新規試験法提案書

皮膚感作性試験代替法
角化細胞株レポーターアッセイに関する提案

平成27年 8 月

国立医薬品食品衛生研究所

新規試験法提案書

平成 27 年 8 月 20 日

No.　2015-01

皮膚感作性試験代替法角化細胞株レポーターアッセイ
に関する提案

平成 27 年 4 月 23 日に東京、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：　角化細胞株レポーターアッセイで陽性の結果が得られた場合、その化学物質を強い感作性物質である GHS(Globally Harmonized System of Classification and Labelling of Chemicals)区分 1 に分類することは行政上可能である。しかしながら、稀に偽陽性の結果が生じることに留意しなければならない。一方、本試験法で陰性の結果が得られた場合、その化学物質の皮膚感作性は偽陰性の可能性があり、本試験法単独でその皮膚感作性を判定することは難しい。本試験法は、そ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源と組み合わせて適切に評価することが、行政的な受け入れに必要であると考える。

この提案書は、OECD TG 442D; *In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Methodおよび JRC Scientific and Policy report,　EURL ECVAM Recommendation on the KeratinoSensTM assay for skin sensitisation testing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　ペプチド結合性試験の使用を提案するものである。

大野泰雄

JaCVAM 評価会議　議長

西川秋佳

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

大野泰雄	(運営委員会推薦)：座長
五十嵐良明	(国立医薬品食品衛生研究所　生活衛生化学部)
飯塚尚文	(独立行政法人　医薬品医療機器総合機構)
金子和弘	(日本化学工業協会)
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)
谷川浩子	(日本動物実験代替法学会)
西川秋佳	(国立医薬品食品衛生研究所　安全性生物試験研究センター)
牧　栄二	(日本免疫毒性学会)
森田　健	(日本環境変異原学会)
山田隆志	(独立行政法人　製品評価技術基盤機構)
横関博雄	(日本皮膚アレルギー・接触皮膚炎学会)
吉田武美	(日本毒性学会)
吉田　緑	(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)
吉村　功	(座長推薦)
渡部一人	(日本製薬工業協会)

任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日

JaCVAM 運営委員会

西川秋佳	（国立医薬品食品衛生研究所　安全性生物試験研究センター）：委員長
川西　徹	（国立医薬品食品衛生研究所）
植村展生	（厚生労働省　医薬食品局　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）
菅野　純	（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）
倉持憲路	（厚生労働省　医薬食品局　化学物質安全対策室）
関野祐子	（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　薬理部）
高木篤也	（国立医薬品食品衛生研究所　安全性生物試験研究センター　毒性部 動物管理室）
束野正明	（厚生労働省　医薬食品局　化学物質安全対策室）
中村高敏	（独立行政法人　医薬品医療機器総合機構）
日田　充	（厚生労働省　医薬食品局　化学物質安全対策室）
広瀬明彦	（国立医薬品食品衛生研究所　安全性生物試験研究センター　安全性予測評価部）
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 ARE-Nrf2 Luciferase Test Method assay for skin sensitisation testing

At a meeting held on 23 April 2015 at the National Institute of Health Sciences (NIHS) in Tokyo, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Proposal: When using the ARE-Nrf2 Luciferase Test Method in a regulatory context, it is reasonable for substances that give positive results to be classified as a strong sensitiser, i.e., a Category 1 substance under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). It is also necessary, however, to bear in mind that this assay occasionally yields false positive results for certain substances. Conversely, it is quite possible that the ARE-Nrf2 Luciferase Test Method will yield false negative results for some chemicals, which means that it would be unreasonable to use it as a standalone test for assessing skin sensitisation potency. We therefore conclude that the use of the ARE-Nrf2 Luciferase Test Method in a regulatory context requires a thorough understanding of the assay's strengths and weaknesses as a prerequisite to its application within an integrated approach to testing and assessment (IATA) that will also take into account information from other sources.

This statement was prepared following a review of OECD TG 442D "*In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method" as well as a JRC Scientific and Policy report "EURL ECVAM Recommendation on the KeratinoSens™ assay for skin sensitisation testing". The JaCVAM Regulatory Acceptance Board acknowledges that the results of this review as well as of a study of materials prepared by the JaCVAM Editorial Committee indicate that this assay is useful in a regulatory context.

Based on the above, we propose that regulatory agencies performing safety assessment of skin sensitisation potency consider using the ARE-Nrf2 Luciferase Test Method as an alternative to testing with laboratory animals.

Yasuo Ohno
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

20 August 2015

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 (nominee by JaCVAM Steering Committee) : Chairperson
Mr. Naofumi Iizuka (Pharmaceuticals and Medical Devices Agency)
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Kazuhiro Kaneko (Japan Chemical Industry Association)
Mr. Eiji Maki (Japanese Society of Immunotoxicology)
Mr. Takeshi Morita (Japanese Environmental Mutagen Society)
Mr. Akiyoshi Nishikawa (NIHS)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Ms. Koko Tanigawa (Japanese Society for Alternatives to Animal Experiments)
Mr. Takashi Yamada (National Institute of Technology and Evaluation)
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)
Ms. Midori Yoshida (NIHS)
Mr. Takemi Yoshida (Japanese Society of Toxicology)
Mr. Isao Yoshimura (nominee by Chairperson)
Mr. Kazuto Watanabe (Japan Pharmaceutical Manufacturers Association)

Term: From 1st April 2014 to 31st March 2016

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

Mr. Akiyoshi Nishikawa (BSRC, NIHS): Chairperson
Mr. Toru Kawanishi (NIHS)
Mr. Mitsuru Hida (Ministry of Health, Labour and Welfare)
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)
Mr. Jun Kanno (Division of Cellular and Molecular Toxicology, BSRC, NIHS)
Mr. Kenji Kuramochi (Ministry of Health, Labour and Welfare)
Mr. Takatoshi Nakamura (Pharmaceutical & Medical Devices Agency)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Ms. Yuko Sekino (Division of Pharmacology, BSRC, NIHS)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Mr. Atsuya Takagi (Animal Management Section of the Division of Cellular and Molecular Toxicology, BSRC, NIHS)
Mr. Masaaki Tsukano (Ministry of Health, Labour and Welfare)
Mr. Nobuo Uemura (Ministry of Health, Labour and Welfare)
Mr. Hajime Kojima (Section for the Evaluation of Novel Methods, Division of Risk Assessment, BSRC, NIHS): Secretary

皮膚感作性試験代替法角化細胞株レポーターアッセイ

目　　次

評価会議報告書

ARE-Nrf2 Luciferase Test Method
（角化細胞株レポーターアッセイ）

JaCVAM 評価会議

平成 27 年（2015 年）4 月 30 日

1

JaCVAM 評価会議

大野泰雄　（運営委員会推薦）：座長

五十嵐良明　（国立医薬品食品衛生研究所　生活衛生化学部）

金子和弘　（日本化学工業協会）

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）

谷川浩子　（日本動物実験代替法学会）

西川秋佳　（国立医薬品食品衛生研究所　安全性生物試験研究センター）

平賀秀明　（独立行政法人　医薬品医療機器総合機構）

牧　栄二　（日本免疫毒性学会）

森田　健　（日本環境変異原学会）

山田隆志　（独立行政法人　製品評価技術基盤機構）

横関博雄　（日本皮膚アレルギー・接触皮膚炎学会）

吉田武美　（日本毒性学会）

吉田　緑　（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）

吉村　功　（座長推薦）

渡部一人　（日本製薬工業協会）

任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日

2

角化細胞株レポーターアッセイ：Antioxidant response element (ARE)-Nrf2[1] Luciferase Test Method（以下、本試験法と記す）は、化学物質の皮膚感作性を予測する試験法であり、従来のモルモットを用いる皮膚感作性試験（OECD TG406)、マウスを用いる局所リンパ節試験（Local Lymph Node Assay (LLNA)、OECD TG429）および LLNA の変法である LLNA:DA (OECD TG442A)ならびに LLNA:BrdU-ELISA (OECD TG442B)と比べ、動物を用いないという特徴を有する。本試験法は、皮膚感作性を有する多くの化学物質が ARE により制御されている遺伝子の発現を誘導することから、この誘導活性を培養細胞を用いて測定することにより、皮膚感作性の有無を予測する試験法である。本試験法のバリデーション研究の結果については、European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)による第三者評価が終了している[1)]。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　角化細胞株レポーターアッセイの評価報告書を基に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：　角化細胞株レポーターアッセイ：ARE-Nrf2 Luciferase Test Method

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 [LLNA (OECD TG429)、LLNA:DA (OECD TG442A)、LLNA:BrdU-ELISA (OECD TG442B)]

試験法の概略：　本試験法は、Nrf2-Keap1[2]-ARE pathway を利用し、ケラチノサイト由来 ARE-Nrf2 ルシフェラーゼレポーター遺伝子を用いたレポーターアッセイである[1)]。

Nrf2-Keap1-ARE pathway は、転写因子 Nrf2、Nrf2 の抑制因子 Keap1 および ARE が関係する遺伝子発現経路である。Nrf2 は Keap1 と結合し、ARE に依存して発現する遺伝子群の発現量を制御している。Keap1 のシステイン残基に求電子性の化学物質が結合すると、Nrf2 は Keap1 から解離し、核内へ移行して DNA 上の ARE に結合する。その結果、下流の遺伝子群の発現が誘導され、化学物質による障害から細胞を保護するために機能する。皮膚感作性を有する多くの化学物質が Nrf2-Keap1-ARE pathway を活性化することが知られている。

本試験法では、AKR1C2 遺伝子（樹状細胞において皮膚感作性物質により発現誘導される遺伝子の 1 つ）の ARE を融合させた SV40 プロモーターを有するルシフェラーゼ遺伝子のプラスミドを安定的に導入した HaCaT 細胞（ヒトケラチノサイト由来培養細胞株）を用いる。化学物質により Nrf2-Keap1-ARE pathway が活性化されるとルシフェラーゼ遺伝子が発現する。基質を添加してルシフェラーゼが触媒する反応の発光強度を測定することにより、化学物質の皮膚感作性が評価される。

---

[1] Nrf2：Nuclear factor (erythroid-derived 2)-like 2

[2] Keap1: Kelch-like ECH-associated protein 1

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、KeratinoSens™[3]を用いて実施されたバリデーション研究 [2-5]とそれに続く EURL ECVAM Scientific Advisory Committee により独立した第三者評価が実施され [1]、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いる。本邦においては、JaCVAM 皮膚感作性試験資料編纂委員会が、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、公開されている情報 [1-5]を基に評価した。評価の結果、本試験法は、皮膚感作性を有する多くの化学物質が ARE により制御されている遺伝子の発現を誘導するという皮膚感作性発現機序における重要なイベントを検出しており、皮膚感作性試験の代替試験法として原理的妥当性があると考えられた [6]。

