CD54) on a human monocytic leukemia cell line, THP-1 cells, following 24 hours exposure to the test chemical. These surface molecules are typical markers of monocytic THP-1 activation and may mimic DC activation, which plays a critical role in T-cell priming. The changes of surface marker expression are measured by flow cytometry following cell staining with fluorochrome-tagged antibodies. Cytotoxicity measurement is also conducted concurrently to assess whether upregulation of surface marker expression occurs at sub-cytotoxic concentrations. The relative fluorescence intensity of surface markers compared to solvent/vehicle control are calculated and used in the prediction model (see paragraph 26), to support the discrimination between sensitisers and non-sensitisers

DEMONSTRATION OF PROFICIENCY

8. Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency, using the 10 Proficiency Substances listed in Appendix II. Moreover, test method users should maintain an historical database of data generated with the reactivity checks (see paragraph 11) and with the positive and solvent/vehicle controls (see paragraphs 20-22), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

PROCEDURE

9. This test method is based on the h-CLAT DataBase service on ALternative Methods to animal experimentation (DB-ALM) protocol no. 158 (18) which represents the protocol used for the EURL ECVAM-coordinated validation study. It is recommended that this protocol is used when implementing and using the h-CLAT method in the laboratory. The following is a description of the main components and procedures for the h-CLAT method, which comprises two steps: *dose finding assay* and *CD86/CD54 expression measurement*.

Preparation of cells

10. The human monocytic leukaemia cell line, THP-1, should be used for performing the h-CLAT method. It is recommended that cells (TIB-202™) are obtained from a well-qualified cell bank, such as the American Type Culture Collection.

11. THP-1 cells are cultured, at 37°C under 5% CO_2 and humidified atmosphere, in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, 100 units/mL penicillin and 100 µg/mL streptomycin. The use of penicillin and streptomycin in the culture medium can be avoided. However, in such a case users should verify that the absence of antibiotics in the culture medium has no impact on the results, for example by testing the proficiency substances listed in Appendix II. In any case, in order to minimise the risk of contamination, good cell culture practices should be followed independently of the presence or not of antibiotics in the cell culture medium. THP-1 cells are routinely seeded every 2-3 days at the density of 0.1 to 0.2 × 10^6 cells/mL. They should be maintained at densities from 0.1 to 1.0 × 10^6 cells/mL. Prior to using them for testing, the cells should be qualified by conducting a reactivity check. The reactivity check of the cells should be performed using the positive controls, 2,4-dinitrochlorobenzene (DNCB) (CAS n. 97-00-7, ≥ 99% purity) and nickel sulfate ($NiSO_4$) (CAS n. 10101-97-0, ≥ 99% purity) and the negative control, lactic acid (LA) (CAS n. 50-21-5, ≥ 85% purity), two weeks after thawing. Both DNCB and $NiSO_4$ should produce a positive response of both CD86 and CD54 cell surface markers, and LA should produce a negative response of both CD86 and CD54 cell surface markers. Only the cells which passed the reactivity check are to be used for the assay. Cells can be propagated up to two months after thawing. Passage number should not exceed 30. The reactivity check should be performed according to the procedures described in paragraphs 20-24.

12. For testing, THP-1 cells are seeded at a density of either 0.1 × 10^6 cells/mL or 0.2 × 10^6 cells/mL, and pre-cultured in culture flasks for 72 hours or for 48 hours, respectively. It is important that the cell density in the culture flask just after the pre-culture period be as consistent as possible in each experiment (by using one of the two pre-culture conditions described above), because the cell density in the culture flask just after pre-culture could affect the CD86/CD54 expression induced by allergens (19). On the day of testing, cells harvested from culture flask are resuspended with fresh culture medium at 2 × 10^6 cells/mL. Then, cells are distributed into a 24 well flat-bottom plate with 500 µL (1 × 10^6 cells/well) or a 96-well flat-bottom plate with 80 µL (1.6 × 10^5 cells/well).

Dose finding assay

13. A *dose finding assay* is performed to determine the CV75, being the test chemical concentration that results in 75% cell viability (CV) compared to the solvent/vehicle control. The CV75 value is used to determine the concentration of test chemicals for the *CD86/CD54 expression measurement* (see paragraphs 20-24).

Preparation of test chemicals and control substances

14. The test chemicals and control substances are prepared on the day of testing. For the h-CLAT method, test chemicals are dissolved or stably dispersed (see also paragraph 4) in saline or medium as first solvent/vehicle options or dimethyl sulfoxide (DMSO, ≥ 99% purity) as a second solvent/vehicle option if the test chemical is not soluble or does not form a stable dispersion in the previous two solvents/vehicles, to final concentrations of 100 mg/mL (in saline or medium) or 500 mg/mL (in DMSO). Other solvents/vehicles than those described above may be used if sufficient scientific rationale is provided. Stability of the test chemical in the final solvent/vehicle should be taken into account.

15. Starting from the 100 mg/mL (in saline or medium) or 500 mg/mL (in DMSO) stock solutions of the test chemicals, the following dilution steps should be taken:

- For saline or medium as solvent/vehicle: Eight stock solutions (eight concentrations) are prepared, by two-fold serial dilutions using the corresponding solvent/vehicle. These stock solutions are then further diluted 50-fold into culture medium (working solutions). If the top final concentration in the plate of 1000 μg/mL is non-toxic, the maximum concentration should be re-determined by performing a new cytotoxicity test. The final concentration in the plate should not exceed 5000 μg/mL for test chemicals dissolved or stably dispersed in saline or medium.
- For DMSO as solvent/vehicle: Eight stock solutions (eight concentrations) are prepared, by two-fold serial dilutions using the corresponding solvent/vehicle. These stock solutions are then further diluted 250-fold into culture medium (working solutions).The final concentration in plate should not exceed 1000 μg/mL even if this concentration is non-toxic.

The working solutions are finally used for exposure by adding an equal volume of working solution to the volume of THP-1 cell suspension in the plate (see also paragraph 17) to achieve a further two-fold dilution (usually, the final range of concentrations in the plate is 7.81–1000 μg/mL).

16. The solvent/vehicle control used in the h-CLAT method is culture medium (for test chemicals solubilised or stably dispersed (see paragraph 4) either with medium or saline) or DMSO (for test chemicals solubilised or stably dispersed in DMSO) tested at a single final concentration in the plate of 0.2%. It undergoes the same dilution as described for the working solutions in paragraph 15.

Application of test chemicals and control substances

17. The culture medium or working solutions described in paragraphs 15 and 16 are mixed 1:1 (v/v) with the cell suspensions prepared in the 24-well or 96-well flat-bottom plate (see paragraph 12). The treated plates are then incubated for 24±0.5 hours at 37°C under 5% CO_2. Care should be taken to avoid evaporation of volatile test chemicals and cross-contamination between wells by test chemicals, e.g. by sealing the plate prior to the incubation with the test chemicals (20).

Propidium iodide (PI) staining

18. After 24±0.5 hours of exposure, cells are transferred into sample tubes and collected by centrifugation. The supernatants are discarded and the remaining cells are resuspended with 200 μL (in case of 96-well) or 600 μL (in case of 24-well) of a phosphate buffered saline containing 0.1% bovine serum albumin (staining buffer). 200 μL of cell suspension is transferred into 96-well round-bottom plate (in case of 96-well) or micro tube (in case of 24-well) and washed twice with 200 μL (in case of 96-well) or 600 μL (in case of 24-well) of staining buffer. Finally, cells are resuspended in staining buffer (e.g. 400 μL) and PI solution (e.g. 20 μL) is added (for example, final concentration of PI is 0.625 μg/mL). Other cytotoxicity markers, such as 7-Aminoactinomycin D (7-AAD), Trypan blue or others may be used if the alternative stains can be shown to provide similar results as PI, for example by testing the proficiency substances in Appendix II.

Cytotoxicity measurement by flow cytometry and estimation of CV75 value

19. The PI uptake is analysed using flow cytometry with the acquisition channel FL-3. A total of 10,000 living cells (PI negative) are acquired. The cell viability can be calculated using the following equation by the cytometer analysis program. When the cell viability is low, up to 30,000 cells including dead cells should be acquired. Alternatively, data can be acquired for one minute after the initiation of the analysis.

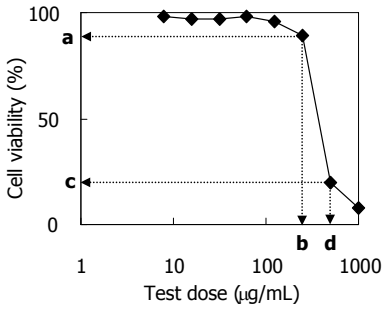
$$\textbf{Cell Viability} = \frac{\textbf{Number of living cells}}{\textbf{Total Number of acquired cells}} \times \textbf{100}$$

The CV75 value (see paragraph 13), i.e. a concentration showing 75% of THP-1 cell survival (25% cytotoxicity), is calculated by log-linear interpolation using the following equation:

$$\textbf{Log CV75} = \frac{\textbf{(75 – c) × Log (b) – (75 – a) × Log (d)}}{\textbf{a – c}}$$

Where:

a is the minimum value of cell viability over 75%
c is the maximum value of cell viability below 75%
b and d are the concentrations showing the value of cell viability a and c respectively



Other approaches to derive the CV75 can be used as long as it is demonstrated that this has no impact on the results (e.g. by testing the proficiency substances).

CD86/CD54 expression measurement

Preparation of the test chemicals and control substances

20. The appropriate solvent/vehicle (saline, medium or DMSO; see paragraph 14) is used to dissolve or stably disperse the test chemicals. The test chemicals are first diluted to the concentration corresponding to 100-fold (for saline or medium) or 500-fold (for DMSO) of the 1.2 × CV75 determined in the *dose finding assay* (see paragraph 19). If the CV75 cannot be determined (i.e. if sufficient cytotoxicity is not observed in the *dose finding assay*), the highest soluble or stably dispersed concentration of test chemical prepared with each solvent/vehicle should be used as starting concentration. Please note that the final concentration in the plate should not exceed 5000 μg/mL (in case of saline or medium) or 1000 μg/mL (in case of DMSO). Then, 1.2-fold serial dilutions are made using the corresponding solvent/vehicle to obtain the stock solutions (eight concentrations ranging from 100×1.2 × CV75 to 100×0.335 × CV75 (for saline or medium) or from 500×1.2 × CV75 to 500×0.335 × CV75 (for DMSO)) to be tested in the h-CLAT method (see DB-ALM protocol N_O. 158 for an example of dosing scheme). The stock solutions are then further diluted 50-fold (for saline or medium) or 250-fold (for DMSO) into the culture medium (working solutions). These working solutions are finally used for exposure with a further final two-fold dilution factor in the plate. If the results do not meet the acceptance criteria described in the paragraphs 29 and 30 regarding cell viability, the *dose finding assay* may be repeated to determine a more precise CV75. Please note that only 24-well plates can be used for CD86/CD54 expression measurement.

21. The solvent/vehicle control is prepared as described in paragraph 16. The positive control used in the h-CLAT method is DNCB (see paragraph 11), for which stock solutions are prepared in DMSO and diluted as described for the stock solutions in paragraph 20. DNCB should be used as the positive control for *CD86/CD54 expression measurement* at a final single concentration in the plate (typically 4.0 μg/mL). To obtain a 4.0 μg/mL concentration of DNCB in the plate, a 2 mg/mL stock solution of DNCB in DMSO is prepared and further diluted 250-fold with culture medium to a 8 μg/mL working solution. Alternatively, the CV75 of DNCB, which is determined in each test facility, could be also used as the positive control concentration. Other suitable positive controls may be used if historical data are available to derive comparable run acceptance criteria. For positive controls, the final single concentration in the plate should not exceed 5000 μg/mL (in case of saline or medium) or 1000 μg/mL (in case of DMSO). The run acceptance criteria are the same as those described for the test chemical (see paragraph 29), except for the last acceptance criterion since the positive control is tested at a single concentration.

Application of test chemicals and control substances

22. For each test chemical and control substance, one experiment is needed to obtain a prediction. Each experiment consists of at least two independent runs for *CD86/CD54 expression measurement* (see paragraphs 26-28). Each independent run is performed on a different day or on the same day provided that for each run: a) independent fresh stock solutions and working solutions of the test chemical and antibody solutions are prepared and b) independently harvested cells are used (i.e. cells are collected from different culture flasks); however, cells may come from the same passage. Test chemicals and control substances prepared as working solutions (500 μL) are mixed with 500 μL of suspended cells ($1x10^6$ cells) at 1:1 ratio, and cells are incubated for 24±0.5 hours as described in paragraphs 20 and 21. In each run, a single replicate for each concentration of the test chemical and control substance is sufficient because a prediction is obtained from at least two independent runs.

Cell staining and analysis

23. After 24±0.5 hours of exposure, cells are transferred from 24 well plate into sample tubes, collected by centrifugation and then washed twice with 1mL of staining buffer (if necessary, additional washing steps may be done). After washing, cells are blocked with 600 µL of blocking solution (staining buffer containing 0.01% (w/v) globulin (Cohn fraction II, III, Human: SIGMA, #G2388-10G)) and incubated at 4°C for 15 min. After blocking, cells are split in three aliquots of 180 µL into a 96-well round-bottom plate or micro tube.

24. After centrifugation, cells are stained with 50 µL of FITC-labelled anti-CD86, anti-CD54 or mouse IgG1 (isotype) antibodies at 4°C for 30 min. The antibodies described in the h-CLAT DB-ALM protocol no. 158 (18) should be used by diluting 3:25 (v/v, for CD86 (BD-PharMingen, #555657; Clone: Fun-1)) or 3:50 (v/v, for CD54 (DAKO, #F7143; Clone: 6.5B5) and IgG1 (DAKO, #X0927)) with staining buffer. These antibody dilution factors were defined by the test method developers as those providing the best signal-to-noise ratio. Based on the experience of the test method developers, the fluorescence intensity of the antibodies is usually consistent between different lots. However, users may consider titrating the antibodies in their own laboratory's conditions to define the best concentrations for use. Other fluorochrome-tagged anti-CD86 and/or anti-CD54 antibodies may be used if they can be shown to provide similar results as FITC-conjugated antibodies, for example by testing the proficiency substances in Appendix II. It should be noted that changing the clone or supplier of the antibodies as described in the h-CLAT DB-ALM protocol no. 158 (18) may affect the results. After washing twice or more with 150 µL of staining buffer, cells are resuspended in staining buffer (e.g. 400 µL), and the PI solution (e.g. 20 µL to obtain a final concentration of 0.625 µg/mL) or another cytotoxicity marker's solution (see paragraph 18) is added. The expression levels of CD86 and CD54, and cell viability are analysed using flow cytometry.

DATA AND REPORTING

Data evaluation

25. The expression of CD86 and CD54 is analysed with flow cytometry with the acquisition channel FL-1. Based on the geometric mean fluorescence intensity (MFI), the relative fluorescence intensity (RFI) of CD86 and CD54 for positive control (ctrl) cells and chemical-treated cells are calculated according to the following equation:

$$\text{RFI} = \frac{\text{MFI of chemical-treated cells} - \text{MFI of chemical-treated isotype control cells}}{\text{MFI of solvent/vehicle-treated ctrl cells} - \text{MFI of solvent/vehicle-treated isotype ctrl cells}} \times 100$$

The cell viability from the isotype control (ctrl) cells (which are stained with mouse IgG1 (isotype) antibodies) is also calculated according to the equation described in paragraph 19.

Prediction model

26. For *CD86/CD54 expression measurement*, each test chemical is tested in at least two independent runs to derive a single prediction (POSITIVE or NEGATIVE). An h-CLAT prediction is considered POSITIVE if at least one of the following conditions is met in 2 of 2 or in at least 2 of 3 independent runs, otherwise the h-CLAT prediction is considered NEGATIVE (Figure 1):

- The RFI of CD86 is equal to or greater than 150% in at least one tested concentration (with cell viability ≥ 50%);
- The RFI of CD54 is equal to or greater than 200% in at least one tested concentration (with cell viability ≥ 50%).