なお、再現性を検討したバリデーション試験において被験物質選択に偏りがあり、施設内・施設間再現性には留意すべきであると評価会議は判断した。

３．本試験法の有用性と適用限界

本試験法は、ヒトケラチノサイト由来培養細胞株を用いる *in vitro* 試験法で、3Rs の精神に合致している。また、１被験物質当たりのランニングコストも、マウスを用いる LLNA と比較して 1/7 程度で実施可能であり、試験期間も LLNA より短期間であることから、本試験法は簡便性および経済性の面から有用といえる。更に、皮膚感作性を有する多くの化学物質が ARE により制御されている遺伝子の発現を誘導することから、本試験法は、この誘導活性を培養細胞を用いて測定することにより、皮膚感作性の有無を予測するものであり、化学物質の感作性を判断する上で重要な情報を与える。

しかしながら、現時点でこの試験に使用できる細胞系は KeratinoSens™ のみであり、使用には細胞系を樹立した Givaudan 社からライセンスを取得することが必要であり、費用も含めて利用可能な条件の詳細は不明である。一方、KeratinoSens™ を使用しない場合は、OECD 作成の本試験法に関する Performance Standard（案）[7]に従い、妥当性を示さなければならない。また、LogP が 7 以上の強疎水性物質は、DMSO と水への溶解特性から試験に適用できない。酸無水物は、リジン残基と反応性を示す特徴があり、システイン基との反応が必要な Nrf2 pathway を誘導しないため、偽陰性と判定される可能性がある。ルシフェラーゼ酵素に干渉する化学物質では、ルシフェラーゼ活性を正しく測定することができないため注意が必要である。酸化反応や酸化的脱アミノ反応を必要とする多くのプレハプテンは正しく判定されるが、シトクロム P450 による活性化を必要とするプロハプテンは検出できない。培養細胞を用いる試験系のため、細胞毒性の強い物質は適切な濃度で試験ができない場合がある。100 物質以上を評価した報告 [4,5]における本試験法の感度は、76.7%と 77%であるため、陰性の結果が得られても偽陰性の可能性を考慮する必要がある。また、特異度も、82.1%

[3] KeratinoSens™：Givaudan 社によって樹立されたケラチノサイト由来 ARE-Nrf2 ルシフェラーゼレポーター遺伝子のプラスミドを安定的に導入したヒトケラチノサイト由来培養細胞株

と 79%であることから、陽性の結果が得られた場合にも、偽陽性の可能性があることに留意しなければならない。

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、細胞培養技術および 96 穴プレート用ルミノメーター操作技術を修得していれば容易に実施できる試験である。ただ、現時点で使用できる細胞系は KeratinoSens™ のみであり、販売元である Givaudan 社からライセンスを取得する必要がある。本試験法の実施と結果の解釈に当たっては、化学物質の性質とその適用限界を見極める必要がある。しかし、本試験法は、皮膚感作性を有する多くの化学物質が ARE により制御されている遺伝子の発現を誘導するという皮膚感作性発現機序における重要なイベントを検出しており、化学物質の皮膚感作性を考える上で重要な情報を与えることから、本試験法の社会的受け入れ性は高いものと考える。

行政上の利用性：

本試験法で陽性の結果が得られた場合、その化学物質を強い感作性物質である GHS(Globally Harmonized System of Classification and Labelling of Chemicals)区分 1 に分類することは行政上可能である。しかしながら、稀に偽陽性の結果が生じることに留意しなければならない。一方、本試験法で陰性の結果が得られた場合、その化学物質の皮膚感作性は偽陰性の可能性があり、本試験法単独でその皮膚感作性を判定することは難しい。本試験法は、そ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源と組み合わせて適切に評価することが、行政的な受け入れに必要であると考える。

引用文献

1) EURL ECVAM (2012). ESAC Working Group Peer Review Consensus Report on Givaudan-coordinated study transferability and reliability of the KeratinoSens assay for skin sensitisation testing.
2) DB-ALM (INVITTOX) (2013). Protocol 155: KeratinoSens™ .
3) Natsch A., Ryan C.A., Foertsch L., Emter R., Jaworska J., Gerberick G.F., Kern P. (2013). A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. Journal of Applied Toxicology, 33, 1337-1352.
4) Emter R., Ellis G., Natsch A. (2010). Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro.* Toxicology and Applied Pharmacology 245, 281-290.
5) EURL-ECVAM (2014). Recommendation on the KeratinoSens™ assay for skin sensitisation testing, 42 pp.
   Available at:
   http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations/recommendation-keratinosens-skin-sensitisation
6) JaCVAM 皮膚感作性試験資料編纂委員会：皮膚感作性試験評価報告書　ARE-Nrf2 Luciferase Test Method（平成 27 年 4 月 28 日）
7) OECD (2014, in preparation). Performance Standards for the assessment of proposed similar or modified in vitro skin sensitisation ARE-Nrf2 luciferase test methods in TG xxx. OECD Environment, Health and Safety publications, Series on Testing and Assessment N. xxx, OECD, Paris.

皮膚感作性試験評価報告書

ARE-Nrf2 Luciferase Test Method

角化細胞株レポーターアッセイ

平成 27 年 4 月 28 日

JaCVAM 皮膚感作性試験資料編纂委員会

JaCVAM 皮膚感作性試験資料編纂委員会

委員長　筒井尚久（田辺三菱製薬株式会社）
委員　　安達玲子（国立医薬品食品衛生研究所）
　　金澤由基子（一般財団法人　食品薬品安全センター）
　　小島幸一（一般財団法人　食品薬品安全センター）
　　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）
　　森本隆史（住友化学株式会社）

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: Quantitative Structure-Activity Relationship)モデルや *in vitro* 試験の代替法が推奨されており、動物実験により安全性評価された成分を含む化粧品の輸入販売が禁止されたことから（2013 年 3 月全面施行)、動物を用いない *in vitro* 試験法の開発が強く望まれている。ARE (Antioxidant response element)-Nrf2[1] Luciferase Test Method は、多くの皮膚感作性物質が ARE により制御される遺伝子の発現を誘導することを利用し、この誘導活性について培養細胞を用いてアッセイする試験法である。本報告書は、この ARE-Nrf2 Luciferase Test Method について、本試験法を開発した Givaudan 社の先行バリデーション試験の成績並びに EURL ECVAM（European Union Reference Laboratory for Alternatives to Animal Testing）科学諮問委員会（ESAC）により実施された第三者評価報告書などを基にその手順を纏め、有用性と限界を評価したものである。

ARE-Nrf2 Luciferase Test Method（以下、本試験法と記す）は、感作性発現機序における第二段階のイベントであるケラチノサイトにおける炎症反応および Nrf2-Keap1[2]-ARE pathway を利用した試験法であり、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。

マウスを用いる試験法である局所リンパ節試験（LLNA:Local Lymph Node Assay）の約 1/7 程度のランニングコストで実施可能であり、*in vitro* 試験法であることから、有用性は高い。しかしながら、現時点で本試験法に使用できることが知られている細胞系は KeratinoSens™ のみであり、その使用には本細胞系を樹立した Givaudan 社からライセンスを受けることが必要なため、新規導入の容易な試験系とは言い難い。

本試験法の先行バリデーション試験における施設内再現性は、5 施設中 1 施設において、GHS(Globally Harmonized System of Classification and Labelling of Chemicals)区分 1B に分類される物質（弱い感作性物質）および非感作性物質で再現性が得られなかったため、目安とした達成基準（85%）に達しておらず、強い感作性物質以外では判定がぶれる懸念がある。一方、施設間再現性は、目安とした達成基準（80%）を上回った。

本試験法の感度は、約 80%であり、陰性の結果が得られた場合は、偽陰性の可能性を考慮し、補完し得る他の試験によって確認しなければならず、本試験法のみで皮膚感作性を陰性と判定することはできない。また、特異度も、約 80%であることから、陽性の結果が得られた場合にも、偽陽性の結果が生じる可能性があることに留意しなければならない。

本試験法では、活性化に代謝系を必要とする化学物質は、正しくその感作性が検出されない可能性がある。また、細胞系であるため、疎水性の高い物質では規定されている最高

[1] Nrf2：Nuclear factor (erythroid-derived 2)-like 2
[2] Keap1: Kelch-like ECH-associated protein 1

濃度（2000 μM）での評価が難しく、陰性判定が下せない場合がある。

以上を踏まえ、本委員会は、本試験法が皮膚感作性評価に汎用されるためには、KeratinoSens™が安価に入手できることが前提と考える。また、本試験法の様々な限界を考慮すると、本試験法単独では皮膚感作性の評価は不十分であり、証拠の重み付けや他の試験法（LLNA、モルモットを用いる皮膚感作性試験など）と組み合わせでの評価を推奨する。

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 TG406）やマウスを用いる局所リンパ節試験（LLNA: Local Lymph Node Assay, OECD TG429）がある。この[^{3}H-Methyl]-thymidine 取込量を測定する LLNA 以外に放射性同位元素（RI）を用いず ATP 量を測定する LLNA:DA（OECD TG442A）や Bromodeoxyuridine 量を測定する LLNA:BrdU-ELISA（OECD TG442B）がある。このように、現在 OECD からガイドラインとして公表されている試験法は、動物を用いた *in vivo* の試験法のみである。

EU における欧州化学品規則（REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals）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: Quantitative Structure-Activity Relationship)モデルや *in vitro* 試験等による代替法が推奨されており、動物実験により安全性が評価された成分を含んだ化粧品の輸入および販売が禁止された（2013 年 3 月全面施行）。そのため、化学物質の皮膚感作性を評価する代替法の開発が強く求められている。

現在、ペプチドとの結合反応を利用した Direct Peptide Reactivity Assay（DPRA）、単球系細胞の活性化を利用した human Cell Line Activation Test（h-CLAT）および Myeloid U937 Skin Sensitization Test（MUSST）、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 などの皮膚感作性試験の動物を用いない動物実験代替法が提案されており、EURL ECVAM（European Union Reference Laboratory for Alternatives to Animal Testing）等においてバリデーション研究が行われている。

ARE(Antioxidant response element)-Nrf2 Luciferase Test Method は、多くの皮膚感作性物質が ARE により制御される遺伝子の発現を誘導することを利用し、この誘導活性について培養細胞を用いて評価する試験法である。KeratinoSens™法は本試験法のために確立された細胞を使用する評価系である。KeratinoSens™法を用いて実施された本試験法のバリデーション研究の結果については、EURL ECVAM 科学諮問委員会（ESAC）による第三者評価が完了している [1)]。

JaCVAM 皮膚感作性試験資料編纂委員会（以下、本委員会）が ARE-Nrf2 Luciferase Test Method 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、KeratinoSens™法を用いて実施された本試験法のバリデーション研究の結果等、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。

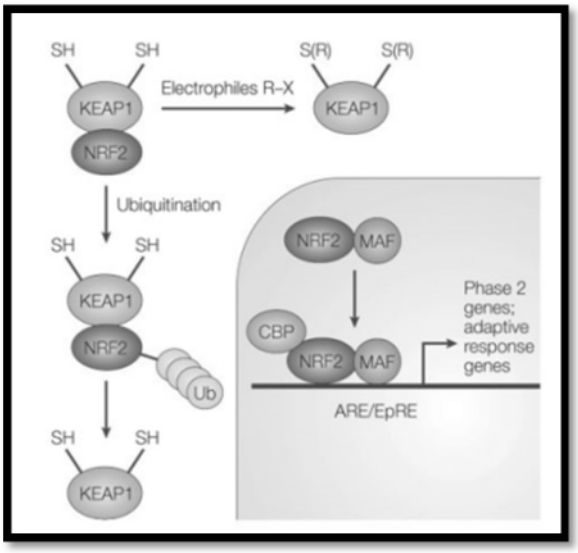
2．試験法の原理

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)では、化学物質による皮膚感作性は次の 4 つの Key event から成るとされている [1)]。

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および 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

ARE-Nrf2[3] Luciferase Test Method は上記の第 2 の Key event に対応する試験法である。その基本的原理は Nrf2-Keap1[4]-ARE pathway（図 1）を利用したレポーターアッセイである [1)]。

図 1．Nrf2-Keap1-ARE pathway の模式図



Nrf2-Keap1-ARE pathway は、転写因子 Nrf2、Nrf2 の抑制因子である Keap1 および ARE が関係する遺伝子発現経路である。Nrf2 は Keap1 と結合し、ARE に依存して発現する遺伝子群の発現量を制御している。Keap1 のシステイン残基に求電子性の化学物質が結合すると、Nrf2 は Keap1 から解離し、核内へ移行して DNA 上の ARE に結合する。その結果、下流の遺伝子群の発現が誘導され、化学物質による障害から細胞を保護するために機能する。多くの皮膚感作性物質が Nrf2-Keap1-ARE pathway を活性化する。

ARE-Nrf2 Luciferase Test Method では、AKR1C2 遺伝子（樹状細胞において皮膚感作性物質により発現誘導される遺伝子の 1 つ）の ARE を融合させた SV40 プロモーターを有するルシフェラーゼ遺伝子のプラスミドを安定的に導入した HaCaT 細胞（ヒトケラチノサイト

[3] Nrf2 :Nuclear factor-erythroid 2-related factor 2
[4] Keap1 : Kelch-like ECH-associated protein 1

系培養細胞株）を用いる。化学物質により Nrf2-Keap1-ARE pathway が活性化されるとルシフェラーゼ遺伝子が発現する。基質を添加し、ルシフェラーゼが触媒する反応の発光強度を測定することにより、化学物質の皮膚感作性を評価する。

3．試験手順／判定

試験手順は特に示さない限り KeratinoSens™ を用いた場合の記載である。ケラチノサイト由来 ARE-Nrf2 ルシフェラーゼレポーター遺伝子（keratinocyte-based ARE-Nrf2 luciferase reporter gene）を用いた本試験法の細胞として KeratinoSens™ 以外の細胞を用いる場合は、OECD 作成の ARE-Nrf2 LuciferaseTest Method に関する Performance Standard（案）[2]に従い KeratinoSens™ を用いた場合と同等かあるいは優る信頼性、正確度、感度、特異度などを示すことを確認したのちに使用しなければならない。いずれの場合も日常的に用いる前に OECD ガイドライン（案）[3]の Annex 2 に従い技術的熟達度を確認することを推奨する。

3-1. 細胞の調製

ARE 制御下のルシフェラーゼレポーター遺伝子を安定的に取り込んだトランスジェニック細胞系を用いる。（現在は、KeratinoSens™ のみであるため、試験法の開発者と使用許諾契約を結んだうえでこの細胞系を入手して使用することになる）。指定された継代数の細胞（2 から 4 代）を増殖し、分割して保管し、これを主ストック細胞とする。主ストック細胞から増殖させ、指定された継代数（25 代）以内で試験に使用する。試験前日に 80～90%コンフルエントになった培養フラスコから調製した均一な細胞液を 4 枚（3 枚はルシフェラーゼ活性測定、1 枚は生細胞数測定）の 96 ウエルプレートに播種（10,000 cells / well）する。

3-2. 被験物質および対照物質の調製

原則として、試験当日の調製とする。

被験物質は DMSO に溶解して 200 mM の溶液を調製する。DMSO に不溶の場合は滅菌水あるいは培養液にて同様に調製し、その溶液は、滅菌（例えば濾過）する。分子量が不明の被験物質の場合は 40 mg/mL あるいは 4%（W/V）の溶液とする。これらの溶液を DMSO（不溶の場合は滅菌水または滅菌培養液）で倍々希釈して 12 段階の濃度（0.098～200 mM）溶液を調製する。

陰性対照（媒体）は 1 プレートあたり 6 ウエル分を同様に調製する。

陽性対照は Cinnamic Aldehyde （CAS No: 14371-10-9, trans-3-Phenyl-2-propenal, trans-Cinnamaldehyde）を用い、DMSO に溶解して 200 mM の溶液を調製し、さらに DMSO で希釈して 6.4 mM とする。この溶液から DMSO で倍々希釈をして 5 段階の濃度（0.4～6.4 mM）溶液を調製する。

さらに、すべての溶液を血清含有培養液で 25 倍希釈する。これらの調製溶液を各ウエルに加える（「被験物質等の適用」参照）と、最終濃度は被験物質で 0.98～2000 μM、陽性対

照で 4〜64 μM となる。陰性対照の DMSO の最終濃度は他の調製液と同じ 1%となる。

少なくとも 2 回の繰り返し測定を行うが、3 回目を行う場合も含めて、それぞれの繰り返し測定は日を変えて行い、被験物質の溶液調製、細胞の前培養（継代数は同じもの）も繰り返し測定ごとに行う。

3-3. 被験物質等の適用

24 時間培養後の 4 枚のプレートの培養液を捨て、1 ウエルあたり 150 μL の血清含有培地（抗生物質不含）で置き換える。調製した被験物質溶液等を 50 μL ずつ各ウエルに加え、48 時間、37±1℃、5%CO_2 インキュベータ内で培養する。ただし、1 ウエルは無処置（無細胞、空ウエル）とする。ウエルからの蒸発や交差汚染を避けるためにプレートごとに遮蔽する。

3-4. ルシフェラーゼ活性の測定

適切なルシフェラーゼ活性の測定には、1) 感度の良いルミノメータ、2) 光の交差による測定の妨害を防ぐに十分なウエルの高さを持ったプレート、3) 十分な感度とバラつきの低い測定値を得るためのルシフェラーゼ基質の選択が重要である。これらを確認するために Annex 3 [3)]に示されたセットアップ方法を試験前に確認することを勧める。

培養終了後、上清を捨て、リン酸緩衝化生理食塩水で一度洗う。細胞溶解用緩衝液を各ウエルに加え、室温で 20 分間処理する。細胞溶解物を含むプレートをルミノメータで測定するため、各ウエルにルシフェラーゼの基質液 50 μL を加え、1 秒待ち、2 秒間の発光量を積算する。

3-5. 細胞生存率の測定

細胞の生存率を測定するプレートは、培養終了後に培地を MTT（3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue tetrazolium bromide; CAS No. 298-93-1）含有新鮮培地に交換する。4 時間、37℃、5%CO_2 インキュベータ内で培養する。培養後、MTT 含有培地を捨て、10%SDS(Sodium Dodecyl Sulphate)溶液などで一夜細胞溶解後、600 nm の吸光度を測定する。

3-6. 測定値から以下のパラメーターを求める。

- 被験物質および陽性対照で観察されたルシフェラーゼ活性の最大誘導倍率：I_{max}
- ルシフェラーゼ活性の誘導が溶媒対照の 1.5 倍の閾値（ルシフェラーゼ活性が 50%増加）を超えた濃度：$EC_{1.5}$
- 細胞生存率が 50%および 30%減となる濃度：IC_{50} および IC_{30}

ルシフェラーゼ活性の誘導倍率（*Fold induction*）は式 1 から求める。全体の最大誘導倍率は、個々の繰り返し測定の平均として求める。

式 1　　$Fold\ induction = (L_{sample} - L_{blank}) / (L_{solvent} - L_{blank})$

L_{sample} ：被験物質の発光強度

L_{blank} ：無細胞、無処置の対照ウエルの発光強度

$L_{solvent}$ ；細胞と溶媒からなるウエルの平均発光強度

$EC_{1.5}$ は式 2 により線形補間に基づいて求める。全体の $EC_{1.5}$ は、個々の繰り返し測定の幾何学的平均で求める。

式 2　$EC_{1.5} = (C_b - C_a) \times \{(1.5 - I_a) / (I_b - I_a)\} + C_a$

C_a：1.5-fold induction を超えた最低濃度（μM）

C_b：1.5-fold induction 未満の最高濃度（μM）

I_a：1.5-fold induction を超えた最低濃度での fold induction（3 回測定の平均）

I_b：1.5-fold induction 未満の最高濃度での fold induction（3 回測定の平均）

生存率（*Viability*）は式 3 から求める。

式 3　$Viability = (V_{sample} - V_{blank}) / (V_{solvent} - V_{blank}) \times 100$

V_{sample}：被験物質のウエルの MTT 吸光度

V_{blank}：無細胞、無処置の対照ウエルの MTT 吸光度

$V_{solvent}$：細胞と溶媒からなるウエルの平均 MTT 吸光度

IC_{50} と IC_{30} は式 4 により線形補間に基づいて求める。全体の IC_{50} と IC_{30} は、個々の繰り返し測定の幾何学的平均で求める。

式 4　$IC_x = (C_b - C_a) \times [\{(100 - x) - V_a\} / (V_b - V_a)] + C_a$

x ：濃度を求める減少率

C_a：細胞生存率 x％減を超えた最低濃度（μM）

C_b：細胞生存率 x％減未満の最高濃度（μM）

V_a：細胞生存率 x％減を超えた最低濃度における生存率

V_b：細胞生存率 x％減未満の最高濃度における生存率

1.5-fold induction を超えた各濃度について、ルシフェラーゼ活性の誘導が統計学的に陰性対照に対して有意（$p < 0.05$）であるかを検証する。グラフを作成して視覚的に確認することも推奨する。明らかな濃度依存性が認められない場合、濃度反応曲線が二相性を示す場合には、測定を繰り返し、被験物質特異的か、測定エラーなのかなどを確認する。二相性であることが確認できた場合は、より低い $EC_{1.5}$ 値を選択する。