27. Based on the above, if the first two runs are both positive for CD86 and/or are both positive for CD54, the h-CLAT prediction is considered POSITIVE and a third run does not need to be conducted. Similarly, if the first two runs are negative for both markers, the h-CLAT prediction is considered NEGATIVE (with due consideration of the provisions of paragraph 30) without the need for a third run. If however, the first two runs are not concordant for at least one of the markers (CD54 or CD86), a third run is needed and the final prediction will be based on the majority result of the three individual runs (i.e. 2 out of 3). In this respect, it should be noted that if two independent runs are conducted and one is only positive for CD86 (hereinafter referred to as P_1) and the other is only positive for CD54 (hereinafter referred to as P_2), a third run is required. If this third run is negative for both markers (hereinafter referred to as N), the h-CLAT prediction is considered NEGATIVE. On the other hand, if the third run is positive for either marker (P_1 or P_2) or for both markers (hereinafter referred to as P_{12}), the h-CLAT prediction is considered POSITIVE.

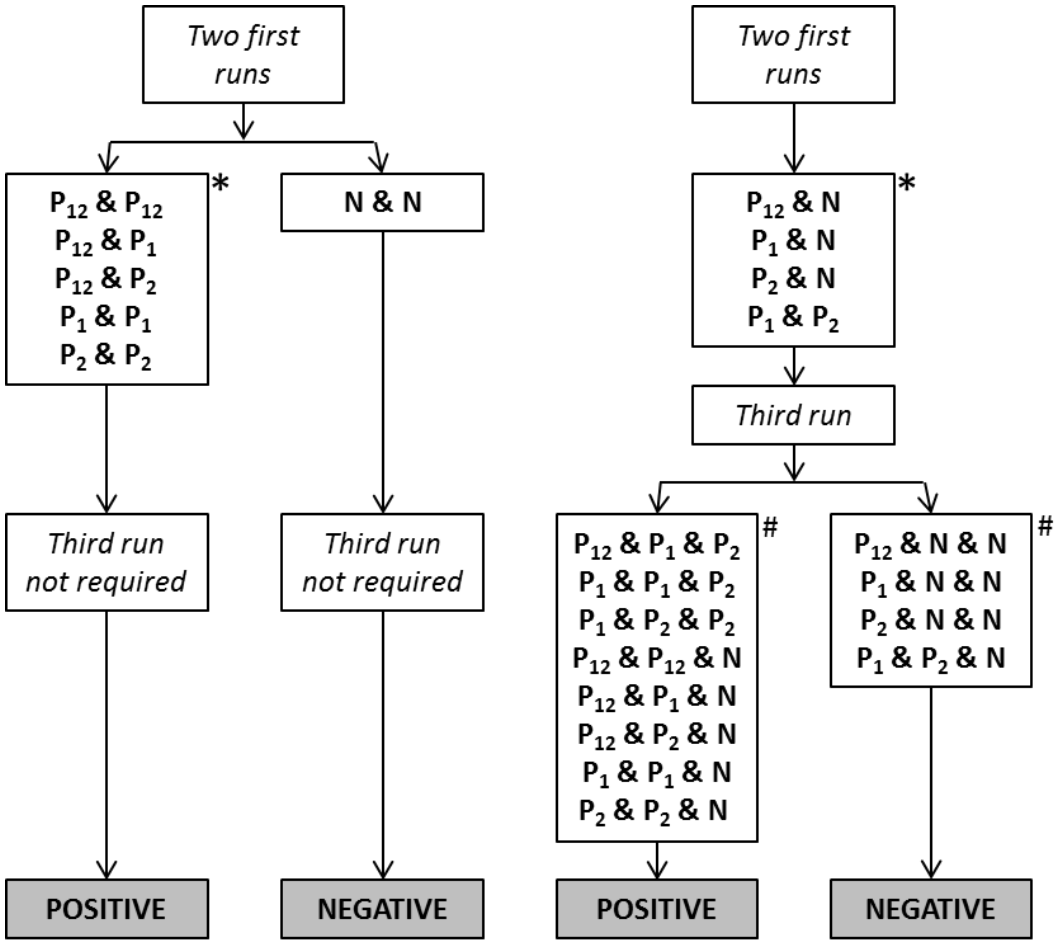


Figure 1: Prediction model used in the h-CLAT test method. An h-CLAT prediction should be considered in the framework of an IATA and in accordance with the provision of paragraphs 7 and 8 in the General introduction. P_1: run with only CD86 positive; P_2; run with only CD54 positive; P_{12}: run with both CD86 and CD54 positive; N: run with neither CD86 nor CD54 positive. *The boxes show the relevant combinations of results from the first two runs, independently of the order in which they may be obtained. #The boxes show the relevant combinations of results from the three runs on the basis of the results obtained in the first two runs shown in the box above, but do not reflect the order in which they may be obtained.

28. For the test chemicals predicted as POSITIVE with the h-CLAT, optionally, two Effective Concentrations (EC) values, the EC150 for CD86 and EC200 for CD54, i.e. the concentration at which the test chemicals induced a RFI of 150 or 200, may be determined. These EC values potentially could contribute to the assessment of sensitising potency (9) when used in integrated approaches such as IATA (4) (5) (6) (7) (8). They can be calculated by the following equations:

$$\mathbf{EC150\ (for\ CD86) = B_{concentration} + [(150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{concentration} - B_{concentration})]}$$

where

$A_{concentration}$is the lowest concentration in µg/mL with RFI > 150 (CD86) or 200 (CD54)

$B_{concentration}$ is the highest concentration in µg/mL with RFI < 150 (CD86) or 200 (CD54)
A_{RFI} is the RFI at the lowest concentration with RFI > 150 (CD86) or 200 (CD54)
B_{RFI} is the RFI at the highest concentration with RFI < 150 (CD86) or 200 (CD54)

For the purpose of more precisely deriving the EC150 and EC200 values, three independent runs for *CD86/CD54 expression measurement* may be required. The final EC150 and EC200 values are then determined as the median value of the ECs calculated from the three independent runs. When only two of three independent runs meet the criteria for positivity (see paragraphs 26-27), the higher EC150 or EC200 of the two calculated values is adopted.

Acceptance criteria

29. The following acceptance criteria should be met when using the h-CLAT method (22) (27).

- The cell viabilities of medium and solvent/vehicle controls should be higher than 90%.

- In the solvent/vehicle control, RFI values of both CD86 and CD54 should not exceed the positive criteria (CD86 RFI ≥ 150% and CD54 RFI ≥ 200%). RFI values of the solvent/vehicle control are calculated by using the formula described in paragraph 25 ("MFI of chemical" should be replaced with "MFI of solvent/vehicle", and "MFI of solvent/vehicle" should be replaced with "MFI of (medium) control").

- For both medium and solvent/vehicle controls, the MFI ratio of both CD86 and CD54 to isotype control should be > 105%.

- In the positive control (DNCB), RFI values of both CD86 and CD54 should meet the positive criteria (CD86 RFI ≥ 150 and CD54 RFI ≥ 200) and cell viability should be more than 50%.

- For the test chemical, the cell viability should be more than 50% in at least four tested concentrations in each run.

30. Negative results are acceptable only for test chemicals exhibiting a cell viability of less than 90% at the highest concentration tested (i.e. 1.2 × CV75 according to the serial dilution scheme described in paragraph 20). If the cell viability at 1.2 × CV75 is equal or above 90% the negative result should be discarded. In such a case it is recommended to try to refine the dose selection by repeating the CV75 determination. It should be noted that when 5000 µg/mL in saline (or medium or other solvents/vehicles), 1000 µg/mL in DMSO or the highest soluble concentration is used as the maximal test concentration of a test chemical, a negative result is acceptable even if the cell viability is above 90%.

Test report

31. The test report should include the following information.

Test chemical

- Mono-constituent substance

    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
    - Physical appearance, Log Kow, water solubility, DMSO solubility, molecular weight, and

additional relevant physicochemical properties, to the extent available;

- Purity, chemical identity of impurities as appropriate and practically feasible, etc.;
- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Concentration(s) tested;
- Storage conditions and stability to the extent available;
- Justification for choice of solvent/vehicle for each test chemical.

- Multi-constituent substance, UVCB and mixture

  - Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
  - Physical appearance, water solubility, DMSO solubility and additional relevant physicochemical properties, to the extent available;
  - Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent/vehicle for each test chemical.

Controls

- Positive control

  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
  - Physical appearance, Log Kow, water solubility, DMSO solubility, molecular weight, and additional relevant physicochemical properties, to the extent available and where applicable;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc.;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available;
  - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.

- Negative and solvent/vehicle control

  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;

- Purity, chemical identity of impurities as appropriate and practically feasible, etc.;
- Physical appearance, molecular weight, and additional relevant physicochemical properties in the case other control solvent/vehicle than those mentioned in the Test Guideline are used and to the extent available;
- Storage conditions and stability to the extent available;
- Justification for choice of solvent/vehicle for each test chemical.

Test method conditions

- Name and address of the sponsor, test facility and study director;
- Description of test method used;
- Cell line used, its storage conditions and source (e.g. the facility from which they were obtained);
- Flow cytometry used (e.g. model), including instrument settings, globulin, antibodies and cytotoxicity marker used;
- The procedure used to demonstrate proficiency of the laboratory in performing the test method by testing of proficiency substances, and the procedure used to demonstrate reproducible performance of the test method over time, e.g. historical control data and/or historical reactivity checks' data.

Test acceptance criteria

- Cell viability, MFI and RFI values obtained with the solvent/vehicle control in comparison to the acceptance ranges;
- Cell viability and RFI values obtained with the positive control in comparison to the acceptance ranges;
- Cell viability of all tested concentrations of the tested chemical.

Test procedure

- Number of runs used;
- Test chemical concentrations, application and exposure time used (if different than the one recommended)
- Duration of exposure (if different than the one recommended);
- Description of evaluation and decision criteria used;
- Description of any modifications of the test procedure.

Results

- Tabulation of the data, including CV75 (if applicable), individual geometric MFI, RFI, cell viability values, EC150/EC200 values (if applicable) obtained for the test chemical and for the positive control in each run, and an indication of the rating of the test chemical according to the prediction model;
- Description of any other relevant observations, if applicable.

Discussion of the results

- Discussion of the results obtained with the h-CLAT method;
- Consideration of the test method results within the context of an IATA, if other relevant information is available.

Conclusions

LITERATURE

1. Ashikaga T, Yoshida Y, Hirota M, Yoneyama K, Itagaki H, Sakaguchi H, Miyazawa M, Ito Y, Suzuki H, Toyoda H. (2006), Development of an in vitro skin sensitization test using human cell lines: The human Cell Line Activation Test (h-CLAT) I. Optimization of the h-CLAT protocol. Toxicol. In Vitro 20, 767–773.

2. Miyazawa M, Ito Y, Yoshida Y, Sakaguchi H, Suzuki H. (2007), Phenotypic alterations and cytokine production in THP-1 cells in response to allergens. Toxicol. In Vitro 21, 428-437.

3. EC EURL-ECVAM (2013), Recommendation on the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing. Accessible at: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations

4. Takenouchi O, Fukui S, Okamoto K, Kurotani S, Imai N, Fujishiro M, Kyotani D, Kato Y, Kasahara T, Fujita M, Toyoda A, Sekiya D, Watanabe S, Seto H, Hirota M, Ashikaga T, Miyazawa M. (2015), Test battery with the human cell line activation test, direct peptide reactivity assay and DEREK based on a 139 chemical data set for predicting skin sensitizing potential and potency of chemicals. J Appl Toxicol. 35, 1318-1332.

5. Hirota M, Fukui S, Okamoto K, Kurotani S, Imai N, Fujishiro M, Kyotani D, Kato Y, Kasahara T, Fujita M, Toyoda A, Sekiya D, Watanabe S, Seto H, Takenouchi O, Ashikaga T, Miyazawa M. (2015), Evaluation of combinations of in vitro sensitization test descriptors for the artificial neural network-based risk assessment model of skin sensitization. J Appl Toxicol. 35, 1333-1347.

6. Bauch C, Kolle SN, Ramirez T, Fabian E, Mehling A, Teubner W, van Ravenzwaay B, Landsiedel R. (2012), Putting the parts together: combining in vitro methods to test for skin sensitizing potencials. Regul Toxicol Parmacol. 63, 489-504.

7. Van der Veen JW, Rorije E, Emter R, Natch A, van Loveren H, Ezendam J. (2014), Evaluating the performance of integrated approaches for hazard identification of skin sensitizing chemicals. Regul Toxicol Pharmacol. 69, 371-379.

8. Urbisch D, Mehling A, Guth K, Ramirez T, Honarvar N, Kolle S, Landsiedel R, Jaworska J, Kern PS, Gerberick F, Natsch A, Emter R, Ashikaga T, Miyazawa M, Sakaguchi H. (2015), Assessing skin sensitization hazard in mice and men using non-animal test methods. Regul Toxicol Parmacol. 71, 337-351.

9. Jaworska JS, Natsch A, Ryan C, Strickland J, Ashikaga T, Miyazawa M. (2015), Bayesian integrated testing strategy (ITS) for skin sensitization potency assessment: a decision support system for quantitative weight of evidence and adaptive testing strategy. Arch Toxicol. 89, 2355-2383.

10. Strickland J, Zang Q, Kleinstreuer N, Paris M, Lehmann DM, Choksi N, Matheson J, Jacobs A, Lowit A, Allen D, Casey W. (2016), Integrated decision strategies for skin sensitization hazard. J Appl Toxicol. DOI 10.1002/jat.3281.

11. Nukada Y, Ashikaga T, Miyazawa M, Hirota M, Sakaguchi H, Sasa H, Nishiyama N. (2012), Prediction of skin sensitization potency of chemicals by human Cell Line Activation Test (h-CLAT) and an attempt at classifying skin sensitization potency. Toxicol. In Vitro 26, 1150-60.

12. EC EURL ECVAM (2015), Re-analysis of the within and between laboratory reproducibility of the human Cell Line Activation Test (h-CLAT). Accessible at: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/eurl-ecvam-recommendation-on-the-human-cell-line-activation-test-h-clat-for-skin-sensitisation-testing

13. EC EURL ECVAM (2012), human Cell Line Activation Test (h-CLAT) Validation Study Report Accessible at: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations

14. Takenouchi O, Miyazawa M, Saito K, Ashikaga T, Sakaguchi H. (2013), Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic with high octanol-water partition coefficients. J. Toxicol. Sci. 38, 599-609.

15. Ashikaga T, Sakaguchi H, Sono S, Kosaka N, Ishikawa M, Nukada Y, Miyazawa M, Ito Y, NishiyamaN, Itagaki H. (2010), A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). Altern. Lab. Anim. 38, 275-284.

16. Fabian E., Vogel D., Blatz V., Ramirez T., Kolle S., Eltze T., van Ravenzwaay B., Oesch F., Landsiedel R. (2013), Xenobiotic metabolizin enzyme activities in cells used for testing skin sensitization in vitro. Arch Toxicol 87, 1683-1969.

17. Okamoto K, Kato Y, Kosaka N, Mizuno M, Inaba H, Sono S, Ashikaga T, Nakamura T, Okamoto Y, Sakaguchi H, Kishi M, Kuwahara H, Ohno Y. (2010), The Japanese ring study of a human Cell Line Activation Test (h-CLAT) for predicting skin sensitization potential (6th report): A study for evaluating oxidative hair dye sensitization potential using h-CLAT. AATEX 15, 81-88.

18. DB-ALM (INVITTOX) (2014), Protocol 158: human Cell Line Activation Test (h-CLAT), 23pp. Accessible at: http://ecvam-dbalm.jrc.ec.europa.eu/

19. Mizuno M, Yoshida M, Kodama T, Kosaka N, Okamato K, Sono S, Yamada T, Hasegawa S, Ashikaga T, Kuwahara H, Sakaguchi H, Sato J, Ota N, Okamoto Y, Ohno Y. (2008), Effects of pre-culture conditions on the human Cell Line Activation Test (h-CLAT) results; Results of the 4th Japanese inter-laboratory study. AATEX 13, 70-82.