3-7. 測定成立条件

以下の 3 条件をすべて満たす場合に成立する。

1)　陽性対照の Cinnamic Aldehyde は陽性でなくてはならない。すなわち、陽性対照の誘

導は少なくとも 1 濃度で 1.5 の閾値以上で統計学的に有意でなくてはならない。

2）Cinnamic Aldehyde の $EC_{1.5}$ 値はヒストリカルデータ（7 μM と 30 μM の間のバリデーションデータに基づき定期的に更新すること）の平均の 2 標準偏差値以内であることと、64 μM の Cinnamic Aldehyde の 3 プレートの平均の Fold induction は 2 から 8 の間にあることを確認する。後者が満たされない場合は、Cinnamic Aldehyde によるルシフェラーゼ活性の誘導と濃度依存性の関連を慎重に確認し、濃度依存性が明らかな場合に受け入れられる。

3) 3 プレートの 6 ウエルの溶媒対照（合計 18 ウエル）の平均変動係数が 20%以下であることが必要で、これよりも高い場合は無効とする。

3-8. 陽性の判定

2 回の繰り返し実験の 2 回あるいは 3 回の繰り返し実験の 2 回で、以下のすべての条件に合致した場合に被験物質は感作性物質と判断する。

1) I_{max} 値が 1.5 倍誘導よりも大きく溶媒対照に比較して統計学的に有意であること。

2) 1.5 倍以上のルシフェラーゼ活性の誘導を起こした最低濃度において、細胞生存率は 70%以上であること。

3) $EC_{1.5}$ 値が 1000 μM 未満（分子量未知の場合は 200 mg/mL 未満）であること。

4) ルシフェラーゼの誘導に明らかな全体的濃度依存性があること。

1）から 3）のいずれもが満たされたが、ルシフェラーゼの誘導に明らかな濃度依存性が認められないとき、結論は下せず、 さらに繰り返しの実験が必要となる。1000 μM 未満（分子量未知の場合は 200 mg/mL 未満）で陰性の場合も結論は下せない。細胞毒性を示す濃度領域でルシフェラーゼ活性の誘導を示す物質は、わずかな濃度変化で陽性の判定が覆る例がまれにある。このような物質は、より狭い濃度範囲でより小さい希釈系列（例えば 1.33 あるいは 1.41）を用いて、誘導が細胞毒性濃度で起こるのか否かを決めることが必要である。

4. 精度

Givaudan 社の先行バリデーション研究 [4]において、技術移転性、施設内再現性および施設間再現性が検討されている。

4-1. 技術移転性（表 1）

7 物質を用いて主導施設の Givaudan 社から Procter&Gamble 社、Beiersdorf 社、Institute for in vitro science（IIVS）および BASF の 4 施設への技術移転性について評価が行われた。その結果、直接の技術指導をせずに SOP の提供だけで技術移転は可能であった。

GHS(Globally Harmonized System of Classification and Labelling of Chemicals)で区分 1A に分類される 2,4-Dinitrochlorobenzene および Citral、GHS で区分 1B に分類される Ethylene glycol

dimethacrylate は、全 5 施設 3 回繰り返し行った試験の結果はすべて陽性であった。一方、GHS で区分 1B に分類される Hexyl cinnamic aldehyde は 2 施設で 3 回中 3 回陽性であったが、主導施設を含む 3 施設ではすべて陰性あるいは、陽性 1 回、陰性 2 回の混在した判定結果を示した。非感作性物質である Chlorobenzene、Methyl salicylate および Sulfanilamide はすべての施設で陰性であったが、3 回繰り返し中 1 回陽性の判定を示す施設があった。これらの結果は、主導施設が想定した範囲内の結果であると推測されるため、技術移転性に問題はないと考えられる。

表 1　技術移転性の評価成績

No.	Chemical Name	CAS	GHS potency category	Positve with EC 1.5 up to 1000 μM					
				Lead Lab. Histolocal Data	Lead lab.	Lab.1	Lab.2	Lab.3	Lab.4
1	2,4-Dinitrochlorobenzene	97-00-7	1A	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
2	Citral	5392-40-5	1A	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
3	Ethylne glycol dimethacrylate	97-90-5	1B	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
4	Hexyl cinnamic aldehyde	101-86-0	1B	2 of 2	1 of 3	0 of 3	1 of 3	3 of 3	3 of 3
5	Chlorobenzene	108-90-7	No	0 of 2	0 of 3	0 of 3	0 of 3	0 of 3	1 of 3
6	Methyl salicylate	119-36-8	No	0 of 2	0 of 3	1 of 3	0 of 3	1 of 3	1 of 3
7	Sulfanilamide	64-74-1	No	0 of 2	0 of 3	0 of 3	0 of 3	1 of 3	0 of 3

4-2.　施設内再現性（表 2）

ブラインド下で評価された 21 物質において、5 施設の施設内再現性（3 回の繰り返しの試験で同じ結果）は、主導施設：90.5%、試験施設 1：90.5%、試験施設 2：95.2%、試験施設 3 : 95.2%、試験施設 4 : 81.0%であった。本試験では、達成基準は設定されていないため、DPRA のバリデーション研究で採用された 85%を目安に考えた場合、5 施設中 4 施設がこの基準を上回った。基準に達しなかった試験施設 4 において再現性の得られない物質は、GHS で区分 1B に分類される Eugenol、Phenyl benzoate、および非感作性物質である Diethyl phthalate および Sodium lauryl sulfate であった。

ただし、本評価に使用された皮膚感作性陽性物質の内訳は、GHS で 1A に分類される物質が 11 物質に対し、施設内で再現性が得られない場合のある 1B に分類される物質が 4 物質と偏りがあることは留意すべき点と考える。

4-3. 施設間再現性（表 2）

ブラインド下で評価された 21 物質の 5 施設の施設間再現性（5 施設で同じ結果）は 85.7%であった。本先行バリデーション研究では、達成基準は設定されていないため、DPRA のバリデーション研究で採用された 80%を目安に考えた場合、この基準を上回った。

施設内再現性と同様に、本評価に使用された皮膚感作性陽性物質の内訳に偏りがあることは留意すべき点と考える。

表 2　施設間変動および施設内変動の評価成績

No.	Chemical Name	CAS	GHS potency category	Positve with EC 1.5 up to 1000 μM					Between Laboratory Reproducibility
				Lead lab.	Lab.1	Lab.2	Lab.3	Lab.4	
1	2-Mercaptobenzothiazole	149-30-4	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
2	4-Methylaminophenol sulphate	55-55-0	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
3	4-Nitrobenzylbromide	100-11-8	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
4	4-Phenylenediamine	106-50-3	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
5	(5-Chloro)-methylisothiazolinone	26172-55-4	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
6	Cinnamic aldehyde	104-55-2	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
7	Isoeugenol	97-54-1	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
8	Glyoxal	107-22-2	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
9	Methyldibromo glutaronitrile	35691-65-7	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
10	Oxazolone	15646-46-5	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
11	Tetramethylthiuramdisulfide	137-26-8	1A	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
12	Cinnamyl alcohol	104-54-1	1B	3 of 3	2 of 3	3 of 3	3 of 3	3 of 3	Y
13	Eugenol	97-53-0	1B	1 of 3	1 of 3	3 of 3	3 of 3	1 of 3	N
14	Imidazolidinyl urea	39236-46-9	1B	2 of 3	3 of 3	3 of 3	3 of 3	3 of 3	Y
15	Phenyl benzoate	93-99-2	1B	0 of 3	0 of 3	0 of 3	0 of 3	1 of 3	Y
16	Diethyl phthalate	84-66-2	No	0 of 3	0 of 3	1 of 3	0 of 3	2 of 3	N
17	Isopropanol	67-63-0	No	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3	Y
18	Glycerol	56-81-5	No	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3	Y
19	Lactic acid	50-21-5	No	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3	Y
20	Salicylic acid	69-72-7	No	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3	Y
21	Sodium lauryl sulfate	151-21-3	No	3 at cytotox,conc.	1 at cytotox,conc.	1 at cytotox,conc.	1 at cytotox,conc.	3 of 3	N
Within Laboratory Reproducibility				90.5%(19/21)	90.5%(19/21)	95.2%(20/21)	100%(21/21)	85.7%(18/21)	85.7%(18/21)

5．正確度（感度および特異度）

Givaudan 社の先行バリデーション研究[4]においてブラインド下で評価された 21 物質を基に GHS 分類との一致度、感度および特異度に関する評価を行った。

実施 5 施設の成績の積算による感度（Sensitivity）は 89.3%、特異度（Specificity）は 93.3%、正確度（Accuracy）は 90.5%であった。試験施設毎の成績は、主導施設および試験施設 1：感度 86.7%、特異度 100%、正確度 90.5%、試験施設 2 および 3：感度 93.3%、特異度 100%、正確度 95.2%、試験施設 4：感度 86.7%、特異度 66.7%、正確度 81.0%であった。

本試験法の正確度に関して開発施設である Givaudan 社から提出されたヒストリカルデータでは 92.9%と報告されている。また、初期の評価物質数が不十分であるとの ESAC のコメントを受け、Givaudan 社が実施した 67 物質 （感作性物質 44、非感作性物質 23）と、さらに追加で実施した物質を含む 114 物質（感作性物質 86、非感作性物質 28）での評価結果は[4]、67 物質：感度 86.4%、特異度 82.6%、正確度 85.1%、114 物質：感度 76.7%、特異度 82.1%、正確度 78.1%であった。さらに、Natsch ら[5]の 145 物質を用いた試験では、感度 77%、特異度 79%、正確度 72%であった。また、表 3 に示す通り、GHS で 1A に分類される物質を誤って陰性と判断することはなかった。

表 3　GHS 区分 1A 物質の成績

Chemical	Cas no.	GHS 分類	Imax			
			2008[6]	2010[7]	2011[8]	INVITTOX[4]
Citral	5392-40-5	1A	9.8	96.4	22.3-104.4	96.4
Tetramethylthiuram disulfide	137-26-8	1A	ND	6.8	8.1-67.9	6.8
Cinnamic aldehyde	104-55-2	1A	31.6	16.2	9.8-44.7	16.2
2-Mercaptobenzothiazole	149-30-4	1A	10.9	8.8	4.9-64.1	8.8
Glyoxal	107-22-2	1A	ND	28.2	14.5-195.0	28.2
Isoeugenol	97-54-1	1A	60.2	6.4	9.5-56.8	6.4
1,2-Dibromo-2,4-dicyanobutane	35691-65-7	1A	ND	4.0	2.1-7.8	4.0
4-(Methylamino)phenol sulfate	55-55-0	1A	32.4	5.9	4.5-12.2	10.3
1,4-Phenylenediamine	106-50-3	1A	12.7	26.8	19.4-45.2	26.8
4-Nitrobenzyl bromide	100-11-8	1A	ND	6.9	4.7-14.0	6.9
1-Chloro-2,4-dinitrobenzene	97-00-7	1A	12.3	14.8	4.3-19.5	14.8
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	1A	7.2	7.2	4.8-13.5	7.2
Oxazolone	15646-46-5	1A	2.3	2.4	6.4-46.3	2.4

ND: Not determined

6. 評価可能な物質の範囲

Emter らが報告しているように[6]、様々な構造を有する化学物質の皮膚感作性の予測が可能である（表 4)。ただし、溶解性や溶媒中での安定性（例えば、溶媒中での加水分解）が問題で評価不可能となる物質が存在する。具体的には、LogP が 7 以上の強疎水性分子は DMSO と水への溶解特性から試験ができない。一方、LogP が 5 までの化学物質は、水あるいは DMSO に可溶なため、容易に試験が可能である。酸無水物は、システイン残基ではなくリジン残基と反応特徴があり、システイン残基との反応が必要な Nrf2 pathway を誘導しないことが推察され、偽陰性と判定されることが考えられる。さらに、酸化反応や酸化的脱アミノ反応を必要とする多くのプレハプテンを正しく判定できるが、P450 による活性化が必要と推定されるプロハプテンは検出できない（KeratinoSens™ の由来細胞である HaCaT 細胞は薬物代謝酵素活性を有するが限定的である)。ルシフェラーゼ酵素に干渉する化学物質も評価に影響する[3]。

表 4　Emter らの報告

Chemical Name	LLNA EC3	KeratinoSens results			Chemical Name	LLNA EC3	KeratinoSens results		
		ARE I_{max}	ARE $EC_{1.5}$	ARE IC_{50}			ARE I_{max}	ARE $EC_{1.5}$	ARE IC_{50}
Sensitizers					***Non-sensitizers***				
Oxazolone	0.003	2.4	175.5	1370.9	Sodium lauryl sulfate	var.	1.2	n.i.	44.7
Benzoquinone	0.01	15.2	6.5	104.5	Salicylic acid	var.	1.1	n.i.	>2000
(5-Chloro)-Methylisothiazolinone	0.01	7.2	8.7	7.1	Methyl salicylate	var.	1.2	n.i.	>2000
2,4-Dinitrochlorobenzene	0.05	14.8	2.5	8.2	Sulfanilamide	NC	1.4	n.i.	>2000
4-nitrobenzylbromide	0.05	6.9	1.3	9.1	Diethyl phthalate	>100%	1.1	n.i.	>2000
4-phenylenediamine	0.11	26.8	5.0	438.9	Glycerol	>100%	1.2	n.i.	>2000
Glutaraldehyde	0.12	80.7	24.3	242.6	Propylene glycol	>100%	1.2	n.i.	>2000
Benzoyl peroxide	0.22	1.4	n.i.	567.6	Benzoic acid	>20%	1.1	n.i.	>2000
Glyoxal	0.75	28.2	89.1	677.9	1-Butanol	>20%	1.1	n.i.	>2000
4-Methylaminophenol sulphate	0.80	5.9	9.4	11.7	4-Hydroxybenzoic acid	>25%	1.1	n.i.	>2000
Formaldehyde	0.84	16.9	63.2	201.6	Sulfanilic acid	>25%	1.3	n.i.	>1000
Methyldibromo glutaronitrile	0.90	4.0	7.8	25.6	Tartaric acid	>25%	1.2	n.i.	>2000
Cinnamic aldehyde	1.3	16.2	16.1	194.4	Propylparaben	>25%	9.7	14.5	813.1
2-Hydroxyethyl acrylate	1.4	54.9	32.3	207.2	Ethyl vanillin	>50%	5.4	161.7	>2000
Isoeugenol	1.5	6.4	16.1	731.4	Isopropanol	>50%	1.2	n.i.	>2000
Ethylenediamine	2.2	13.2	99.9	>2000	Benzyl alcohol	>50%	1.2	n.i.	>2000
Benzylidene Acetone	2.2	503.9	9.7	174.5	Dimethylisophtalate	NC	2.1	694.9	>2000
Methyl-2-nonynoate	2.5	33.1	1.8	121.9	Dextran	NC	1.5	n.i.	>2000
2-Mercaptobenzothiazole	2.5	8.8	48.1	1003.1	Tween 80	NC	2.7	19.3	399.8
Benzyl salicylate	2.9	5.5	8.4	111.0	Chlorobenzene	Neg.	1.2	n.i.	>2000
Tetramethylthiuramdisulfide	3.1	6.8	0.8	39.1	Lactic acid	Neg.	1.3	n.i.	>2000
Diethylenetriamine	3.3	1.7	1259.4	>2000	Phenol	Neg.	1.3	n.i.	>2000
Thioglycerol	3.5	1.5	n.i.	>2000	Benzaldehyde	>25	2.3	443.1	>2000
Phenylacetaldehyde	4.5	11.3	28.5	116.2	Octanoic acid	>50	1.1	n.i.	>2000
Resorcinol	5.9	1.0	n.i.	>2000	n.i.: no significant induction above threshold				
Dihydroeugenol	6.8	1.5	462.0	759.2	ver.: Variable results				
Benzoisothiazolione	7.8	24.0	3.2	50.9	NC: Level not specified				
Citral	9.8	96.4	23.2	182.8	Neg: Negative reference according to D. Basketter. Food Chem. Toxicol. 37, 1167-1174				
Hexyl cinnamic aldehyde	9.9	2.7	17.3	26.3					
Eugenol	10.1	1.3	n.i.	1505.7					
Abietic acid	11.6	11.4	16.6	104.6					
Phenyl benzoate	13.6	1.3	n.i.	191.6					
Lyral HMPCC	17.1	16.1	79.6	355.4					
Benzocaine	17.1	3.0	18.2	>2000					
Benzyl cinnamate	18.4	8.7	11.0	>2000					
2,4-Dichloronitrobenzene	20.0	2.9	68.3	816.0					
Cinnamyl alcohol	21.0	1.7	123.6	774.6					
Hydroxycitronellal	23.0	137.1	79.4	>2000					
Imidazolidinyl urea	24.0	2.9	45.4	90.4					
Butyl glycidyl ether	30.9	340.7	218.5	>2000					
Ethylene glycol dimethacrylate	32.9	188.4	57.4	1655.8					
Cobalt chloride	Pos.	23.3	298.6	1330.2					
Nickel sulfate	var.	4.2	329.0	998.7					

7. 有用性と限界

本試験法は、細胞培養の技術と 96 ウエル対応のルミノメーターの使用技術があれば容易に実施可能である。KeratinoSens™ 法および LLNA に関する費用の試算では、LLNA では 1 物質当たりのランニングコストが約 10 万円であるのに対し、KeratinoSens™ 法では 1 物質について 12 濃度で三重測定、かつ 3 回の繰り返し実験の場合のランニングコストは約 1.3 から 1.5 万円である。実験期間も LLNA より短期間で実施可能であることから、経済性・簡便性の面から有用と考えられる。しかしながら、現時点でこの測定に使用できることが知られている細胞系は KeratinoSens™ のみである。その使用には、本細胞系を樹立した Givaudan 社からライセンスを受けることが必要と考えられるが、費用も含めその条件の詳細はわかっていない。一方、KeratinoSens™ を使用しない場合には、同等の細胞系を樹立し、OECD 作成の本試験法に関する performance standard（案）[2)]に従い、妥当性を評価した上で使用しなければならなく、相応の費用と時間が必要なことが容易に想像される。

細胞培養技術に熟練した研究者であれば、自動化した機器を使用しなくても、少なくとも 1 物質 12 段階の濃度で 42 物質の三重測定の実験を 1 週間で実施でき、さらにこれを 3

回の繰り返しで実施すると、42 物質について 3 週間で完全な結果を得ることができるため、ハイスループットの試験法と考えられる。自動化された機器を用いれば更なる効率化も可能である。

EURL ECVAM が実施した LLNA との感作性の有無の比較[7]では、正確度が 77%（155/201）、感度 78%（71/91）、特異度 76%（84/110）であり、Natsch ら[6]の 145 物質を用いた試験でも同様の結果（77%, 79%, 72%）が得られている。本試験法は感作性物質と非感作性物質を分別するのに加えて、用量-反応情報を得るのにも寄与する可能性があるとの報告があるが、現時点でその根拠は十分とは言えない。

本試験法は多成分あるいは混合物でも技術的には適用可能であるが、実施例がないため、慎重な判断が必要である。また、2000 μM を最高濃度として試験を実施するが、貧溶解性を示す物質において、この最高濃度での評価が困難で 1000 μM 未満で得られた陰性の結果を基に、感作性が陰性とは判定できない。

8. 結論

ARE-Nrf2 Luciferase Test Method は、感作性発現機序における第二段階のイベントであるケラチノサイトにおける炎症反応および Nrf2-Keap1-ARE pathway を利用したレポーターアッセイであり、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。

マウスを用いる LLNA の 1/7 程度のランニングコストで実施可能であり、*in vitro* 試験法であることから有用性は高い。しかしながら、現時点で本試験法に使用できることが知られている細胞系は KeratinoSens™ のみであり、その使用には細胞系を樹立した Givaudan 社からライセンスを受けることが必要なため、導入の容易な試験系とは言い難い。

本試験法の先行バリデーション研究における施設内再現性は、5 施設中 1 施設において、GHS 区分 1B に分類される物質（弱い感作性物質）および非感作性物質で再現性が得られなかったため、目安とした達成基準（85%）に達しておらず、強い感作性物質以外では判定がぶれる懸念がある。一方、施設間再現性は、目安とした達成基準（80%）を上回ったものの、本評価に使用された皮膚感作性陽性物質の内訳は、GHS で区分 1A に分類される物質が 11 物質に対し、施設内で再現性が得られない場合のある GHS で区分 1B に分類される物質が 4 物質と偏りがあることは留意点と考える。

100 物質以上を評価した二つの報告における本試験法の感度は、76.7%と 77%であり、陰性の結果が得られた場合は、偽陰性の可能性を考慮し、補完し得る他の試験より確認しなければならなく、本試験法のみで皮膚感作性を陰性と判定することはできない。また、特異度も、82.1%と 79%であることから、陽性の結果が得られた場合にも、偽陽性の結果が生じる可能性があることに留意しなければならない。

本試験法では、活性化に代謝系を必要とする化学物質は、正しくその感作性が検出されない可能性がある。また、細胞を用いた評価系であるため、細胞毒性や疎水性の高い物質では規定されている最高濃度（2000 μM）での評価が難しく、陰性判定が下せない場合があ

る。

以上を踏まえ、本委員会は、本試験法が皮膚感作性評価に汎用されるためには、KeratinoSens™が安価に入手できることが前提と考える。また、本試験法の様々な限界を考慮すると、本試験法単独では皮膚感作性の評価は不十分であり、証拠の重み付けや他の試験法（LLNA、モルモットを用いる皮膚感作性試験など）と組み合わせでの評価を推奨する。

引用文献

1) EURL ECVAM (2012) ESAC Working Group Peer Review Consensus Report on Givaudan-coordinated study transferability and reliability of the KeratinoSens assay for skin sensitisation testing

2) OECD (2014, in preparation) Performance Standards for the assessment of proposed similar or modified in vitro skin sensitization ARE-Nrf2 luciferase test methods in TG xxx. OECD Environment, Health and Safety publications, Series on Testing and Assessment N.XXX, OECD, Paris.