20. Sono S, Mizuno M, Kosaka N, Okamoto K, Kato Y, Inaba H, , Nakamura T, Kishi M, Kuwahara H, Sakaguchi H, Okamoto Y, Ashikaga T, Ohno Y. (2010), The Japanese ring study of a human Cell Line Activation Test (h-CLAT) for predicting skin sensitization potential (7th report): Evaluation of volatile, poorly soluble fragrance materials. AATEX 15, 89-96.

21. OECD (2005), Guidance Document No.34 on "the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment". OECD Series on Testing and Assessment. Organization for Economic Cooperation and Development, Paris, France, 2005, 96 pp.

22. OECD (2012), The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168. Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2012)10/PART1&docLanguage=En

23. United Nations UN (2013), Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Fifth revised edition. New York & Geneva: United Nations Publications. ISBN: 978-92-1-

117006-1. Available at: [http://www.unece.org/trans/danger/publi/ghs/ghs_rev05/05files_e.html].

24. ECETOC (2003). Contact sensitization: Classification according to potency. European Centre for Ecotoxicology & Toxicology of Chemicals (Technical Report No. 87).

25. Ashikaga T, Sakaguchi H, Okamoto K, Mizuno M, Sato J, Yamada T, Yoshida M, Ota N, Hasegawa S, Kodama T, Okamoto Y, Kuwahara H, Kosaka N, Sono S, Ohno Y. (2008), Assessment of the human Cell Line Activation Test (h-CLAT) for Skin Sensitization; Results of the First Japanese Inter-laboratory Study. AATEX 13, 27-35.

APPENDIX I

DEFINITIONS

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

AOP (Adverse Outcome Pathway): sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an *in vivo* outcome of interest (22).

CV75: The estimated concentration showing 75% cell viability.

EC150: the concentrations showing the RFI values of 150 in CD86 expression

EC200: the concentrations showing the RFI values of 200 in CD54 expression

Flow cytometry: a cytometric technique in which cells suspended in a fluid flow one at a time through a focus of exciting light, which is scattered in patterns characteristic to the cells and their components; cells are frequently labeled with fluorescent markers so that light is first absorbed and then emitted at altered frequencies.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

IATA (Integrated Approach to Testing and Assessment): A structured approach used for hazard identification (potential), hazard characterisation (potency) and/or safety assessment (potential/potency and exposure) of a chemical or group of chemicals, which strategically integrates and weights all relevant data to inform regulatory decision regarding potential hazard and/or risk and/or the need for further targeted and therefore minimal testing.

Medium control: An untreated replicate containing all components of a test system. This sample is processed with test chemical-treated samples and other control samples to determine whether the solvent/vehicle interacts with the test system.

Mixture: A mixture or a 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).

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.

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.

Pre-haptens: chemicals which become sensitisers through abiotic transformation

Pro-haptens: chemicals requiring enzymatic activation to exert skin sensitisation potential

Relative fluorescence intensity (RFI): Relative values of geometric mean fluorescence intensity (MFI) in chemical-treated cells compared to MFI in solvent/vehicle-treated cells.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (21).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (21).

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

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (21).

Staining buffer: A phosphate buffered saline containing 0.1% bovine serum albumin.

Solvent/vehicle control: An untreated sample containing all components of a test system except of the test chemical, but including the solvent/vehicle that is used. It is used to establish the baseline response for the samples treated with the test chemical dissolved or stably dispersed in the same solvent/vehicle. When tested with a concurrent medium control, this sample also demonstrates whether the solvent/vehicle interacts with the test system.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (21).

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities

deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing it composition.

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

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (23).

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

Valid test method: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (21).

APPENDIX II

PROFICIENCY SUBSTANCES

Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency by correctly obtaining the expected h-CLAT prediction for the 10 substances recommended in Table 1 and by obtaining CV75, EC150 and EC200 values that fall within the respective reference range for at least 8 out of the 10 proficiency substances. Proficiency substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were that the substances are commercially available, and that high-quality *in vivo* reference data as well as high quality *in vitro* data generated with the h-CLAT method are available. Also, published reference data are available for the h-CLAT method (3) (14).

Table 1: Recommended substances for demonstrating technical proficiency with the h-CLAT method

Proficiency substances	**CASRN**	**Physical state**	***In vivo* prediction[1]**	**CV75 Reference Range in µg/mL[2]**	**h-CLAT results for CD86 (EC150 Reference Range in µg/mL)[2]**	**h-CLAT results for CD54 (EC200 Reference Range in µg/mL)[2]**
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (extreme)	2-12	Positive (0.5-10)	Positive (0.5-15)
4-Phenylenediamine	106-50-3	Solid	Sensitiser (strong)	5-95	Positive (<40)	Negative (>1.5)[3]
Nickel sulfate	10101-97-0	Solid	Sensitiser (moderate)	30-500	Positive (<100)	Positive (10-100)
2-Mercaptbenzothiazole	149-30-4	Solid	Sensitiser (moderate)	30-400	Negative (>10)[3]	Positive (10-140)
R(+)-Limonene	5989-27-5	Liquid	Sensitiser (weak)	>20	Negative (>5)[3]	Positive (<250)
Imidazolidinyl urea	39236-46-9	Solid	Sensitiser (weak)	25-100	Positive (20-90)	Positive (20-75)
Isopropanol	67-63-0	Liquid	Non-sensitiser	>5000	Negative (>5000)	Negative (>5000)
Glycerol	56-81-5	Liquid	Non-sensitiser	>5000	Negative (>5000)	Negative (>5000)
Lactic acid	50-21-5	Liquid	Non-sensitiser	1500-5000	Negative (>5000)	Negative (>5000)
4-Aminobenzoic acid	150-13-0	Solid	Non-sensitiser	>1000	Negative (>1000)	Negative (>1000)

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

[1] The *in vivo* hazard and (potency) prediction is based on LLNA data (3) (14). The *in vivo* potency is derived using the criteria proposed by ECETOC (24).

[2] Based on historical observed values (13) (25).

[3] Historically, a majority of negative results have been obtained for this marker and therefore a negative result is mostly expected. The range provided was defined on the basis of the few historical positive results observed. In case a positive result is obtained, the EC value should be within the reported reference range.

ANNEX II: *IN VITRO* SKIN SENSITISATION: U937 CELL LINE ACTIVATION TEST (U-SENS™)

INITIAL CONSIDERATIONS AND LIMITATIONS

1. The U-SENS™ method quantifies the change in the expression of a cell surface marker associated with the process of activation of monocytes and dendritic cells (DC) (i.e. CD86), in the human histiocytic lymphoma cell line U937, following exposure to sensitisers (1). The measured expression levels of CD86 cell surface marker in the cell line U937 is then used for supporting the discrimination between skin sensitisers and non-sensitisers.

2. The U-SENS™ method has been evaluated in a validation study (2) coordinated by L'Oreal and subsequently independent peer reviewed by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) Scientific Advisory Committee (ESAC) (3). Considering all available evidence and input from regulators and stakeholders, the U-SENS™ was recommended by EURL ECVAM (4) to be used as part of an IATA to support the discrimination between sensitisers and non-sensitisers for the purpose of hazard classification and labelling. In its guidance document on the reporting of structured approaches to data integration and individual information sources used within IATA for skin sensitisation, the OECD currently discusses a number of case studies describing different testing strategies and prediction models. One of the different defined approaches is based on the U-SENS assay (5). Examples of the use of U-SENS™ data in combination with other information, including historical data and existing valid human data (6), are also reported elsewhere in the literature (4) (5) (7).

3. The U-SENS™ method proved to be transferable to laboratories experienced in cell culture techniques and flow cytometry analysis. The level of reproducibility in predictions that can be expected from the test method is in the order of 90% and 84% within and between laboratories, respectively (8). Results generated in the validation study (8) and other published studies (1) overall indicate that, compared with LLNA results, the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers is 86% (N=166) with a sensitivity of 91% (118/129) and a specificity of 65% (24/37). Compared with human results, the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers is 77% (N=101) with a sensitivity of 100% (58/58) and a specificity of 47% (20/43). False negative predictions compared to LLNA with the U-SENS™ are more likely to concern chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A) (1) (8) (9). Taken together, this information indicates the usefulness of the U-SENS™ method to contribute to the identification of skin sensitisation hazards. However, the accuracy values given here for the U-SENS™ as a stand-alone test method are only indicative, since the test method should be considered in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General introduction. Furthermore, when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in humans.

4. On the basis of the data currently available, the U-SENS™ method was shown to be applicable to test chemicals (including cosmetics ingredients e.g. preservatives, surfactants, actives, dyes) covering a variety of organic functional groups, of physicochemical properties, skin sensitisation potency (as determined in in vivo studies) and the spectrum of reaction mechanisms known to be associated with skin sensitisation (i.e. Michael acceptor, Schiff base formation, acyl transfer agent, substitution nucleophilic bi-molecular [SN2], or nucleophilic aromatic substitution [SNAr]) (1) (8) (9) (10). The U-SENS™ method is

applicable to test chemicals that are soluble or that form a stable dispersion (i.e. a colloid or suspension in which the test chemical does not settle or separate from the solvent/vehicle into different phases) in an appropriate solvent/vehicle (see paragraph 13). Chemicals in the dataset reported to be pre-haptens (i.e. substances activated by oxidation) or pro-haptens (i.e. substances requiring enzymatic activation for example via P450 enzymes) were correctly predicted by the U-SENS™ (1) (10). Membrane disrupting substances can lead to false positive results due to a non-specific increase of CD86 expression, as 3 out of 7 false positives relative to the *in vivo* reference classification were surfactants (1). As such positive results with surfactants should be considered with caution whereas negative results with surfactants could still be used to support the identification of the test chemical as a non-sensitiser. Fluorescent test chemicals can be assessed with the U-SENS™ (1), nevertheless, strong fluorescent test chemicals emitting at the same wavelength as fluorescein isothiocyanate (FITC) or as propidium iodide (PI), will interfere with the flow cytometric detection and thus cannot be correctly evaluated using FITC-conjugated antibodies (potential false negative) or PI (viability not measurable). In such a case, other fluorochrome-tagged antibodies or other cytotoxicity markers, respectively, can be used as long as it can be shown they provide similar results as the FITC-tagged antibodies or PI (see paragraph 18) e.g. by testing the proficiency substances in Appendix II. In the light of the above, positive results with surfactants and negative results with strong fluorescent test chemicals should be interpreted in the context of the stated limitations and together with other information sources within the framework of IATA. In cases where there is evidence demonstrating the non-applicability of the U-SENS™ method to other specific categories of test chemicals, it should not be used for those specific categories.

5. As described above, the U-SENS™ method supports the discrimination between skin sensitisers from non-sensitisers. However, it may also potentially contribute to the assessment of sensitising potency when used in integrated approaches such as IATA. Nevertheless, further work, preferably based on human data, is required to determine how U-SENS™ results may possibly inform potency assessment.

6. Definitions are provided in Appendix I.

PRINCIPLE OF THE TEST

7. The U-SENS™ method is an *in vitro* assay that quantifies changes of CD86 cell surface marker expression on a human histiocytic lymphoma cell line, U937 cells, following 45±3 hours exposure to the test chemical. The CD86 surface marker is one typical marker of U937 activation. CD86 is known to be a co-stimulatory molecule that may mimic monocytic activation, which plays a critical role in T-cell priming. The changes of CD86 cell surface marker expression are measured by flow cytometry following cell staining typically with fluorescein isothiocyanate (FITC)-labelled antibodies. Cytotoxicity measurement is also conducted (e.g. by using PI) concurrently to assess whether upregulation of CD86 cell surface marker expression occurs at sub-cytotoxic concentrations. The stimulation index (S.I.) of CD86 cell surface marker compared to solvent/vehicle control is calculated and used in the prediction model (see paragraph 19), to support the discrimination between sensitisers and non-sensitisers.

DEMONSTRATION OF PROFICIENCY

8. Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency, using the 10 Proficiency Substances listed in Appendix II in compliance with the Good *in vitro* Method Practices (11). Moreover, test method users

should maintain a historical database of data generated with the reactivity checks (see paragraph 11) and with the positive and solvent/vehicle controls (see paragraphs 15-16), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

PROCEDURE

9. This test method is based on the U-SENS™ DataBase service on ALternative Methods to animal experimentation (DB-ALM) protocol no. 183 (12). The Standard Operating Procedures (SOP) should be employed when implementing and using the U-SENS™ method in the laboratory. An automated system to run the U-SENS™ can be used if it can be shown to provide similar results, for example by testing the proficiency substances in Appendix II. The following is a description of the main components and procedures for the U-SENS™ method.

Preparation of cells

10. The human histiocytic lymphoma cell line, U937 (13) should be used for performing the U-SENS™ method. Cells (clone CRL1593.2) should be obtained from a well-qualified cell bank such as the American Type Culture Collection.

11. U937 cells are cultured, at 37°C under 5% CO_2 and humidified atmosphere, in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (complete medium). U937 cells are routinely passaged every 2-3 days at the density of 1.5 or 3 × 10^5 cells/mL, respectively. The cell density should not exceed 2 × 10^6 cells/mL and the cell viability measured by trypan blue exclusion should be ≥ 90% (not to be applied at the first passage after thawing). Prior to using them for testing, every batch of cells, FCS or antibodies should be qualified by conducting a reactivity check. The reactivity check of the cells should be performed using the positive control, picrylsulfonic acid (2,4,6-Trinitro-benzene-sulfonic acid: TNBS) (CASRN 2508-19-2, ≥ 99% purity) and the negative control lactic acid (LA) (CASRN 50-21-5, ≥ 85% purity), at least one week after thawing. For the reactivity check, six final concentrations should be tested for each of the 2 controls (TNBS: 1, 12.5, 25, 50, 75, 100µg/mL and LA: 1, 10, 20, 50, 100, 200µg/mL). TNBS solubilised in complete medium should produce a positive and concentration-related response of CD86 (e.g. when a positive concentration, CD86 S.I. ≥ 150, is followed by a concentration with an increasing CD86 S.I), and LA solubilised in complete medium should produce negative response of CD86 (see paragraph 21). Only the batch of cells which passed the reactivity check 2 times should be used for the assay. Cells can be propagated up to seven weeks after thawing. Passage number should not exceed 21. The reactivity check should be performed according to the procedures described in paragraphs 18-22.

12. For testing, U937 cells are seeded at a density of either 3 x 10^5 cells/mL or 6 × 10^5 cells/mL, and pre-cultured in culture flasks for 2 days or 1 day, respectively. Other pre-cultured conditions than those described above may be used if sufficient scientific rationale is provided and if it can be shown to provide similar results, for example by testing the proficiency substances in Appendix II. In the day of testing, cells harvested from culture flask are resuspended with fresh culture medium at 5 × 10^5 cells/mL. Then, cells are distributed into a 96-well flat-bottom plate with 100 µL (final cell density of 0.5 × 10^5 cells/well).

Preparation of test chemicals and control substances

13. Assessment of solubility is conducted prior to testing. For this purpose, test chemicals are dissolved or stably dispersed at a concentration of 50 mg/mL in complete medium as first solvent option or dimethyl sulfoxide (DMSO, ≥ 99% purity) as a second solvent/vehicle option if the test chemical is not soluble in the complete medium solvent/vehicle. For the testing, the test chemical is dissolved to a final concentration of 0.4 mg/mL in complete medium if the chemical is soluble in this solvent/vehicle. If the chemical is soluble only in DMSO, the chemical is dissolved at a concentration of 50 mg/mL. Other solvents/vehicles than those described above may be used if sufficient scientific rationale is provided. Stability of the test chemical in the final solvent/vehicle should be taken into account.

14. The test chemicals and control substances are prepared on the day of testing. Because a dose finding assay is not conducted, for the first run, 6 final concentrations should be tested (1, 10, 20, 50, 100 and 200 µg/mL) into the corresponding solvent/vehicle either in complete medium or in 0.4% DMSO in medium. For the subsequent runs, starting from the 0.4 mg/mL in complete medium or 50 mg/mL in DMSO, solutions of the test chemicals, at least 4 working solutions (i.e. at least 4 concentrations), are prepared using the corresponding solvent/vehicle. The working solutions are finally used for treatment by adding an equal volume of U937 cell suspension (see paragraph 11 above) to the volume of working solution in the plate to achieve a further 2-fold dilution (12). The concentrations (at least 4 concentrations) for any further run are chosen based on the individual results of all previous runs (8). The usable final concentrations are 1, 2, 3, 4, 5, 7.5, 10, 12.5, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180 and 200 µg/mL. The maximum final concentration is 200 µg/mL. In the case of a CD86 positive value at 1 µg/mL is observed, then 0.1 µg/mL is evaluated in order to find the concentration of the test chemical that does not induce CD86 above the positive threshold. For each run, the EC150 (concentration at which a chemical reaches the CD86 positive threshold of 150%, see paragraph 19) is calculated if a CD86 positive concentration-response is observed. Where the test chemical induces a positive CD86 response not concentration related, the calculation of the EC150 might not be relevant as described in the U-SENS™ DB-ALM protocol no. 183 (12). For each run, CV70 (concentration at which a chemical reaches the cytotoxicity threshold of 70%, see paragraph 19) is calculated whenever possible (12). To investigate the concentration response effect of CD86 increase, any concentrations from the usable concentrations should be chosen evenly spread between the EC150 (or the highest CD86 negative non cytotoxic concentration) and the CV70 (or the highest concentration allowed i.e. 200 µg/mL). A minimum of 4 concentrations should be tested per run with at least 2 concentrations being common with the previous run(s), for comparison purposes.

15. The solvent/vehicle control used in the U-SENS™ method is complete medium (for test chemicals solubilised or stably dispersed) (see paragraph 4) or 0.4% DMSO in complete medium (for test chemicals solubilised or stably dispersed in DMSO).

16. The positive control used in the U-SENS™ method is TNBS (see paragraph 11), prepared in complete medium. TNBS should be used as the positive control for CD86 expression measurement at a final single concentration in plate (50 µg/mL) yielding > 70% of cell viability. To obtain a 50 µg/mL concentration of TNBS in plate, a 1 M (i.e. 293 mg/mL) stock solution of TNBS in complete medium is prepared and further diluted 2930-fold with complete medium to a 100 µg/mL working solution. Lactic acid (LA, CAS 50-21-5) should be used as the negative control at 200 µg/mL solubilised in complete medium (from a 0.4 mg/mL stock solution). In each plate of each run, three replicates of complete medium untreated control, solvent/vehicle control, negative and positive controls are prepared (12). Other suitable positive controls may be used if historical data are available to derive comparable run acceptance criteria. The run acceptance criteria are the same as described for the test chemical (see paragraph 12).

Application of test chemicals and control substances

17. The solvent/vehicle control or working solutions described in paragraphs 14-16 are mixed 1:1 (v/v) with the cell suspensions prepared in the 96-well flat-bottom plate (see paragraph 12). The treated plates are then incubated for 45±3 hours at 37°C under 5% CO_2. Prior to incubation, plates are sealed with semi permeable membrane, to avoid evaporation of volatile test chemicals and cross-contamination between cells treated with test chemicals (12).

Cell staining

18. After 45±3 hours of exposure, cells are transferred into V-shaped microtiter plate and collected by centrifugation. Solubility interference is defined as crystals or drops observed under the microscope at 45 ± 3 hours post treatment (before the cell staining). The supernatants are discarded and the remaining cells are washed once with 100 µL of an ice-cold phosphate buffered saline (PBS) containing 5 % foetal calf serum (staining buffer). After centrifugation, cells are re-suspended with 100 µL of staining buffer and stained with 5 µL (e.g. 0.25 µg) of FITC-labelled anti-CD86 or mouse IgG1 (isotype) antibodies at 4°C for 30 min protected from light. The antibodies described in the U-SENS™ DB-ALM protocol no. 183 (12) should be used (for CD86: BD-PharMingen #555657 Clone: Fun-1, or Caltag/Invitrogen # MHCD8601 Clone: BU63; and for IgG1: BD-PharMingen #555748, or Caltag/Invitrogen # GM4992). Based on the experience of the test method developers, the fluorescence intensity of the antibodies is usually consistent between different lots. Other clones or supplier of the antibodies which passed the reactivity check may be used for the assay (see paragraph 11). However, users may consider titrating the antibodies in their own laboratory's conditions to define the best concentration for use. Other detection system e.g. fluorochrome-tagged anti-CD86 antibodies may be used if they can be shown to provide similar results as FITC-conjugated antibodies, for example by testing the proficiency substances in Appendix II. After washing with 100 µL of staining buffer two times and once with 100 µL of an ice-cold PBS, cells are resuspended in ice-cold PBS (e.g. 125 µL for samples being analysed manually tube by tube, or 50 µL using an auto-sampler plate) and PI solution is added (final concentration of 3 µg/mL). Other cytotoxicity markers, such as 7-Aminoactinomycin D (7-AAD) or Trypan blue may be used if the alternative stains can be shown to provide similar results as PI, for example by testing the proficiency substances in Appendix II.

Flow cytometry analysis

19. Expression level of CD86 and cell viability are analysed using flow cytometry. Cells are displayed within a size (FSC) and granularity (SSC) dot plot set to log scale in order to clearly identify the population in a first gate R1 and eliminate the debris. A targeting total of 10,000 cells in gate R1 are acquired for each well. Cells from the same R1 gate are displayed within a FL3 or FL4 / SSC dot plot. Viable cells are delineated by placing a second gate R2 selecting the population of propidium iodide-negative cells (FL3 or FL4 channel). The cell viability can be calculated using the following equation by the cytometer analysis program. When the cell viability is low, up to 20,000 cells including dead cells could be acquired. Alternatively, data can be acquired for one minute after the initiation of the analysis.

$$\text{Cell Viability} = \frac{\text{Number of living cells}}{\text{Total number of acquired cells}} \times 100$$

Percentage of FL1-positive cells is then measured among these viable cells gated on R2 (within R1). Cell

surface expression of CD86 is analysed in a FL1 / SSC dot plot gated on viable cells (R2).
For the complete medium / IgG1 wells, the analysis marker is set close to the main population so that the complete medium controls have IgG1 within the target zone of 0.6 to 0.9%.
Colour interference is defined as a shift of the FITC-labelled IgG1 dot-plot (IgG1 FL1 Geo Mean S.I. ≥ 150%).

The stimulation index (S.I.) of CD86 for controls cells (untreated or in 0.4% DMSO) and chemical-treated cells are calculated according to the following equation:

$$S.I. = \frac{\%\ of\ CD86^{+}\ treated\ cells - \%\ of\ IgG1^{+}\ treated\ cells}{\%\ of\ CD86^{+}\ control\ cells - \%\ of\ IgG1^{+}\ control\ cells} \times 100$$

% of $IgG1^{+}$ untreated control cells: referred to as percentage of FL1-positive IgG1 cells defined with the analysis marker (accepted range of ≥ 0.6% and < 1.5%, see paragraph 22) among the viable untreated cells.
% of $IgG1^{+}/CD86^{+}$ control/treated cells: referred to as percentage of FL1-positive IgG1/CD86 cells measured without moving the analysis marker among the viable control/treated cells.

DATA AND REPORTING

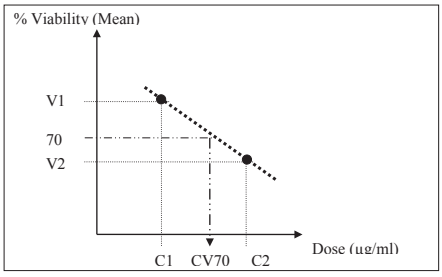
Data evaluation

20. The following parameters are calculated in the U-SENS™ test method: CV70 value, i.e. a concentration showing 70% of U937 cell survival (30% cytotoxicity) and the EC150 value, i.e. the concentration at which the test chemicals induced a CD86 stimulation index (S.I.) of 150%.

CV70 is calculated by log-linear interpolation using the following equation:

$$CV70 = C1 + [(V1 - 70) / (V1 - V2) * (C2 - C1)]$$

Where:
V1 is the minimum value of cell viability over 70%
V2 is the maximum value of cell viability below 70%
C1 and C2 are the concentrations showing the value of cell viability V1 and V2 respectively.

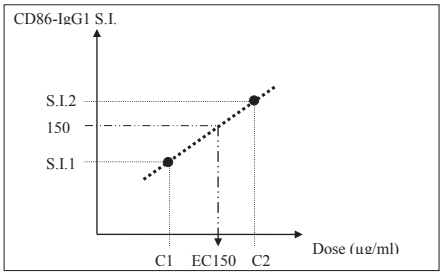


Other approaches to derive the CV70 can be used as long as it is demonstrated that this has no impact on the results (e.g. by testing the proficiency substances).

EC150 is calculated by log-linear interpolation using the following equation:

EC150 = C1 + [(150 – S.I.1) / (S.I.2 – S.I.1) * (C2 – C1)]

Where:
C1 is the highest concentration in µg/mL with a CD86 S.I. < 150% (S.I. 1)
C2 is the lowest concentration in µg/mL with a CD86 S.I. ≥ 150% (S.I. 2).



The EC150 and CV70 values are calculated

- for each run : the individual EC150 and CV70 values are used as tools to investigate the concentration response effect of CD86 increase (see paragraph 14),
- based on the average viabilities, the overall CV70 is determined (12) ,
- based on the average S.I. of CD86 values, the overall EC150 is determined for the test chemical predicted as POSITIVE with the U-SENS™ (see paragraph 21) (12).

Prediction model

21. For CD86 expression measurement, each test chemical is tested in at least four concentrations and in at least two independent runs (performed on a different day) to derive a single prediction (NEGATIVE or POSITIVE).

- The individual conclusion of an U-SENS™ run is considered Negative (hereinafter referred to as N) if the S.I. of CD86 is less than 150% at all non-cytotoxic concentrations (cell viability ≥ 70%) and if no interference is observed (cytotoxicity, solubility: see paragraph 18 or colour: see paragraph 19 regardless of the non-cytotoxic concentrations at which the interference is detected). In all other cases: S.I. of CD86 higher or equal to 150% and/or interferences observed, the individual conclusion of an U-SENS™ run is considered Positive (hereinafter referred to as P).

- An U-SENS™ prediction is considered NEGATIVE if at least two independent runs are negative (N) (Figure 1). If the first two runs are both negative (N), the U-SENS™ prediction is considered NEGATIVE and a third run does not need to be conducted.

- An U-SENS™ prediction is considered POSITIVE if at least two independent runs are positive (P) (Figure 1). If the first two runs are both positive (P), the U-SENS™ prediction is considered POSITIVE and a third run does not need to be conducted.

- Because a dose finding assay is not conducted, there is an exception if, in the first run, the S.I. of CD86 is higher or equal to 150% at the highest non-cytotoxic concentration only. The run is then considered to be NOT CONCLUSIVE (NC), and additional concentrations (between the highest non cytotoxicity concentration and the lowest cytotoxicity concentration - see paragraph 20) should be tested in additional runs. In case a run is identified as NC, at least 2 additional runs should be conducted, and a fourth run in case runs 2 and 3 are not concordant (N and/or P independently) (Figure 1). Follow up runs will be considered positive even if only one non cytotoxic concentration gives a CD86 equal or above 150%, since

the concentration setting has been adjusted for the specific test chemical. The final prediction will be based on the majority result of the three or four individual runs (i.e. 2 out of 3 or 2 out of 4) (Figure 1).

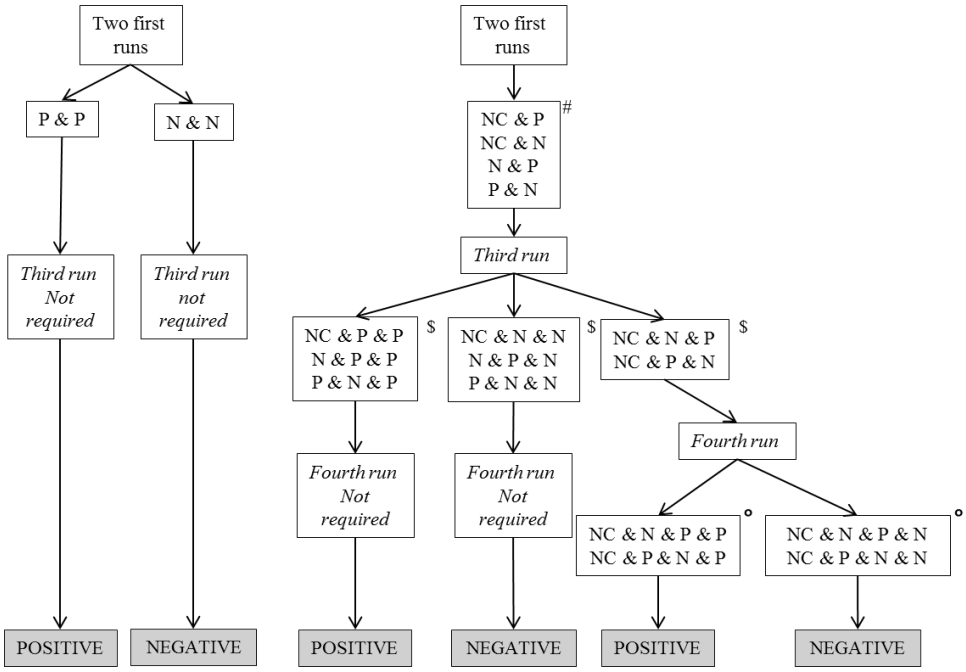


Figure 1: Prediction model used in the U-SENS™ test method. An U-SENS™ prediction should be considered in the framework of an IATA and in accordance with the provision of paragraph 4 and of the General introduction paragraphs 7, 8 and 9.

N: Run with no CD86 positive or interference observed;
P: Run with CD86 positive and/or interference(s) observed;
NC: Not Conclusive. First run with No Conclusion when CD86 is positive at the highest non-cytotoxic concentration only;
#: A Not Conclusive (NC) individual conclusion attributed only to the first run conducts automatically to the need of a third run to reach a majority of Positive (P) or Negative (N) conclusions in at least 2 of 3 independent runs.
$: The boxes show the relevant combinations of results from the three runs on the basis of the results obtained in the first two runs shown in the box above.
°: The boxes show the relevant combinations of results from the four runs on the basis of the results obtained in the first three runs shown in the box above.

Acceptance criteria

22. The following acceptance criteria should be met when using the U-SENS™ method (12).

- At the end of the 45±3 hours exposure period, the mean viability of the triplicate untreated U937 cells had to be > 90% and no drift in CD86 expression is observed. The CD86 basal expression of untreated U937 cells had to be comprised within the range of ≥ 2% and ≤ 25%.

- When DMSO is used as a solvent, the validity of the DMSO vehicle control is assessed by calculating a DMSO S.I. compared to untreated cells, and the mean viability of the triplicate cells had to be > 90%. The DMSO vehicle control is valid if the mean value of its triplicate CD86 S.I. was smaller than 250% of the mean of the triplicate CD86 S.I. of untreated U937 cells.

- The runs are considered valid if at least two out of three IgG1 values of untreated U937 cells fell within the range of ≥ 0.6% and < 1.5%.

- The concurrent tested negative control (lactic acid) is considered valid if at least two out of the three replicates were negative (CD86 S.I. < 150%) and non-cytotoxic (cell viability ≥ 70%).

- The positive control (TNBS) was considered as valid if at least two out of the three replicates were positive (CD86 S.I. ≥ 150%) and non-cytotoxic (cell viability ≥ 70%).

Test report

23. The test report should include the following information.

Test Chemical

- Mono-constituent substance

    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
    - Physical appearance, complete medium solubility, DMSO solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc.;
    - Treatment prior to testing, if applicable (e.g. warming, grinding);
    - Concentration(s) tested;
    - Storage conditions and stability to the extent available;
    - Justification for choice of solvent/vehicle for each test chemical.

- Multi-constituent substance, UVCB and mixture:

    - Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
    - Physical appearance, complete medium solubility, DMSO solubility and additional relevant physicochemical properties, to the extent available;

- Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;
- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Concentration(s) tested;
- Storage conditions and stability to the extent available;
- Justification for choice of solvent/vehicle for each test chemical.

Controls

- Positive control

  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
  - Physical appearance, DMSO solubility, molecular weight, and additional relevant physicochemical properties, to the extent available and where applicable;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc.;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available;
  - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.