3) OECD (2015) In Vitro Skin Sensitization: ARE-Nrf2 Luciferase Test Method, OECD Guidelines for the Testing of Chemicals No. 442D.

4) DB-ALM (INVITTOX) (2013) Protocol 155: KeratinoSens™

5) Natsch A., Ryan C.A., Foertsch L., Emter R., Jaworska J., Gerberick F. and Kern P. (2013) A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. Journal of Applied Toxicology, 33, 1337-1352.

6) Natsch A. and Emter R. (2008) Skin sensitizers induce antioxidant response element dependent genes: application to the *in vitro* testing of the sensitization potential of chemicals. Toxicological Science, 102, 110-119.

7) Emter R., Ellis G. and Natsch A. (2010) Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*. Toxicology and Applied Pharmacology, 245, 281-290.

8) Natsch A., Bauch C., Foerttsch L., Gerberick F., Norman K., Hilberer A., Inglis H., Landsiedel R., Onken S., Reuter H., Schepky A. and Emter R. (2011) The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSens assay to predict skin sensitizers in vitro: results of a ring-study in five laboratories. Toxicology in Vitro, 25, 733-744.

9) EURL ECVAM (2014). Recommendation on the KerationSens™ assay for skin sensitization testing, 42 pp.
http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations/recommendation-keratinosens-skin-sensitisation.

OECD/OCDE TG 442D

Adopted:
February 2015

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method

INTRODUCTION

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). This Test Guideline (TG) provides an *in vitro* procedure (the ARE-Nrf2 luciferase test method) to be used for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (1).

2. There is general agreement regarding the key biological events underlying skin sensitisation. The existing knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised in the form of an Adverse Outcome Pathway (AOP) (2), going from the molecular initiating event through the intermediate events up to the adverse health effect, i.e. allergic contact dermatitis in humans or contact hypersensitivity in rodents (2) (3). The molecular initiating 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 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, typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation, which is indirectly assessed in the murine Local Lymph Node Assay (4).

3. The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods based on guinea-pigs, the Magnusson Kligman Guinea Pig Maximisation Test (GMPT) and the Buehler Test TG 406 (5), study both the induction and elicitation phases of skin sensitisation. A murine test, the Local Lymph Node Assay (LLNA) (TG 429) (4) and its two non-radioactive modifications, LLNA: DA (TG 442A) (6) and LLNA: BrdU-ELISA (TG 442B) (7), which all assess the induction response exclusively, have also gained acceptance since they provide advantages over the guinea pig tests in terms of both animal welfare and objective measurement of the induction phase of skin sensitisation.

4. More recently, mechanistically-based *in chemico* and *in vitro* test methods have been considered scientifically valid for the evaluation of the skin sensitisation hazard of chemicals. However, combinations of non-animal methods (*in silico*, *in chemico*, *in vitro*) within Integrated Approaches to Testing and Assessment (IATA) will be needed to be able to fully substitute for the animal tests currently in use given the restricted AOP mechanistic coverage of each of the currently available non-animal test methods (2) (3).

5. The test method described in this Test Guideline (ARE-Nrf2 luciferase test method) is proposed to address the second key event as explained in paragraph 2. Skin sensitisers have been reported to induce genes that are regulated by the antioxidant response element (ARE) (8) (9). Small electrophilic substances

such as skin sensitisers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by e.g. covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes (8) (10) (11).

6. Currently, the only *in vitro* ARE-Nrf2 luciferase test method covered by this Test Guideline is the KeratinoSens™ test method for which validation studies have been completed (9) (12) (13) followed by an independent peer review conducted by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) (14). The KeratinoSens™ test method was considered scientifically valid to be used as part of an IATA, to support the discrimination between skin sensitisers and non-sensitisers for the purpose of hazard classification and labelling (14). Laboratories willing to implement the test method can obtain the recombinant cell line used in the KeratinoSens™ test method by establishing a licence agreement with the test method developer (15).

7. Definitions are provided in Annex 1.

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

8. Since activation of the Keap1-Nrf2-ARE pathway addresses only the second key event of the skin sensitisation AOP, information from test methods based on the activation of this pathway is unlikely to be sufficient when used on its own to conclude on the skin sensitisation potential of chemicals. Therefore data generated with the present Test Guideline should be considered in the context of integrated approaches, such as IATA, combining them with other 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. Examples on how to use the ARE-Nrf2 luciferase test method in combination with other information are reported in literature (13) (16) (17) (18) (19).

9. The test method described in this Test Guideline can be used to support the discrimination between skin sensitisers (i.e. UN GHS Category 1) and non-sensitisers in the context of IATA. This TG cannot be used on its own, neither to sub-categorise skin sensitisers into subcategories 1A and 1B as defined by the UN GHS (1), for authorities implementing these two optional subcategories, nor to predict potency for safety assessment decisions. However, depending on the regulatory framework, a positive result may be used on its own to classify a chemical into UN GHS category 1.

10. Based on the dataset from the validation study and in-house testing used for the independent peer-review of the test method, the KeratinoSens™ test method proved to be transferable to laboratories experienced in cell culture. The level of reproducibility in predictions that can be expected from the test method is in the order of 85% within and between laboratories (14). The accuracy (77% - 155/201), sensitivity (78% - 71/91) and specificity (76% - 84/110) of the KeratinoSens™ for discriminating skin sensitisers (i.e. UN GHS Cat. 1) from non-sensitisers when compared to LLNA results were calculated by considering all of the data submitted to EURL ECVAM for evaluation and peer-review of the test method (14). These figures are similar to those recently published based on in-house testing of about 145 test substances (77% accuracy, 79% sensitivity, 72% specificity) (13). The KeratinoSens™ is more likely to under predict 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) (13) (14). Taken together, this information indicates the usefulness of the KeratinoSens™ assay to contribute to the identification of skin sensitisation hazard. However, the accuracy values given here for the KeratinoSens™ 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 paragraph 9 above. Furthermore when evaluating non-animal methods for skin sensitisation, it should be

kept in mind that the LLNA as well as other animal tests, may not fully reflect the situation in the species of interest i.e. humans.

11. 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 ARE-Nrf2 luciferase test method to the testing of substances and/or mixtures. On the basis of the current data available the KeratinoSens™ test method was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined with *in vivo* studies) and physico-chemical properties (9) (12) (13) (14). Mainly mono-constituent substances were tested, although a limited amount of data also exist on the testing of mixtures (20). The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. However, before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture. Moreover, when testing multi-constituent substances or mixtures, consideration should be given to possible interference of cytotoxic constituents with the observed responses. The test 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 into different phases) either in water or DMSO (including all of the test chemical components in the case of testing a multi-constituent substance or a mixture). Test chemicals that do not fulfil these conditions at the highest final required concentration of 2000 μM (cf. paragraph 22) may still be tested at lower concentrations. In such a case, results fulfilling the criteria for positivity described in paragraph 39 could still be used to support the identification of the test chemical as a skin sensitiser, whereas a negative result obtained with concentrations < 1000 μM should be considered as inconclusive (see prediction model in paragraph 39). In general test substances with a LogP of up to 5 have been successfully tested whereas extremely hydrophobic substances with a LogP above 7 are outside the known applicability of the test method (14). For test substances having a LogP falling between 5 and 7, only limited information is available.

12. Negative results should be interpreted with caution as substances with an exclusive reactivity towards lysine-residues can be detected as negative by the test method. Furthermore, because of the limited metabolic capability of the cell line used (21) and because of the experimental conditions, pro-haptens (i.e. chemicals requiring enzymatic activation for example via P450 enzymes) and pre-haptens (i.e. chemicals activated by auto-oxidation) in particular with a slow oxidation rate may also provide negative results. Test chemicals that do not act as a sensitiser but are nevertheless chemical stressors may lead on the other hand to false positive results (14). Furthermore, highly cytotoxic test chemicals cannot always be reliably assessed. Finally, test chemicals that interfere with the luciferase enzyme can confound the activity of luciferase in cell-based assays causing either apparent inhibition or increased luminescence (22). For example, phytoestrogen concentrations higher than 1 μM were reported to interfere with the luminescence signals in other luciferase-based reporter gene assays due to over-activation of the luciferase reporter gene (23). As a consequence, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like over-activation of the luciferase reporter gene needs to be examined carefully (23). In cases where evidence can be demonstrated on the non-applicability of the Test Guideline to other specific categories of test chemicals, the test method should not be used for those specific categories.

13. In addition to supporting discrimination between skin sensitisers and non-sensitisers, the KeratinoSens™ assay also provides concentration-response information that may potentially contribute to the assessment of sensitising potency when used in integrated approaches such as IATA (19). However,

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

further work preferably based on reliable human data is required to determine how KeratinoSens™ results can contribute to potency assessment (24) and to sub-categorisation of sensitisers according to UN GHS (1).

PRINCIPLE OF THE TEST

14. The ARE-Nrf2 luciferase test method makes use of an immortalised adherent cell line derived from HaCaT human keratinocytes stably transfected with a selectable plasmid. The cell line contains the luciferase gene under the transcriptional control of a constitutive promoter fused with an ARE element from a gene that is known to be up-regulated by contact sensitisers (25) (26). The luciferase signal reflects the activation by sensitisers of endogenous Nrf2 dependent genes, and the dependence of the luciferase signal in the recombinant cell line on Nrf2 has been demonstrated (27). This allows quantitative measurement (by luminescence detection) of luciferase gene induction, using well established light producing luciferase substrates, as an indicator of the activity of the Nrf2 transcription factor in cells following exposure to electrophilic test substances.