- Negative and solvent/vehicle control

  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc.;
  - Physical appearance, molecular weight, and additional relevant physicochemical properties in the case other control solvent/vehicle than those mentioned in the Test Guideline are used and to the extent available;
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent/vehicle for each test chemical.

Test method Conditions

- Name and address of the sponsor, test facility and study director;
- Description of test method used;
- Cell line used, its storage conditions and source (e.g. the facility from which they were obtained);

- Flow cytometry used (e.g. model), including instrument settings, antibodies and cytotoxicity marker used;
- The procedure used to demonstrate proficiency of the laboratory in performing the test method by testing of proficiency substances, and the procedure used to demonstrate reproducible performance of the test method over time, e.g. historical control data and/or historical reactivity checks' data.

Test Acceptance Criteria

- Cell viability and CD86 S.I values obtained with the solvent/vehicle control in comparison to the acceptance ranges;
- Cell viability and S.I. values obtained with the positive control in comparison to the acceptance ranges;
- Cell viability of all tested concentrations of the tested chemical.

Test procedure

- Number of runs used;
- Test chemical concentrations, application and exposure time used (if different than the one recommended)
- Duration of exposure;
- Description of evaluation and decision criteria used;
- Description of any modifications of the test procedure.

Results

- Tabulation of the data, including CV70 (if applicable), S.I., cell viability values, EC150 values (if applicable) obtained for the test chemical and for the positive control in each run, and an indication of the rating of the test chemical according to the prediction model;
- Description of any other relevant observations, if applicable.

Discussion of the Results

- Discussion of the results obtained with the U-SENS™ method;
- Consideration of the test method results within the context of an IATA, if other relevant information is available.

Conclusions

LITERATURE

1. Piroird, C., Ovigne, J.M., Rousset, F., Martinozzi-Teissier, S., Gomes, C., Cotovio, J., Alépée, N. (2015). The Myeloid U937 Skin Sensitization Test (U-SENS) addresses the activation of dendritic cell event in the adverse outcome pathway for skin sensitization. *Toxicol. In Vitro* 29, 901-916.
2. EURL ECVAM (2017). The U-SENS™ test method Validation Study Report. Accessible at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations
3. EC EURL ECVAM (2016). ESAC Opinion No. 2016-03 on the L'Oréal-coordinated study on the transferability and reliability of the U-SENS™ test method for skin sensitisation testing. EUR 28178 EN; doi 10.2787/815737. Available at: [http://publications.jrc.ec.europa.eu/repository/handle/JRC103705].
4. EC EURL ECVAM (2017). EURL ECVAM Recommendation on the use of non-animal approaches for skin sensitisation testing. EUR 28553 EN; doi 10.2760/588955. Available at: [https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/eurl-ecvam-recommendation-use-non-animal-approaches-skin-sensitisation-testing].
5. Steiling, W. (2016). Safety Evaluation of Cosmetic Ingredients Regarding their Skin Sensitization Potential. doi:10.3390/cosmetics3020014.*Cosmetics* 3, 14.
6. OECD (2016). Guidance Document On The Reporting Of Defined Approaches And Individual Information Sources To Be Used Within Integrated Approaches To Testing And Assessment (IATA) For Skin Sensitisation, Series on Testing & Assessment No. 256, ENV/JM/MONO(2016)29. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/series-testing-assessment-publications-number.htm].
7. Urbisch, D., Mehling, A., Guth, K., Ramirez, T., Honarvar, N., Kolle, S., Landsiedel, R., Jaworska, J., Kern, P.S., Gerberick, F., Natsch, A., Emter, R., Ashikaga, T., Miyazawa, M., Sakaguchi, H. (2015). Assessing skin sensitization hazard in mice and men using non-animal test methods. *Regul. Toxicol. Pharmacol.* 71, 337-351.
8. Alépée, N., Piroird, C., Aujoulat, M., Dreyfuss, S., Hoffmann, S., Hohenstein, A., Meloni, M., Nardelli, L., Gerbeix, C., Cotovio, J. (2015). Prospective multicentre study of the U-SENS test method for skin sensitization testing. *Toxicol In Vitro* 30, 373-382.
9. Reisinger, K., Hoffmann, S., Alépée, N., Ashikaga, T., Barroso, J., Elcombe, C., Gellatly, N., Galbiati, V., Gibbs, S., Groux, H., Hibatallah, J., Keller, D., Kern, P., Klaric, M., Kolle, S., Kuehnl, J., Lambrechts, N., Lindstedt, M., Millet, M., Martinozzi-Teissier, S., Natsch, A., Petersohn, D., Pike, I., Sakaguchi, H., Schepky, A., Tailhardat, M., Templier, M., van Vliet, E., Maxwell, G. (2014). Systematic evaluation of non-animal test methods for skin sensitisation safety assessment. *Toxicol. In Vitro* 29, 259-270.
10. Fabian, E., Vogel, D., Blatz, V., Ramirez, T., Kolle, S., Eltze, T., van Ravenzwaay, B., Oesch, F., Landsiedel, R. (2013). Xenobiotic metabolizin enzyme activities in cells used for testing skin sensitization *in vitro*. *Arch. Toxicol.* 87, 1683-1696.
11. OECD. (2017). Draft Guidance document: Good *In Vitro* Method Practices (GIVIMP) for the Development and Implementation of *In Vitro* Methods for Regulatory Use in Human Safety Assessment. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/OECD%20Draft%20GIVIMP_v05%20-%20clean.pdf].

12. DB-ALM (2016). Protocol no. 183: Myeloid U937 Skin Sensitization Test (U-SENS™), 33pp. Accessible at: [http://ecvam-dbalm.jrc.ec.europa.eu/].

13. Sundström, C., Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer* 17, 565-577.

14. OECD (2005). Series on Testing and Assessment No. 34: Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/series-testing-assessment-publications-number.htm].

15. United Nations UN (2015). Globally Harmonized System of Classification and Labelling of Chemicals (GHS). ST/SG/AC.10/30/Rev.6, Sixth Revised Edition,New York & Geneva: United Nations Publications. Available at: [http://www.unece.org/fileadmin/DAM/trans/danger/publi/ghs/ghs_rev06/English/ST-SG-AC10-30-Rev6e.pdf].

16. OECD (2012). Series on Testing and Assessment No. 168: The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/series-testing-assessment-publications-number.htm].

17. ECETOC (2003). Technical Report No. 87: Contact sensitization: Classification according to potency. European Centre for Ecotoxicology & Toxicology of Chemicals, Brussels. Available at: [https://ftp.cdc.gov/pub/Documents/OEL/06.%20Dotson/References/ECETOC_2003-TR87.pdf].

APPENDIX I

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with concordance to mean the proportion of correct outcomes of a test method (14).

AOP (Adverse Outcome Pathway): sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an *in vivo* outcome of interest (15).

CD86 Concentration response: There is concentration-dependency (or concentration response) when a positive concentration (CD86 S.I. ≥ 150) is followed by a concentration with an increasing CD86 S.I.

CV70: The estimated concentration showing 70% cell viability.

Drift: A drift is defined by i) the corrected %$CD86^+$ value of the untreated control replicate 3 is less than 50% of the mean of the corrected %$CD86^+$ value of untreated control replicates 1and 2; and ii) the corrected %$CD86^+$ value of the negative control replicate 3 is less than 50% of mean of the corrected %$CD86^+$ value of negative control replicates 1and 2.

EC150: the estimated concentrations showing the 150% S.I. of CD86 expression.

Flow cytometry: a cytometric technique in which cells suspended in a fluid flow one at a time through a focus of exciting light, which is scattered in patterns characteristic to the cells and their components; cells are frequently labeled with fluorescent markers so that light is first absorbed and then emitted at altered frequencies.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

IATA (Integrated Approach to Testing and Assessment): A structured approach used for hazard identification (potential), hazard characterisation (potency) and/or safety assessment (potential/potency and exposure) of a chemical or group of chemicals, which strategically integrates and weights all relevant data to inform regulatory decision regarding potential hazard and/or risk and/or the need for further targeted and therefore minimal testing.

Mixture: A mixture or a 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).

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.

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.

Pre-haptens: chemicals which become sensitisers through abiotic transformation, e.g. through oxidation.

Pro-haptens: chemicals requiring enzymatic activation to exert skin sensitisation potential.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (14).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (14).

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

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (14).

S.I.: Stimulation Index. Relative values of geometric mean fluorescence intensity in chemical-treated cells compared to solvent-treated cells.

Solvent/vehicle control: An untreated sample containing all components of a test system except of the test chemical, but including the solvent/vehicle that is used. It is used to establish the baseline response for the samples treated with the test chemical dissolved or stably dispersed in the same solvent/vehicle. When tested with a concurrent medium control, this sample also demonstrates whether the solvent/vehicle interacts with the test system.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (14).

Staining buffer: A phosphate buffered saline containing 5% foetal calf serum.

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing it composition.

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

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (16).

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

Valid test method: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (14).

APPENDIX II

PROFICIENCY SUBSTANCES

Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency by correctly obtaining the expected U-SENS™ prediction for the 10 substances recommended in Table 1 and by obtaining CV70 and EC150 values that fall within the respective reference range for at least 8 out of the 10 proficiency substances. Proficiency substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were that the substances are commercially available, and that high-quality *in vivo* reference data as well as high quality *in vitro* data generated with the U-SENS™ method are available. Also, published reference data are available for the U-SENS™ method (1) (8).

Table 1: Recommended substances for demonstrating technical proficiency with the U-SENS™ method

Proficiency substances	**CASRN**	**Physical state**	***In vivo* prediction[1]**	**U-SENS™ Solvent/ Vehicle**	**U-SENS™ CV70 Reference Range in µg/mL[2]**	**U-SENS™ EC150 Reference Range in µg/mL[2]**
4-Phenylenediamine	106-50-3	Solid	Sensitiser (strong)	Complete medium[3]	<30	Positive (≤10)
Picryl sulfonic acid	2508-19-2	Liquid	Sensitizer (strong)	Complete medium	>50	Positive (≤50)
Diethyl maleate	141-05-9	Liquid	Sensitiser (moderate)	DMSO	10-100	Positive (≤20)
Resorcinol	108-46-3	Solid	Sensitiser (moderate)	Complete medium	>100	Positive (≤50)
Cinnamic alcohol	104-54-1	Solid	Sensitiser (weak)	DMSO	>100	Positive (10-100)
4-Allylanisole	140-67-0	Liquid	Sensitiser (weak)	DMSO	>100	Positive (<200)
Saccharin	81-07-2	Solid	Non-sensitiser	DMSO	>200	Negative (>200)
Glycerol	56-81-5	Liquid	Non-sensitiser	Complete medium	>200	Negative (>200)
Lactic acid	50-21-5	Liquid	Non-sensitiser	Complete medium	>200	Negative (>200)
Salicylic acid	69-72-7	Solid	Non-sensitiser	DMSO	>200	Negative (>200)

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

[1] The *in vivo* hazard and (potency) prediction is based on LLNA data (1) (8). The *in vivo* potency is derived using the criteria proposed by ECETOC (17).

[2] Based on historical observed values (1) (8).

[3] Complete medium: RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (8).

ANNEX III: IN VITRO SKIN SENSITISATION: IL-8 LUC ASSAY

INITIAL CONSIDERATIONS AND LIMITATIONS

1. In contrast to assays analysing the expression of cell surface markers, the IL8-Luc assay quantifies changes in IL-8 expression, a cytokine associated with the activation of dendritic cells (DC). In the THP-1-derived IL-8 reporter cell line (THP-G8, established from the human acute monocytic leukemia cell line THP-1), IL-8 expression is measured following exposure to sensitisers (1). The expression of luciferase is then used to aid discrimination between skin sensitisers and non-sensitisers.

2. The IL-8 Luc method has been evaluated in a validation study (2) conducted by the Japanese Centre for the Validation of Alternatives Methods (JaCVAM), the **Ministry of Economy, Trade and Industry** (METI), and the Japanese Society **for Alternatives to Animal Experiments (JSAAE**) and subsequently subjected to independent peer review (3) under the auspices of JaCVAM and the Ministry of Health, Labour and Welfare (MHLW) with the support of the **International Cooperation on Alternative Test** Methods (ICATM). Considering all available evidence and input from regulators and stakeholders, the IL-8 Luc assay is considered useful as part of IATA to discriminate sensitisers from non-sensitisers for the purpose of hazard classification and labelling. Examples of the use of IL-8 Luc assay data in combination with other information are reported in the literature (4) (5) (6).

3. The IL-8 Luc assay proved to be transferable to laboratories experienced in cell culture and luciferase measurement. Within and between laboratory reproducibilities were 87.7% and 87.5%, respectively (2). Data generated in the validation study (2) and other published work (1) (6) show that versus the LLNA, the IL-8 Luc assay judged 118 out of 143 chemicals as positive or negative and judged 25 chemicals as inconclusive and the accuracy of the IL-8 Luc assay in distinguishing skin sensitisers (UN GHS Cat. 1) from non-sensitisers (UN GHS No Cat.) is 86% (101/118) with a sensitivity of 96% (92/96) and specificity of 41% (9/22). Excluding substances outside the applicability domain described below (paragraph 5), the IL-8 Luc assay judged 113 out of 136 chemicals as positive or negative and judged 23 chemicals as inconclusive and the accuracy of the IL-8 Luc assay is 89% (101/113) with sensitivity of 96% (92/96) and specificity of 53% (9/17). Using human data cited in Urbisch et al. (7), the IL-8 Luc assay judged 76 out of 90 chemicals as positive or negative and judged 14 chemicals as inconclusive and the accuracy is 80% (61/76), sensitivity is 93% (54/58) and specificity is 39% (7/18). Excluding substances outside the applicability domain, the IL-8 Luc assay judged 71 out of 84 chemicals as positive or negative and judged 13 chemicals as inconclusive and the accuracy is 86% (61/71） with sensitivity of 93% (54/58) and specificity of 54% (7/13). False negative predictions with the IL-8 Luc assay are more likely to occur with chemicals showing low/moderate skin sensitisation potency (UN GHS subcategory 1B) than those with high potency (UN GHS subcategory 1A) (6). Together, the information supports a role for the IL-8 Luc assay in the identification of skin sensitisation hazards. The accuracy given for the IL-8 Luc assay as a standalone test method is only for guidance, as the method should be considered in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General introduction. Furthermore, when evaluating non-animal methods for skin sensitisation, it should be remembered that the LLNA and other animal tests may not fully reflect the situation in humans.

4. On the basis of the data currently available, the IL-8 Luc assay was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined in *in vivo* studies) and physicochemical properties (2) (6).

5. Although the IL-8 Luc assay uses X-VIVO™ 15 as a solvent, it correctly evaluated chemicals with a Log $K_{o/w}$ >3.5 and those with a water solubility of around 100 µg/ mL as calculated by EPI Suite™ and its performance to detect sensitisers with poor water solubility is better than that of the IL-8 Luc assay using dimethyl sulfoxide (DMSO) as a solvent (2). However, negative results for test chemicals that are not dissolved at 20 mg/ml may produce false negative results due to their inability to dissolve in X-VIVO™ 15. Therefore, negative results for these chemicals should not be considered. A high false negative rate for anhydrides was seen in the validation study. Furthermore, because of the limited metabolic capability of the cell line (8) and the experimental conditions, pro-haptens (substances requiring metabolic activation) and pre-haptens (substances activated by air oxidation) might give negative results in the assay. However, although negative results for suspected pre/prohaptens should be interpreted with caution, the IL-8 Luc assay correctly judged 11 out of 11 pre-haptens, 6/6 pro-haptens, and 6/8 pre/pro-haptens in the IL-8 Luc assay data set (2). Based on the recent comprehensive review on three non-animal methods (the DPRA, the KeratinoSens™ and the h-CLAT) to detect pre and prohaptens (9), and based on the fact that THP-G8 cells used in the IL-8 Luc assay is a cell line derived from THP-1 that is used in the h-CLAT, the IL-8 Luc assay may also contribute to increase the sensitivity of non-animal methods to detect pre and pro-haptens in the combination of other methods. Surfactants tested so far gave (false) positive results irrespective of their type (e.g. cationic, anionic or on-ionic). Finally, chemicals that interfere with luciferase can confound its activity/measurement, causing apparent inhibition or increased luminescence (10). For example, phytoestrogen concentrations higher than 1µM were reported to interfere with luminescence signals in other luciferase-based reporter gene assays due to over-activation of the luciferase reporter gene. Consequently, luciferase expression obtained at high concentrations of phytoestrogens or compounds suspected of producing phytoestrogen-like activation of the luciferase reporter gene needs to be examined carefully (11). Based on the above, surfactants, anhydrides and chemicals interfering with luciferase are outside the applicability domain of this assay. In cases where there is evidence demonstrating the non-applicability of the IL-8 Luc assay to other specific categories of test chemicals, the method should not be used for those specific categories.

6. As described above, the IL-8 Luc assay supports discrimination of skin sensitisers from non-sensitisers. Further work, preferably based on human data, is required to determine whether IL-8 Luc results can contribute to potency assessment when considered in combination with other information sources.

7. Definitions are provided in Appendix I.

PRINCIPLE OF THE TEST

8. The IL-8 Luc assay makes use of a human monocytic leukemia cell line THP-1 that was obtained from the American Type Culture Collection (Manassas, VA, USA). Using this cell line, the Dept. of Dermatology, Tohoku University School of Medicine, established a THP-1-derived IL-8 reporter cell line, THP-G8, that harbours the Stable Luciferase Orange (SLO) and Stable Luciferase Red (SLR) luciferase genes under the control of the IL-8 and **glyceraldehyde 3-phosphate dehydrogenase (**GAPDH) promoters, respectively (1). This allows quantitative measurement of luciferase gene induction by detecting luminescence from well-established light producing luciferase substrates as an indicator of the activity of the IL-8 and GAPDH in cells following exposure to sensitising chemicals.

9. The dual-colour assay system comprises an orange-emitting luciferase (SLO; λmax = 580 nm) (12) for the gene expression of the IL-8 promoter as well as a red-emitting luciferase (SLR; λmax = 630 nm) (13) for the gene expression of the internal control promoter, GAPDH. The two luciferases emit different colours upon reacting with firefly D-luciferin and their luminescence is measured simultaneously in a one-step reaction by dividing the emission from the assay mixture using an optical filter (14) (Appendix II).

10. THP-G8 cells are treated for 16 hours with the test chemical, after which SLO luciferase activity (SLO-LA) reflecting IL-8 promoter activity and SLR luciferase activity (SLR-LA) reflecting GAPDH promoter activity are measured. To make the abbreviations easy to understand, SLO-LA and SLR-LA are designated as IL8LA and GAPLA, respectively. Table 1 gives a description of the terms associated with luciferase activity in the IL-8 Luc assay. The measured values are used to calculate the normalised IL8LA (nIL8LA), which is the ratio of IL8LA to GAPLA; the induction of nIL8LA (Ind-IL8LA), which is the ratio of the arithmetic means of quadruple-measured values of the nIL8LA of THP-G8 cells treated with a test chemical and the values of the nIL8LA of untreated THP-G8 cells; and the inhibition of GAPLA (Inh-GAPLA), which is the ratio of the arithmetic means of quadruple-measured values of the GAPLA of THP-G8 cells treated with a test chemical and the values of the GAPLA of untreated THP-G8 cells, and used as an indicator for cytotoxicity.

Table 1. Description of terms associated with the luciferase activity in the IL-8 Luc assay

Abbreviations	Definition
GAPLA	SLR luciferase activity reflecting GAPDH promoter activity
IL8LA	SLO luciferase activity reflecting IL-8 promoter activity
nIL8LA	IL8LA / GAPLA
Ind-IL8LA	nIL8LA of THP-G8 cells treated with chemicals / nIL8LA of untreated cells
Inh-GAPLA	GAPLA of THP-G8 treated with chemicals / GAPLA of untreated cells
CV05	The lowest concentration of the chemical at which Inh-GAPLA becomes < 0.05.

11. Performance standards (PS) (15) are available to facilitate the validation of modified *in vitro* IL-8 luciferase test methods similar to the IL-8 Luc assay and allow for timely amendment of this Test Guideline for their inclusion. Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to the PS, if these test methods have been reviewed and included in this Test Guideline by the OECD (16).

DEMONSTRATION OF PROFICIENCY

12. Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency, using the 9 Proficiency Substances listed in Appendix III in compliance with the Good in vitro Method Practices (17). Moreover, test method users should maintain a historical database of data generated with the reactivity checks (see paragraph 15) and with the positive and solvent/vehicle controls (see paragraphs 21-24), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

PROCEDURE

13. The Standard Operating Procedure (SOP) for the IL-8 Luc assay is available and should be employed when performing the test (18). Laboratories willing to perform the test can obtain the recombinant THP-G8 cell line from GPC Lab. Co. Ltd., Tottori, Japan, upon signing a Material Transfer Agreement (MTA) in line with the conditions of the OECD template. The following paragraphs provide a description of the main components and procedures of the assay.

Preparation of cells

14. The THP-G8 cell line from GPC Lab. Co. Ltd., Tottori, Japan, should be used for performing the IL-8 Luc assay (see paragraphs 8 and 13). On receipt, cells are propagated (2-4 passages) and stored frozen as a homogeneous stock. Cells from this stock can be propagated up to a maximum of 12 passages or a maximum of 6 weeks. The medium used for propagation is the RPMI-1640 culture medium containing 10% foetal bovine serum (FBS), antibiotic/antimycotic solution (100U/mL of penicillin G, 100µg/mL of streptomycin and 0.25µg/mL of amphotericin B in 0.85% saline) (e.g. GIBCO Cat#15240-062), 0.15µg/mL Puromycin (e.g. CAS:58-58-2) and 300µg/mL G418 (e.g. CAS:108321-42-2).

15. Prior to use for testing, the cells should be qualified by conducting a reactivity check. This check should be performed 1-2 weeks or 2-4 passages after thawing, using the positive control, 4-nitrobenzyl bromide (4-NBB) (CAS:100-11-8, ≥ 99% purity) and the negative control, lactic acid (LA) (CAS:50-21-5, ≥85% purity). 4-NBB should produce a positive response to Ind-IL8LA (≥1.4), while LA should produce a negative response to Ind-IL8LA (<1.4). Only cells that pass the reactivity check are used for the assay. The check should be performed according to the procedures described in paragraphs 22-24.

16. For testing, THP-G8 cells are seeded at a density of 2 to 5 × 10^5 cells/mL, and pre-cultured in culture flasks for 48 to 96 hours. On the day of the test, cells harvested from the culture flask are washed with RPMI-1640 containing 10% FBS without any antibiotics, and then, resuspended with RPMI-1640 containing 10% FBS without any antibiotics at 1 × 10^6 cells/mL. Then, cells are distributed into a 96-well flat-bottom black plate (e.g. Costar Cat#3603) with 50µL (5 × 10^4 cells/well).

Preparation of the test chemical and control substances

17. The test chemical and control substances are prepared on the day of testing. For the IL-8 Luc assay, test chemicals are dissolved in X-VIVO™ 15, a commercially available serum-free medium (Lonza, 04-418Q), to the final concentration of 20 mg/mL. X-VIVO™ 15 is added to 20 mg of test chemical (regardless of the chemical's solubility) in a microcentrifuge tube and brought to a volume of 1mL and then vortexed vigorously and shaken on a rotor at a maximum speed of 8 rpm for 30 min at an ambient temperature of about 20°C. Furthermore, if solid chemicals are still insoluble, the tube is sonicated until the chemical is dissolved completely or stably dispersed. For test chemicals soluble in X-VIVO™ 15, the solution is diluted by a factor of 5 with X-VIVO™ 15 and used as an X-VIVO™ 15 stock solution of the test chemical (4 mg/mL). For test chemicals not soluble in X-VIVO™ 15, the mixture is rotated again for at least 30 min, then centrifuged at 15,000 rpm (≈20,000*g*) for 5 min; the resulting supernatant is used as an X-VIVO™ 15 stock solution of the test chemical. A scientific rationale should be provided for the use of other solvents, such as DMSO, water, or the culture medium. The detailed procedure for dissolving chemicals is shown in Appendix V. The X-VIVO™ 15 solutions described in paragraphs 18-23 are mixed 1:1 (v/v) with the cell suspensions prepared in a 96-well flat-bottom black plate (see paragraph 16).

18. The first test run is aimed to determine the cytotoxic concentration and to examine the skin sensitising potential of chemicals. Using X-VIVO™ 15, serial dilutions of the X-VIVO™ 15 stock solutions of the test chemicals are made at a dilution factor of two (see Appendix V) using a 96-well assay block (e.g. Costar Cat#EW-01729-03). Next, 50 µl/well of diluted solution is added to 50 µl of the cell suspension in a 96-well flat-bottom black plate. Thus for test chemicals that are soluble in X-VIVO ™ 15, the final concentrations of the test chemicals range from 0.002 to 2 mg/mL (Appendix V). For test chemicals that are not soluble in X-VIVO ™ 15 at 20 mg/mL, only dilution factors that range from 2 to 2^{10}, are determined, although the actual final concentrations of the test chemicals remain uncertain and are dependent on the saturated concentration of the test chemicals in the X-VIVO ™ 15 stock solution.

19. In subsequent test runs (i.e. the second, third, and fourth replicates), the X-VIVO™ 15 stock solution is made at the concentration 4 times higher than the concentration of cell viability 05 (CV05; the lowest concentration at which the Inh-GAPLA becomes <0.05) in the first experiment. If Inh-GAPLA does not decrease below 0.05 at the highest concentration in the first run, the X-VIVO™ 15 stock solution is made at the first run highest concentration. The concentration of CV05 is calculated by dividing the concentration of the stock solution in the first run by dilution factor for CV05 (X) (dilution factor CV05 (X); the dilution factor required to dilute stock solution to CV05) (see Appendix V). For test substances not soluble in X-VIVO at 20 mg/ml, CV05 is determined by the concentration of the stock solution x 1/X. For run 2 to 4, a second stock solution is prepared as 4 x CV50 (Appendix V).

20. Serial dilutions of the X-VIVO™ 15 second stock solutions are made at a dilution factor of 1.5 using a 96-well assay block. Next, 50 µl/well of diluted solution is added to 50 µl of the cell suspension in the wells of a 96-well flat-bottom black plate. Each concentration of each test chemical should be tested in 4 wells. The samples are then mixed on a plate shaker and incubated for 16 hours at 37°C and 5% CO_2, after which the luciferase activity is measured as described below.

21. The solvent control is the mixture of 50 µL/well of X-VIVO™ 15 and 50 µL/well of cell suspension in RPMI-1640 containing 10% FBS.

22. The recommended positive control is 4-NBB. 20 mg of 4-NBB is prepared in a 1.5-mL microfuge tube, to which X-VIVO™ 15 is added up to 1 mL. The tube is vortexed vigorously and shaken on a rotor at a maximum speed of 8 rpm for at least 30 min. After centrifugation at 20,000g for 5 min, the supernatant is diluted by a factor of 4 with X-VIVO™ 15, and 500 µl of the diluted supernatant is transferred to a well in a 96-well assay block. The diluted supernatant is further diluted with X-VIVO™ 15 at factors of 2 and 4, and 50 µl of the solution is added to 50 µl of THP-G8 cell suspension in the wells of a 96-well flat-bottom black plate (Appendix VI). Each concentration of the positive control should be tested in 4 wells. The plate is agitated on a plateshaker, and incubated in a CO_2 incubator for 16 hours (37°C, 5% CO_2), after which the luciferase activity is measured as described in paragraph 29.

23. The recommended negative control is LA. 20 mg of LA prepared in a 1.5-mL microfuge tube, to which X-VIVO™ 15 is added up to 1 mL (20 mg/ mL). Twenty mg/mL of LA solution is diluted by a factor of 5 with X-VIVO™ 15 (4 mg/mL); 500 µl of this 4 mg/mL LA solution is transferred to a well of a 96-well assay block. This solution is diluted by a factor of 2 with X-VIVO™ 15 and then diluted again by a factor of 2 to produce 2 mg/mL and 1 mg/mL solutions. 50 µl of these 3 solutions and vehicle control (X-VIVO™ 15) are added to 50 µl of THP-G8 cell suspension in the wells of a 96-well flat-bottom black plate. Each concentration of the negative control is tested in 4 wells. The plate is agitated on a plateshaker and incubated in a CO_2 incubator for 16 hours (37°C, 5% CO_2), after which the luciferase activity is measured as described in paragraph 29.

24. Other suitable positive or negative controls may be used if historical data are available to derive comparable run acceptance criteria.

25. Care should be taken to avoid evaporation of volatile test chemicals and cross-contamination between wells by test chemicals, e.g. by sealing the plate prior to the incubation with the test chemicals.

26. The test chemicals and solvent control require 2 to 4 runs to derive a positive or negative prediction (see Table 2). Each run is performed on a different day with fresh X-VIVO™ 15 stock solution of test chemicals and independently harvested cells. Cells may come from the same passage.

Luciferase activity measurements

27. Luminescence is measured using a 96-well microplate luminometer equipped with optical filters, e.g. Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany) and the ARVO series (PerkinElmer, Waltham, MA, USA). The luminometer must be calibrated for each test to ensure reproducibility (19). Recombinant orange and red emitting luciferases are available for this calibration.

28. 100µL of pre-warmed Tripluc® Luciferase assay reagent (Tripluc) is transferred to each well of the plate containing the cell suspension treated with or without chemical. The plate is shaken for 10 min at an ambient temperature of about 20°C. The plate is placed in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter. Justification should be provided for the use of alternative settings, e.g. depending on the model of luminometer used.

29. Parameters for each concentration are calculated from the measured values, e.g. IL8LA, GAPLA, nIL8LA, Ind-IL8LA, Inh-GAPLA, the mean ±SD of IL8LA, the mean ±SD of GAPLA, the mean ±SD of nIL8LA, the mean ±SD of Ind-IL8LA, the mean ±SD of Inh-GAPLA, and the 95% confidence interval of Ind-IL8LA. Definitions of the parameters used in this paragraph are provided in Appendices I and IV, respectively.

30. Prior to measurement, colour discrimination in multi-colour reporter assays is generally achieved using detectors (luminometer and plate reader) equipped with optical filters, such as sharp-cut (long-pass or short-pass) filters or band-pass filters. The transmission coefficients of the filters for each bioluminescence signal colour should be calibrated prior to testing, per Appendix II.

DATA AND REPORTING

Data evaluation

31. Criteria for a positive/negative decision require that in each run:

- an IL-8 Luc assay prediction is judged positive if a test chemical has a Ind-IL8LA ≥ 1.4 and the lower limit of the 95% confidence interval of Ind-IL8LA ≥ 1.0
- an IL-8 Luc assay prediction is judged negative if a test chemical has a Ind-IL8LA < 1.4 and/or the lower limit of the 95% confidence interval of Ind-IL8LA < 1.0

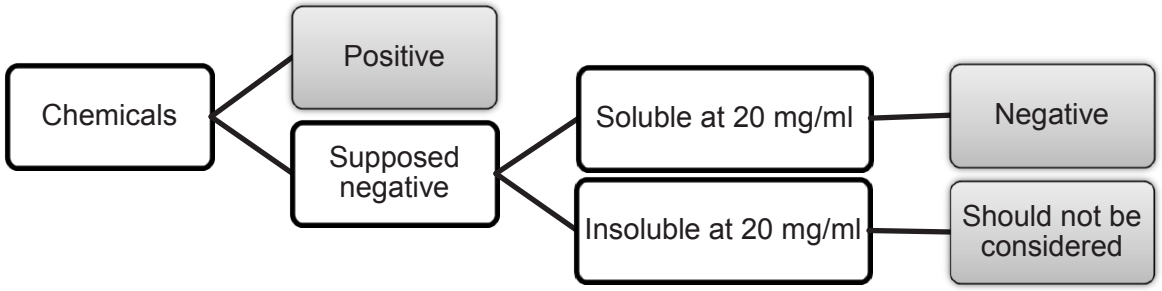
Prediction model

32. Test chemicals that provide two positive results from among the 1st, 2nd, 3rd or 4th runs are identified as positives whereas those that give three negative results from among the 1st, 2nd, 3rd or 4th runs are identified as supposed negative (Table 2). Among supposed negative chemicals, chemicals that are dissolved at 20 mg/ml of X-VOVO™ 15 are judged as negative, while chemicals that are not dissolved at 20 mg/ml of X-VOVO™ 15 should not be considered (Figure 1).