15. Test chemicals are considered positive in the KeratinoSens™ if they induce a statistically significant induction of the luciferase activity above a given threshold (i.e. > 1.5 fold or 50% increase), below a defined concentration which does not significantly affect cell viability (i.e. below 1000 μM and at a concentration at which the cellular viability is above 70% (9) (12)). For this purpose, the maximal fold induction of the luciferase activity over solvent (negative) control (I_{max}) is determined. Furthermore, since cells are exposed to series of concentrations of the test chemicals, the concentration needed for a statistically significant induction of luciferase activity above the threshold (i.e. $EC_{1.5}$ value) should be interpolated from the dose-response curve (see paragraph 32 for calculations). Finally, parallel cytotoxicity measurements should be conducted to assess whether luciferase activity induction levels occur at sub-cytotoxic concentrations.

16. Prior to routine use of the ARE-Nrf2 luciferase test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency, using the ten Proficiency Substances listed in Annex 2.

17. Performance standards (PS) (28) are available to facilitate the validation of new or modified *in vitro* ARE-Nrf2 luciferase test methods similar to the KeratinoSens™ 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.

PROCEDURE

18. Currently, the only test method covered by this Test Guideline is the scientifically valid KeratinoSens™ test method (9) (12) (13) (14). The Standard Operating Procedures (SOP) for the KeratinoSens™ is available and should be employed when implementing and using the test method in the laboratory (15). Laboratories willing to implement the test method can obtain the recombinant cell line used in the KeratinoSens™ test method by establishing a licence agreement with the test method developer. The following paragraphs provide with a description of the main components and procedures of the ARE-Nrf2 luciferase test method.

Preparation of the keratinocyte cultures

19. A transgenic cell line having a stable insertion of the luciferase reporter gene under the control of the ARE-element should be used (e.g. the KeratinoSens™ cell line). Upon receipt, cells are propagated

(e.g. 2 to 4 passages) and stored frozen as a homogeneous stock. Cells from this original stock can be propagated up to a maximum passage number (i.e. 25 in the case of KeratinoSens™) and are employed for routine testing using the appropriate maintenance medium (in the case of KeratinoSens™ this represents DMEM containing serum and Geneticin).

20. For testing, cells should be 80-90% confluent, and care should be taken to ensure that cells are never grown to full confluence. One day prior to testing cells are harvested, and distributed into 96-well plates (10,000 cells/well in the case of KeratinoSens™). Attention should be paid to avoid sedimentation of the cells during seeding to ensure homogeneous cell number distribution across wells. If this is not the case, this step may give raise to high well-to-well variability. For each repetition, three replicates are used for the luciferase activity measurements, and one parallel replicate used for the cell viability assay.

Preparation of the test chemical and control substances

21. The test chemical and control substances are prepared on the day of testing. For the KeratinoSens™ test method, test chemical are dissolved in dimethyl sulfoxide (DMSO) to the final desired concentration (e.g. 200 mM). The DMSO solutions can be considered self-sterilising, so that no sterile filtration is needed. Test chemical not soluble in DMSO is dissolved in sterile water or culture medium, and the solutions sterilised by e.g. filtration. For a test chemical which has no defined molecular weight (MW), a stock solution is prepared to a default concentration (40 mg/mL or 4% (w/v)) in the KeratinoSens™ assay. In case solvents other than DMSO, water or the culture medium are used, sufficient scientific rationale should be provided.

22. Based on the stock DMSO solutions of the test chemical, serial dilutions are made using DMSO to obtain 12 master concentrations of the chemical to be tested (from 0.098 to 200 mM in the KeratinoSens™ test method). For a test chemical not soluble in DMSO, the dilutions to obtain the master concentrations are made using sterile water or sterile culture medium. Independent of the solvent used, the master concentrations, are then further diluted 25 fold into culture medium containing serum, and finally used for treatment with a further 4 fold dilution factor so that the final concentrations of the tested chemical range from 0.98 to 2000 μM in the KeratinoSens™ test method. Alternative concentrations may be used upon justification (e.g. in case of cytotoxicity or poor solubility).

23. The negative (solvent) control used in the KeratinoSens™ test method is DMSO (CAS No. 67-68-5, ≥ 99% purity), for which six wells per plate are prepared. It undergoes the same dilution as described for the master concentrations in paragraph 22, so that the final negative (solvent) control concentration is 1%, known not to affect cell viability and corresponding to the same concentration of DMSO found in the tested chemical and in the positive control. For a test chemical not soluble in DMSO, for which the dilutions were made in water, the DMSO level in all wells of the final test solution must be adjusted to 1% as for the other test chemicals and control substances.

24. The positive control used in the case of KeratinoSens™ is cinnamic aldehyde (CAS No. 14371-10-9, ≥ 98% purity), for which a series of 5 master concentrations ranging from 0.4 to 6.4 mM are prepared in DMSO (from a 6.4 mM stock solution) and diluted as described for the master concentrations in paragraph 22, so that the final concentration of the positive control range from 4 to 64 μM. Other suitable positive controls, preferentially providing $EC_{1.5}$ values in the mid-range, may be used if historical data are available to derive comparable run acceptance criteria.

Application of the test chemical and control substances

25. For each test chemical and positive control substance, one experiment is needed to derive a prediction (positive or negative), consisting of at least two independent repetitions containing each three

replicates (i.e. n=6). In case of discordant results between the two independent repetitions, a third repetition containing three replicates should be performed (i.e. n=9). Each independent repetition is performed on a different day with fresh stock solution of test chemicals and independently harvested cells. Cells may come from the same passage however.

26. After seeding as described in paragraph 20, cells are grown for 24 hours in the 96-wells microtiter plates. The medium is then removed and replaced with fresh culture medium (150 µl culture medium containing serum but without Geneticin in the case of KeratinoSens™) to which 50 µl of the 25 fold diluted test chemical and control substances are added. At least one well per plate should be left empty (no cells and no treatment) to assess background values.

27. The treated plates are then incubated for about 48 hours at 37±1°C in the presence of 5% CO_2 in the KeratinoSens™ test method. Care should be taken to avoid evaporation of volatile test chemicals and cross-contamination between wells by test chemicals by e.g. covering the plates with a foil prior to the incubation with the test chemicals.

Luciferase activity measurements

28. Three factors are critical to ensure appropriate luminescence readings:

- the choice of a sensitive luminometer,
- the use of a plate format with sufficient height to avoid light-cross-contamination; and
- the use of a luciferase substrate with sufficient light output to ensure sufficient sensitivity and low variability.

Prior to testing, a control experiment setup as described in Annex 3 should be carried out to ensure that these three points are met.

29. After the 48 hour exposure time with the test chemical and control substances in the KeratinoSens™ test method, cells are washed with a phosphate buffered saline, and the relevant lysis buffer for luminescence readings added to each well for 20 min at room temperature.

30. Plates with the cell lysate are then placed in the luminometer for reading which in the KeratinoSens™ test method is programmed to: (i) add the luciferase substrate to each well (i.e. 50 µl), (ii) wait for 1 second, and (iii) integrate the luciferase activity for 2 seconds. In case alternative settings are used, e.g. depending on the model of luminometer used, these should be justified. Furthermore, a glow substrate may also be used provided that the quality control experiment of Annex 3 is successfully fulfilled."

Cytotoxicity Assessment

31. For the KeratinoSens™ cell viability assay, medium is replaced after the 48 hour exposure time with fresh medium containing MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue tetrazolium bromide; CAS No. 298-93-1) and cells incubated for 4 hours at 37°C in the presence of 5% CO_2. The MTT medium is then removed and cells are lysed (e.g. by adding 10% SDS solution to each well) overnight. After shaking, the absorption is measured at i.e. 600 nm with a photometer.

DATA AND REPORTING

Data evaluation

32. The following parameters are calculated in the KeratinoSens^{TM} test method:

- - the maximal average fold induction of luciferase activity (I_{max}) value observed at any concentration of the tested chemical and positive control;
- - the $EC_{1.5}$ value representing the concentration for which induction of luciferase activity is above the 1.5 fold threshold (i.e. 50% enhanced luciferase activity) was obtained; and
- - the IC_{50} and IC_{30} concentration values for 50% and 30% reduction of cellular viability.

Fold luciferase activity induction is calculated by Equation 1, and the overall maximal fold induction (I_{max}) is calculated as the average of the individual repetitions.

Equation 1: $$Fold\ induction = \frac{(L_{sample} - L_{blank})}{(L_{solvent} - L_{blank})}$$

where

L_{sample} is the luminescence reading in the test chemical well
L_{blank} is the luminescence reading in the blank well containing no cells and no treatment
$L_{solvent}$ is the average luminescence reading in the wells containing cells and solvent (negative) control

$EC_{1.5}$ is calculated by linear interpolation according to Equation 2, and the overall $EC_{1.5}$ is calculated as the geometric mean of the individual repetitions.

Equation 2: $$EC1.5 = (C_b - C_a) \times \left(\frac{1.5 - I_a}{I_b - I_a}\right) + C_a$$

where

C_a is the lowest concentration in µM with > 1.5 fold induction
C_b is the highest concentration in µM with < 1.5 fold induction
I_a is the fold induction measured at the lowest concentration with > 1.5 fold induction (mean of three replicate wells)
I_b is the fold induction at the highest concentration with < 1.5 fold induction (mean of three replicate wells)

Viability is calculated by Equation 3:

Equation 3: $$Viability = \frac{(V_{sample} - V_{blank})}{(V_{solvent} - V_{blank})} \times 100$$

where

V_{sample} is the MTT-absorbance reading in the test chemical well
V_{blank} is the MTT-absorbance reading in the blank well containing no cells and no treatment
$V_{solvent}$ is the average MTT-absorbance reading in the wells containing cells and solvent (negative) control

IC_{50} and IC_{30} are calculated by linear interpolation according to Equation 4, and the overall IC_{50} and IC_{30} are calculated as the geometric mean of the individual repetitions.

Equation 4: $IC_x = (C_b - C_a) \times \left(\frac{(100-x)-V_a}{V_b-V_a}\right) + C_a$

where
X is the % reduction at the concentration to be calculated (50 and 30 for IC_{50} and IC_{30})
C_a is the lowest concentration in µM with > x% reduction in viability
C_b is the highest concentration in µM with < x% reduction in viability
V_a is the % viability at the lowest concentration with > x% reduction in viability
V_b is the % viability at the highest concentration with < x% reduction in viability

For each concentration showing > 1.5 fold luciferase activity induction, statistical significance is calculated (e.g. by a two-tailed Student's t-test), comparing the luminescence values for the three replicate samples with the luminescence values in the solvent (negative) control wells to determine whether the luciferase activity induction is statistically significant (p <0.05). The lowest concentration with > 1.5 fold luciferase activity induction is the value determining the $EC_{1.5}$ value. It is checked in each case whether this value is below the IC_{30} value, indicating that there is less than 30% reduction in cellular viability at the $EC_{1.5}$ determining concentration.