Table 2. Criteria for identifying positive and supposed negative

1st run	2nd run	3rd run	4th run	Final prediction
Positive	Positive	-	-	Positive
	Negative	Positive	-	Positive
		Negative	Positive	Positive
			Negative	Supposed negative
Negative	Positive	Positive	-	Positive
		Negative	Positive	Positive
			Negative	Supposed negative
	Negative	Positive	Positive	Positive
			Negative	Supposed negative
		Negative	-	Supposed negative

Figure 1. Prediction model for final judgment



Acceptance criteria

33. The following acceptance criteria should be met when using the IL-8 Luc assay:

- Ind-IL8LA should be more than 5.0 at least in one concentration of the positive control, 4-NBB, in each run.
- Ind-IL8LA should be less than 1.4 at any concentration of the negative control, lactic acid, in each run.
- Data from plates for which the GAPLA of control wells with cells and Tripluc but without chemicals is less than 5 times of that of well containing test medium only (50 µL/well of RPMI-1640 containing 10% FBS and 50 µL/well of X-VIVO™ 15) should be rejected.
- Data from plates for which the Inh-GAPLA of all concentrations of the test or control chemicals is less than 0.05 should be rejected. In this case, the first test should be repeated so the highest final concentration of the repeated test is the lowest final concentration of the previous test.

TEST REPORT

34. The test report should include the following information:

Test chemicals

- Mono-constituent substance:
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
    - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc;

- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Solubility in X-VIVO™ 15. For chemicals that are insoluble in X-VIVO™ 15, whether precipitation or flotation are observed after centrifugation;
- Concentration(s) tested;
- Storage conditions and stability to the extent available;
- Justification for choice of solvent/vehicle for each test chemical if X-VIVO™ 15 has not been used.

- Multi-constituent substance, UVCB and mixture:

- Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
- Physical appearance, water solubility, and additional relevant physicochemical properties, to the extent available;
- Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant relevant for the conduct of the study;
- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Solubility in X-VIVO™ 15. For chemicals that are insoluble in X-VIVO™ 15, whether precipitation or flotation are observed after centrifugation;
- Concentration(s) tested;
- Storage conditions and stability to the extent available.
- Justification for choice of solvent/vehicle for each test chemical, if X-VIVO™ 15 has not been used.

Controls

- Positive control:

- Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
- Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available and where applicable;
- Purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Concentration(s) tested;
- Storage conditions and stability to the extent available;
- Reference to historical positive control results demonstrating suitable acceptance criteria, if applicable.

- Negative control:

    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers;
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
    - Physical appearance, molecular weight, and additional relevant physicochemical properties in the case other negative controls than those mentioned in the Test Guideline are used and to the extent available;
    - Storage conditions and stability to the extent available;
    - Justification for choice of solvent for each test chemical.

Test method conditions

- Name and address of the sponsor, test facility and study director;
- Description of test method used;
- Cell line used, its storage conditions, and source (e.g. the facility from which it was obtained);
- Lot number and origin of FBC, supplier name, lot number of 96-well flat-bottom black plate, and lot number of Tripluc reagent;
- Passage number and cell density used for testing;
- Cell counting method used for seeding prior to testing and measures taken to ensure homogeneous cell number distribution;
- Luminometer used (e.g. model), including instrument settings, luciferase substrate used, and demonstration of appropriate luminescence measurements based on the control test described in Appendix II;
- The procedure used to demonstrate proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or to demonstrate reproducible performance of the test method over time.

Test procedure

- Number of replicates and runs performed;
- Test chemical concentrations, application procedure and exposure time (if different from those recommended);
- Description of evaluation and decision criteria used;
- Description of study acceptance criteria used;
- Description of any modifications of the test procedure.

Results

- Measurements of IL8LA and GAPLA;
- Calculations for nIL8LA, Ind-IL8LA, and Inh-GAPLA;
- The 95% confidence interval of Ind-IL8LA;
- A graph depicting dose-response curves for induction of luciferase activity and viability;
- Description of any other relevant observations, if applicable.

Discussion of the results

- Discussion of the results obtained with the IL-8 Luc assay;
- Consideration of the assay results in the context of an IATA, if other relevant information is available.

Conclusion

LITERATURE

1. Takahashi T, Kimura Y, Saito R, Nakajima Y, Ohmiya Y, Yamasaki K, and Aiba S. 2011. An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. Toxicol Sci 124:359-69.

2. OECD (2017), Validation report for the international validation study on the IL-8 Luc assay as a test evaluating the skin sensitizing potential of chemicals conducted by the IL-8 Luc Assay. Series on Testing and Assessment No. 267, ENV/JM/MONO(2017)19. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/series-testing-assessment-publications-number.htm].

3. OECD (2017), Report of the Peer Review Panel for the IL-8 Luciferase (IL-8 Luc) Assay for in vitro skin sensitisation. Series on Testing and Assessment No. 258, ENV/JM/MONO(2017)20. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/series-testing-assessment-publications-number.htm].

2. OECD (2016), Guidance Document On The Reporting Of Defined Approaches And Individual Information Sources To Be Used Within Integrated Approaches To Testing And Assessment (IATA) For Skin Sensitisation, Series on Testing & Assessment No. 256, ENV/JM/MONO(2016)29. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/series-testing-assessment-publications-number.htm].

3. van der Veen JW, Rorije E, Emter R, Natsch A, van Loveren H, and Ezendam J. 2014. Evaluating the performance of integrated approaches for hazard identification of skin sensitizing chemicals. Regul Toxicol Pharmacol 69:371-9.

4. Kimura Y, Fujimura C, Ito Y, Takahashi T, Nakajima Y, Ohmiya Y, and Aiba S. 2015. Optimization of the IL-8 Luc assay as an in vitro test for skin sensitization. Toxicol In Vitro 29:1816-30.

5. Urbisch D, Mehling A, Guth K, Ramirez T, Honarvar N, Kolle S, Landsiedel R, Jaworska J, Kern PS, Gerberick F, et al. 2015. Assessing skin sensitization hazard in mice and men using non-animal test methods. Regul Toxicol Pharmacol 71:337-51.

6. Ashikaga T, Sakaguchi H, Sono S, Kosaka N, Ishikawa M, Nukada Y, Miyazawa M, Ito Y, Nishiyama N, and Itagaki H. 2010. A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). Alternatives to laboratory animals : ATLA 38:275-84.

7. Patlewicz G, Casati S, Basketter DA, Asturiol D, Roberts DW, Lepoittevin J-P, Worth A and Aschberger K (2016) Can currently available non-animal methods detect pre and pro haptens relevant for skin sensitisation? Regul Toxicol Pharmacol, online.

8. Thorne N, Inglese J, and Auld DS. 2010. Illuminating insights into firefly luciferase and other bioluminescent reporters used in chemical biology. Chem Biol 17:646-57.

9. OECD (2016), *Test No. 455: Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and Antagonists*, OECD Publishing, Paris. http://dx.doi.org/10.1787/9789264265295-en.

10. Viviani V, Uchida A, Suenaga N, Ryufuku M, and Ohmiya Y. 2001. Thr226 is a key residue for bioluminescence spectra determination in beetle luciferases. Biochem Biophys Res Commun 280:1286-91.

11. Viviani VR, Bechara EJ, and Ohmiya Y. 1999. Cloning, sequence analysis, and expression of active Phrixothrix railroad-worms luciferases: relationship between bioluminescence spectra and primary structures. Biochemistry 38:8271-9.

12. Nakajima Y, Kimura T, Sugata K, Enomoto T, Asakawa A, Kubota H, Ikeda M, and Ohmiya Y. 2005. Multicolor luciferase assay system: one-step monitoring of multiple gene expressions with a single substrate. Biotechniques 38:891-4.

13. OECD (2017), To be published - Performance Standards for the assessment of proposed similar or modified *in vitro* skin sensitisation IL-8 luc test methods. OECD Environment, Health and Safety Publications, Series on Testing and Assessment. OECD, Paris, France.

14. OECD (2005), Guidance Document the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. OECD Environment, Health and Safety publications, OECD Series on Testing and Assessment No.34. OECD, Paris, France.

15. OECD (2017), Draft Guidance document: Good *In Vitro* Method Practices (GIVIMP) for the Development and Implementation of *In Vitro* Methods for Regulatory Use in Human Safety Assessment. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/OECD%20Draft%20GIVIMP_v05%20-%20clean.pdf].

16. JaCVAM (2016), IL-8 Luc assay protocol, Available at. http://www.jacvam.jp/en_effort/effort02.html.

17. Niwa K, Ichino Y, Kumata S, Nakajima Y, Hiraishi Y, Kato D, Viviani VR, and Ohmiya Y. 2010. Quantum yields and kinetics of the firefly bioluminescence reaction of beetle luciferases. Photochem Photobiol 86:1046-9.

18. OECD (2012), The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins, Part 1: Scientific Evidence. OECD Environment, Health and Safety Publications, Series on Testing and Assessment No. 168. OECD, Paris, France.

19. United Nations (2015), Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Sixth revised edition. New York & Geneva: United Nations Publications. ISBN: 978-92-1-117006-1. Available at: http://www.unece.org/trans/danger/publi/ghs/ghs_rev05/05files_e.html.

APPENDIX I

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with concordance to mean the proportion of correct outcomes of a test method (16).

AOP (Adverse Outcome Pathway): Sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an *in vivo* outcome of interest (20).

CV05: Cell viability 05. Minimum concentration at which chemicals show less than 0.05 of Inh-GAPLA.

FInSLO-LA: Abbreviation used in the validation report and in previous publications regarding the IL-8 Luc assay to refer to Ind-IL8LA. See Ind-IL8LA for definition.

GAPLA: Luciferase Activity of Stable Luciferase Red (SLR) (λmax = 630 nm), regulated by GAPDH promoter and demonstrates cell viability and viable cell number.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

IATA (Integrated Approach to Testing and Assessment): A structured approach used for hazard identification (potential), hazard characterisation (potency) and/or safety assessment (potential/potency and exposure) of a chemical or group of chemicals, which strategically integrates and weights all relevant data to inform regulatory decision regarding potential hazard and/or risk and/or the need for further targeted and therefore minimal testing.

II-SLR-LA: Abbreviation used in the validation report and in previous publications regarding the IL-8 Luc assay to refer to Inh-GAPLA. See Inh-GAPLA for definition

IL-8 (Interleukin-8): A cytokine derived from endothelial cells, fibroblasts, keratinocytes, macrophages, and monocytes that causes chemotaxis of neutrophils and T-cell lymphocytes.

IL8LA: Luciferase Activity of Stable Luciferase Orange (SLO) (λmax = 580 nm), regulated by IL-8 promoter.

Ind-IL8LA: Fold induction of IL8LA. It is obtained by dividing the nIL8LA of THP-G8 cells treated with chemicals by that of non-stimulated THP-G8 cells and represents the induction of IL-8 promoter activity by chemicals.

Inh-GAPLA: Inhibition of GAPLA. It is obtained by dividing GAPLA of THP-G8 treated with chemicals with GAPLA of non-treated THP-G8 and represents cytotoxicity of chemicals.

Minimum induction threshold (MIT): the lowest concentration at which a chemical satisfies the positive criteria

Mixture: A mixture or a 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).

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one of the main constituents 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.

nIL8LA: The SLO luciferase activity reflecting IL-8 promoter activity (IL8LA) normalised by the SLR luciferase activity reflecting GAPDH promoter activity (GALPA). It represents IL-8 promoter activity after considering cell viability or cell number.

nSLO-LA: Abbreviation used in the validation report and in previous publications regarding the IL-8 Luc assay to refer to nIL8LA. See nIL8LA for definition

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.

Pre-haptens: Chemicals which become sensitisers through abiotic transformation.

Pro-haptens: Chemicals requiring enzymatic activation to exert skin sensitisation potential.

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

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (16).

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

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

SLO-LA: Abbreviation used in the validation report and in previous publications regarding the IL-8 Luc assay to refer to IL8LA. See IL8LA for definition.

SLR-LA: Abbreviation used in the validation report and in previous publications regarding the IL-8 Luc assay to refer to GAPLA. See GAPLA for definition.

Solvent/vehicle control: An untreated sample containing all components of a test system except of the test chemical, but including the solvent/vehicle that is used. It is used to establish the baseline response

for the samples treated with the test chemical dissolved or stably dispersed in the same solvent/vehicle. When tested with a concurrent medium control, this sample also demonstrates whether the solvent/vehicle interacts with the test system.

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

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, inducing any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing it composition.

Surfactant: Also called surface-active agent, this is a substance, such as a detergent, that can reduce the surface tension of a liquid and thus allow it to foam or penetrate solids; it is also known as a wetting agent. (TG437)

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

THP-G8: An IL-8 reporter cell line used in IL-8 Luc assay. The human macrophage-like cell line THP-1 was transfected the SLO and SLR luciferase genes under the control of the IL-8 and GAPDH promoters, respectively.

United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (21).

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

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

APPENDIX II

PRINCIPLE OF MEASUREMENT OF LUCIFERASE ACTIVITY AND DETERMINATION OF THE TRANSMISSION COEFFICIENTS OF OPTICAL FILTER FOR SLO AND SLR

MultiReporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-colour detection system, which can equip an optical filter (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)). The optical filter used in measurement is 600–620 nm long or short pass filter, or 600–700 nm band pass filter.

(1) Measurement of two-colour luciferases with an optical filter.
This is an example using Phelios AB-2350 (ATTO). This luminometer is equipped with a 600 nm long pass filter (R60 HOYA Co.), 600 nm LP, Filter 1) for splitting SLO (λmax = 580 nm) and SLR (λmax = 630 nm) luminescence.

To determine transmission coefficients of the 600 nm LP, first, using purified SLO and SLR luciferase enzymes, measure i) the intensity of SLO and SLR bioluminescence intensity without filter (F0), ii) the SLO and SLR bioluminescence intensity that passed through 600 nm LP (Filter 1), and iii) calculate the transmission coefficients of 600 nm LP for SLO and SLR listed below.

Transmission coefficients		Abbreviation	Definition
SLO	Filter 1 Transmission coefficients	κO_{R60}	The filter's transmission coefficient for the SLO
SLR	Filter 1 Transmission coefficients	κR_{R60}	The filter's transmission coefficient for the SLR

When the intensity of SLO and SLR in test sample are defined as O and R, respectively, i) the intensity of light without filter (all optical) F0 and ii) the intensity of light that transmits through 600 nm LP (Filter 1) F1 are described as below.

$$F0=O+R$$

$$F1=\kappa O_{R60} \times O + \kappa R_{R60} \times R$$

These formulas can be rephrased as follows:

$$\begin{pmatrix} F0 \\ F1 \end{pmatrix} = \begin{pmatrix} 1 & 1 \\ \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} O \\ R \end{pmatrix}$$

Then using calculated transmittance factors (κO_{R60} and κR_{R60}) and measured F0 and F1, you can calculate O and R-value as follows:

$$\begin{pmatrix} O \\ R \end{pmatrix} = \begin{pmatrix} 1 & 1 \\ \kappa O_{R60} & \kappa R_{R60} \end{pmatrix}^{-1} \begin{pmatrix} F0 \\ F1 \end{pmatrix}$$

Materials and methods for determining transmittance factor

(1) Reagents
· Single purified luciferase enzymes:
Lyophilised purified SLO enzyme
Lyophilised purified SLR enzyme
(which for the validation work were obtained from GPC Lab. Co. Ltd., Tottori, Japan with THP-G8 cell line)
· Assay reagent:
Tripluc® Luciferase assay reagent (for example from TOYOBO Cat#MRA-301)
· Medium: for luciferase assay (30 ml, stored at 2 – 8°C)

Reagent	Conc.	Final conc. in medium	Required amount
RPMI-1640	-	-	27 ml
FBS	-	10 %	3 ml

(2) Preparation of enzyme solution
Dissolve lyophilised purified luciferase enzyme in tube by adding 200 µl of 10 ~ 100 mM Tris/HCl or Hepes/HCl (pH 7.5 ~ 8.0) supplemented with 10% (w/v) glycerol, divide the enzyme solution into 10 µl aliquots in 1.5 ml disposable tubes and store them in a freezer at -80°C. The frozen enzyme solution can be used for up to 6 months. When used, add 1 ml of medium for luciferase assay (RPMI-1640 with 10% FBS) to each tube containing the enzyme solutions (diluted enzyme solution) and keep them on ice to prevent deactivation.

(3) Bioluminescence measurement
Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to allow the photomultiplier to stabilise. Transfer 100 µl of the diluted enzyme solution to a black 96 well plate (flat bottom) (the SLO reference sample to #B1, #B2, #B3, the SLR reference sample to #D1, #D2, #D3). Then, transfer 100 µl of pre-warmed Tripluc to each well of the plate containing the diluted enzyme solution using a pipetman. Shake the plate for 10 min at room temperature (about 25°C) using a plate shaker. Remove bubbles from the solutions in wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter.

Transmission coefficient of the optical filter was calculated as follows:

Transmission coefficient (SLO (κO_{R60}))= (#B1 of F1+ #B2 of F1+ #B3 of F1) / (#B1 of F0+ #B2 of F0+ #B3 of F0)

Transmission coefficient (SLR (κR_{R60}))= (#D1 of F1+ #D2 of F1+ #D3 of F1) / (#D1 of F0+ #D2 of F0+ #D3 of F0)

Calculated transmittance factors are used for all the measurements executed using the same luminometer.

Quality control of equipment

The procedures described in the IL-8 Luc protocol should be used (18).

.

APPENDIX III

PROFICIENCY SUBSTANCES

Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency by obtaining the expected IL-8 Luc assay prediction for the 9 substances recommended in Table 1 and by obtaining values that fall within the respective reference range for at least 8 out of the 9 proficiency substances (selected to represent the range of responses for skin sensitisation hazards). Other selection criteria were that the substances are commercially available, and that high-quality *in vivo* reference data as well as high quality *in vitro* data generated with the IL-8 Luc assay are available. Also, published reference data are available for the IL-8 Luc assay (6) (1).

Table 1: Recommended substances for demonstrating technical proficiency with the IL-8 Luc assay

Proficiency substances	CAS no.	State	Solubility in X-VIVO15 at 20 mg/mL	*In vivo* prediction[1]	IL-8 Luc prediction[2]	Reference range (µg/mL)[3]	
						CV05[4]	IL-8 Luc MIT[5]
2,4-Dinitrochlorobenzene	97-00-7	Solid	Insoluble	Sensitiser (Extreme)	Positive	2.3-3.9	0.5-2.3
Formaldehyde	50-00-0	Liquid	Soluble	Sensitiser (Strong)	Positive	9-30	4-9
2-Mercaptobenzothiazole	149-30-4	Solid	Insoluble	Sensitiser (Moderate)	Positive	250-290	60-250
Ethylenediamine	107-15-3	Liquid	Soluble	Sensitiser (Moderate)	Positive	500-700	0.1-0.4
Ethyleneglycol dimethacrylate	97-90-5	Liquid	Insoluble	Sensitiser (Weak)	Positive	>2000	0.04-0.1
4-Allylanisole (Estragol)	140-67-0	Liquid	Insoluble	Sensitiser (Weak)	Positive	>2000	0.01-0.07
Streptomycin sulphate	3810-74-0	Solid	Soluble	Non-sensitiser	Negative	>2000	>2000
Glycerol	56-81-5	Liquid	Soluble	Non-sensitiser	Negative	>2000	>2000
Isopropanol	67-63-0	Liquid	Soluble	Non-sensitiser	Negative	>2000	>2000

Abbreviations: CAS no. = Chemical Abstracts Service Registry Number

[1] The *in vivo* potency is derived using the criteria proposed by ECETOC (19).

[2] Based on historical observed values (1) (6).

[3] CV05 and IL-8 Luc MIT were calculated using water solubility given by EPI Suite™.

[4] CV05: the minimum concentration at which chemicals show less than 0.05 of Inh-GAPLA.

[5] MIT: the lowest concentrations at which a chemical satisfies the positive criteria.

APPENDIX IV

INDEXES AND JUDGMENT CRITERIA

nIL8LA (nSLO-LA)

The j-th repetition (j = 1-4) of the i-th concentration (i = 0-11) is measured for IL8LA (SLO-LA) and GAPLA (SLR-LA) respectively. The normalised IL8LA, referred to as nIL8LA (nSLO-LA), and is defined as:

$nIL8LA_{ij} = IL8LA_{ij} / GAPLA_{ij.}$

This is the basic unit of measurement in this assay.

Ind-IL8LA (FInSLO-LA)

The fold increase of the averaged nIL8LA (nSLO-LA) for the repetition on the i-th concentration compared with it at the 0 concentration, Ind-IL8LA, is the primary measure of this assay. This ratio is written by the following formula:

$$\text{Ind-IL8LA}_i = \left\{(1/4)\times\sum_j nIL8LA_{ij}\right\} / \left\{(1/4)\times\sum_j nIL8LA_{0j}\right\}.$$

The lead laboratory has proposed that a value of 1.4 corresponds to a positive result for the tested chemical. This value is based on the investigation of the historical data of the lead laboratory. Data management team then used this value through all the phases of validation study. The primary outcome, Ind-IL8LA, is the ratio of 2 arithmetic means as shown in equation.

95% confidence interval (95% CI)

The 95% confidence interval (95% CI) based on the ratio can be estimated to show the precision of this primary outcome measure. The lower limit of the 95% CI ≥ 1 indicates that the nIL8LA with the i-th concentration is significantly greater than that with solvent control. There are several ways to construct the 95% CI. We used the method known as Fieller's theorem in this study. This 95% confidence interval theorem is obtained from the following formula:

$$\left[\frac{-B-\sqrt{B^2-4AC}}{2A}, \frac{-B+\sqrt{B^2-4AC}}{2A}\right],$$

where $A = \bar{x}_0^2 - t^2_{0.975(\nu)} \times \frac{sd_0^2}{n_0}$, $B = -2\times\bar{x}\times\bar{y}$, $C = \bar{y}_i^2 - t^2_{0.975(\nu)} \times \frac{sd_{y_i}^2}{n_{y_i}}$, and $n_0 = 4$,

$\bar{x}_0 = (1/n_0)\times\sum_j nIL8LA_{0j}$, $sd_0^2 = \{1/(n_0-1)\}\times\sum_j (nIL8LA_{0j} - \bar{x}_0)^2$,

$n_{y_i} = 4$, $\bar{y}_i = (1/n_{y_i})\times\sum_j (nIL8LA_{ij})$, $sd_{y_i}^2 = \{1/(n_{y_j}-1)\}\times\sum_j (nIL8LA_{ij} - \bar{y}_i)^2$.

$t_{0.975(\nu)}$ is 97.5 percentile of the central t distribution with the ν of the degree of freedom, where

$$\nu = \left(\frac{sd_0^2}{n_0} + \frac{sd_{y_i}^2}{n_{y_i}} \right)^2 / \left\{ \left(\frac{sd_0^2}{n_0} \right)^2 / (n_0 - 1) + \left(\frac{sd_{y_i}^2}{n_{y_i}} \right)^2 / (n_{y_i} - 1) \right\}.$$

Inh-GAPLA (II-SLR-LA)

The Inh-GAPLA is a ratio of the averaged GAPLA (SLR-LA) for the repetition of the i-th concentration compared with that with solvent control, and this is written by

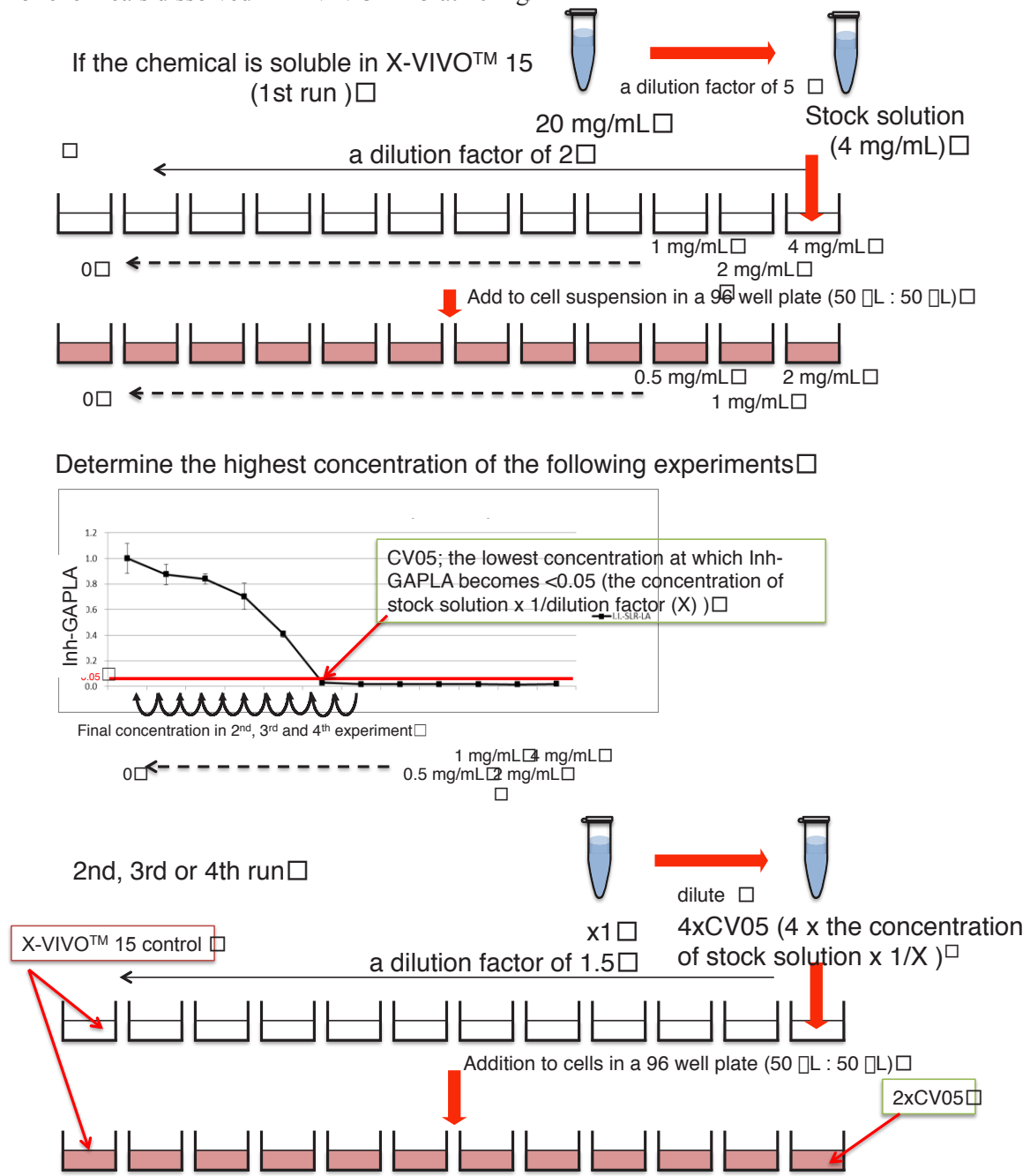
$$\text{Inh-GAPLA}_i = \left\{ (1/4) \times \sum_j GAPLA_{ij} \right\} / \left\{ (1/4) \times \sum_j GAPLA_{0j} \right\}.$$

Since the GAPLA is the denominator of the nIL8LA, an extremely small value causes large variation in the nIL8LA. Therefore, Ind-IL8LA values with an extremely small value of Inh-GAPLA (less than 0.05) might be considered poor precision.

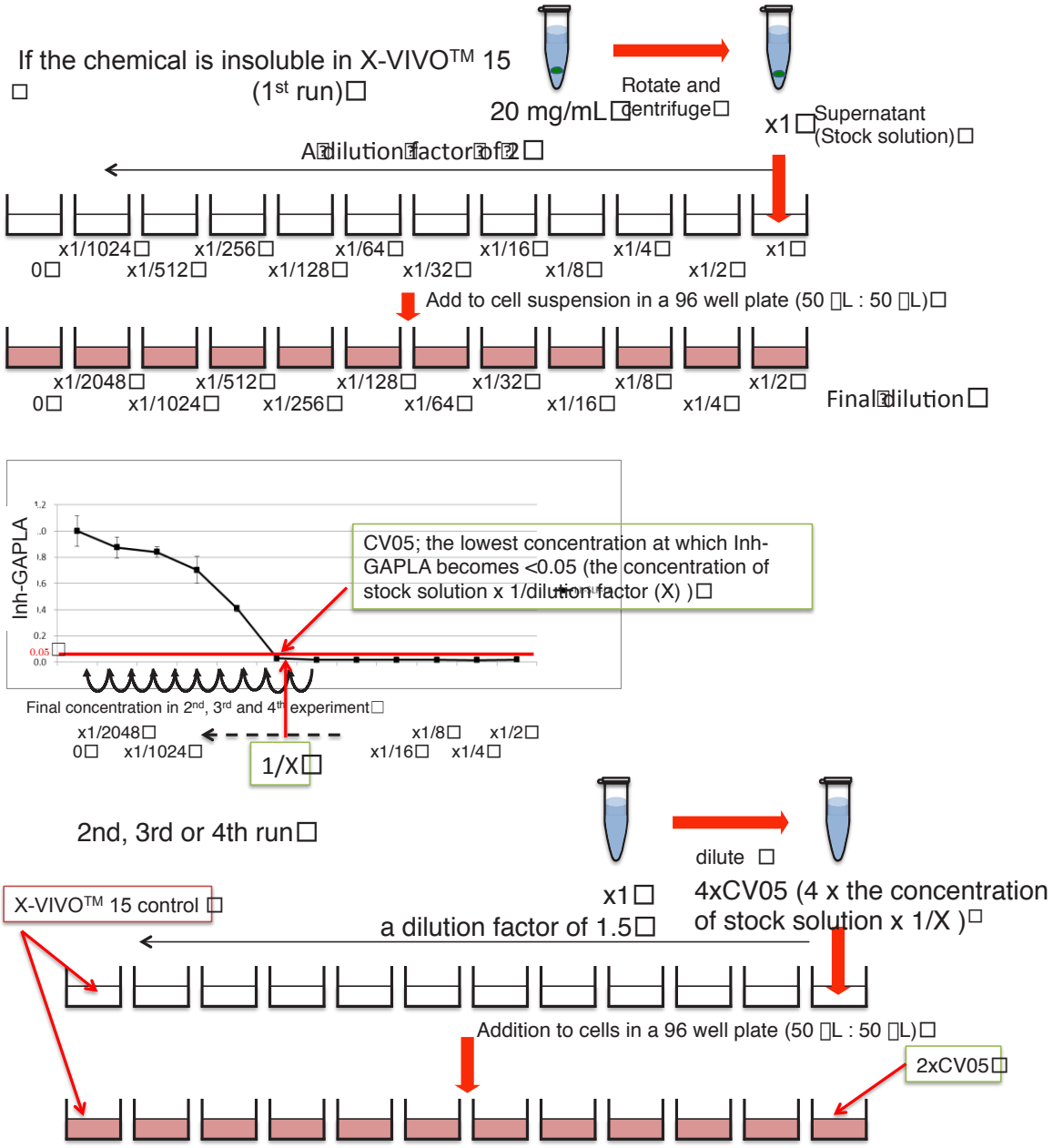
APPENDIX V

THE SCHEME OF THE METHODS TO DISSOLVE CHEMICALS FOR THE IL-8 LUC ASSAY.

(a) For chemicals dissolved in X-VIVO™ 15 at 20 mg/mL



(b) For chemicals insoluble in X-VIVO™ 15 at 20 mg/mL



APPENDIX VI

THE SCHEME OF THE METHOD TO DISSOLVE 4-NBB FOR THE POSITIVE CONTROL OF THE IL-8 LUC ASSAY.