33. It is recommended that data are visually checked with the help of graphs. If no clear dose-response curve is observed, or if the dose-response curve obtained is biphasic (i.e. crossing the threshold of 1.5 twice), the experiment should be repeated to verify whether this is specific to the test chemical or due to an experimental artefact. In case the biphasic response is reproducible in an independent experiment, the lower $EC_{1.5}$ value (the concentration when the threshold of 1.5 is crossed the first time) should be reported.

34. In the rare cases where a statistically non-significant induction above 1.5 fold is observed followed by a higher concentration with a statistically significant induction, results from this repetition are only considered as valid and positive if the statistically significant induction above the threshold of 1.5 was obtained for a non-cytotoxic concentration.

35. Finally, for test chemicals generating a 1.5 fold or higher induction already at the lowest test concentration of 0.98 µM, the $EC_{1.5}$ value of <0.98 is set based on visual inspection of the dose-response curve.

Acceptance criteria

36. The following acceptance criteria should be met when using the KeratinoSens™ test method. First, the luciferase activity induction obtained with the positive control, cinnamic aldehyde, should be statistically significant above the threshold of 1.5 (e.g. using a t-test) in at least one of the tested concentrations (from 4 to 64 µM).

37. Second, the $EC_{1.5}$ value should be within two standard deviations of the historical mean of the testing facility (e.g. between 7 µM and 30 µM based on the validation dataset) which should be regularly updated. In addition, the average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8. If the latter criterion is not fulfilled, the dose-response of cinnamic aldehyde should be carefully checked, and tests may be accepted only if there is a clear dose-response with increasing luciferase activity induction at increasing concentrations for the positive control.

38. Finally, the average coefficient of variation of the luminescence reading for the negative (solvent) control DMSO should be below 20% in each repetition which consists of 6 wells tested in triplicate. If the variability is higher, results should be discarded.

Interpretation of results and prediction model

39. A KeratinoSens™ prediction is considered positive if the following 4 conditions are all met in 2 of 2 or in the same 2 of 3 repetitions, otherwise the KeratinoSens™ prediction is considered negative (Figure 1):

1. the I_{max} is higher than (>) 1.5 fold and statistically significantly different as compared to the solvent (negative) control (as determined by a two-tailed, unpaired Student's T-test);

2. the cellular viability is higher than (>) 70% at the lowest concentration with induction of luciferase activity above 1.5 fold (i.e. at the $EC_{1.5}$ determining concentration);

3. the $EC_{1.5}$ value is less than (<) 1000 µM (or < 200 µg/mL for test chemicals with no defined MW);

4. there is an apparent overall dose-response for luciferase induction (or a biphasic response as mentioned under paragraph 33).

If in a given repetition, all of the three first conditions are met but a clear dose-response for the luciferase induction cannot be observed, then the result of that repetition should be considered inconclusive and further testing may be required (Figure 1). In addition, a negative result obtained with concentrations < 1000 µM (or <200 µg/mL for test chemicals with no defined MW) should also be considered as inconclusive (see paragraph 11).

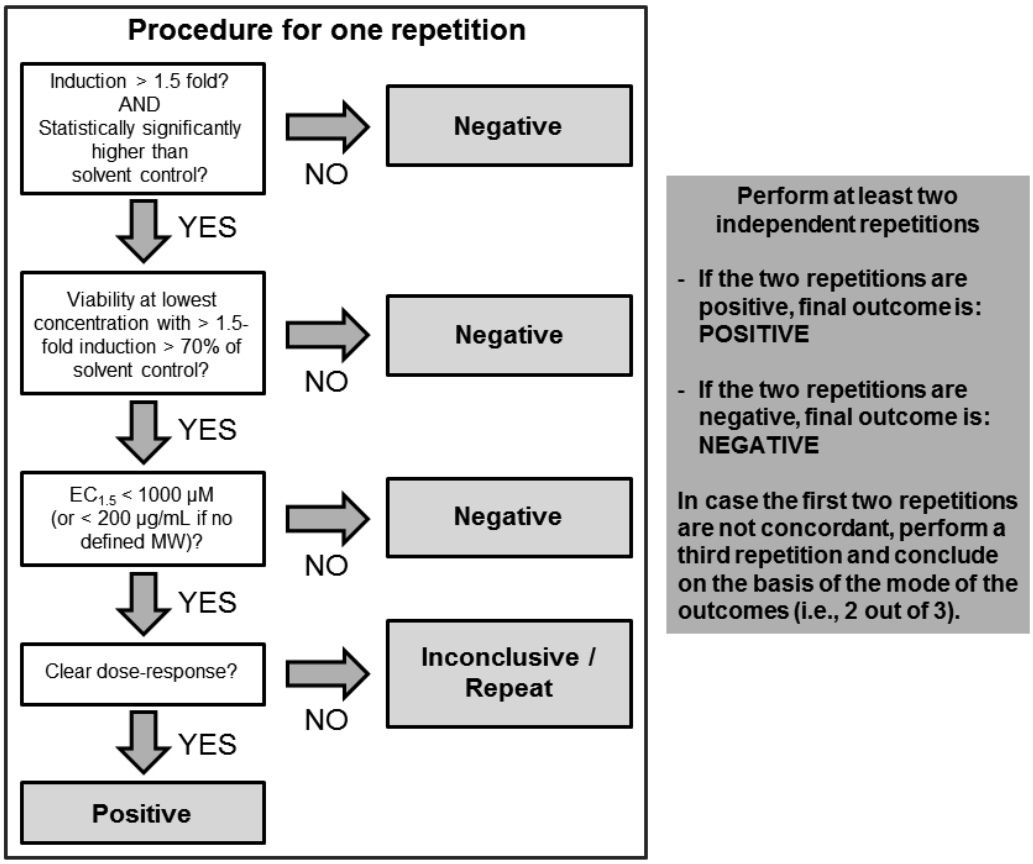


Figure 1: Prediction model used in the KeratinoSens™ test method. A KeratinoSens™ prediction should be considered in the framework of an IATA and in accordance with the provision of paragraphs 9 and 11.

40. In rare cases, test chemicals which induce the luciferase activity very close to the cytotoxic levels can be positive in some repetitions at non-cytotoxic levels (i.e. $EC_{1.5}$ determining concentration below (<) the IC_{30}), and in other repetitions only at cytotoxic levels (i.e. $EC_{1.5}$ determining concentration above (>) the IC_{30}). Such test chemicals shall be retested with more narrow dose-response analysis using a lower dilution factor (e.g. 1.33 or √2 (=1.41) fold dilution between wells), to determine if induction has occurred at cytotoxic levels or not (9).

Test report

41. 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, 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.

- 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.

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, 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 (vehicle) 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 / vehicles 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 they were obtained);
- Passage number and level of confluence of cells used for testing;
- Cell counting method used for seeding prior to testing and measures taken to ensure homogeneous cell number distribution (cf. paragraph 20);
- Luminometer used (e.g. model), including instrument settings, luciferase substrate used, and demonstration of appropriate luminescence measurements based on the control test described in Annex 3;
- 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 repetitions and replicates used;
- Test chemical concentrations, application procedure and exposure time used (if different than the one recommended)

- Description of evaluation and decision criteria used;
- Description of study acceptance criteria used;
- Description of any modifications of the test procedure.

Results

- Tabulation of I_{max}, $EC_{1.5}$ and viability values (i.e. IC_{50}, IC_{30}) obtained for the test chemical and for the positive control for each repetition as well as the mean values (I_{max}: average; $EC_{1.5}$ and viability values: geometric mean) and SD calculated using data from all individual repetitions and an indication of the rating of the test chemical according to the prediction model;
- Coefficient of variation obtained with the luminescence readings for the negative control for each experiment;
- 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 KeratinoSens™ test method;
- Consideration of the test method results within the context of an IATA, if other relevant information is available.

Conclusion

LITERATURE

1. United nations (UN) (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fifth revised edition, UN New York and Geneva, 2013. Available at: [http://www.unece.org/trans/danger/publi/ghs/ghs_rev05/05files_e.html.]

2. 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.

3. Adler S., Basketter D., Creton S., Pelkonen O., van Benthem J., Zuang V., Andersen K.E., Angers-Loustau A., Aptula A., Bal-Price A., Benfenati E., Bernauer U., Bessems J., Bois F.Y., Boobis A., Brandon E., Bremer S., Broschard T., Casati S., Coecke S., Corvi R., Cronin M., Daston G., Dekant W., Felter S., Grignard E., Gundert-Remy U., Heinonen T., Kimber I., Kleinjans J., Komulainen H., Kreiling R., Kreysa J., Leite S.B., Loizou G., Maxwell G., Mazzatorta P., Munn S., Pfuhler S., Phrakonkham P., Piersma A., Poth A., Prieto P., Repetto G., Rogiers V., Schoeters G., Schwarz M., Serafimova R., Tähti H., Testai E., van Delft J., van Loveren H., Vinken M., Worth A., Zaldivar J.M. (2011). Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. Archives of Toxicology 85, 367-485.

4. OECD (2010). Skin sensitization: Local Lymph Node assay. OECD Guidelines for Chemical Testing No. 429. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines].

5. OECD (1992). Skin Sensitisation. OECD Guidelines for the Testing of Chemicals No. 406. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines].

6. OECD (2010). Skin sensitization: Local Lymph Node assay: DA. OECD Guidelines for Chemical Testing No. 442A. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines].

7. OECD (2010). Skin sensitization: Local Lymph Node assay: BrdU-ELISA. OECD Guidelines for Chemical Testing No. 442B. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines].

8. Natsch A. (2010). The Nrf2-Keap1-ARE Toxicity Pathway as a Cellular Sensor for Skin Sensitizers-Functional Relevance and Hypothesis on Innate Reactions to Skin Sensitizers. Toxicological Sciences 113, 284-292.

9. Emter R., Ellis G., Natsch A.(2010). Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*. Toxicology and Applied Pharmacology 245, 281-290.

10. Dinkova-Kostova A.T., Holtzclaw W.D., Kensler T.W. (2005). The role of Keap1 in cellular protective responses. Chem Res Toxicol. 18, 1779-1791.

11. Kansanen E., Kuosmanen S.M., Leinonen H., Levonen A.L. (2013). The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. Redox Biol. 18,1, 45-49.

12. Natsch A., Bauch C., Foertsch L., Gerberick F., Normann K., Hilberer A., Inglis H., Landsiedel R., Onken S., Reuter H., Schepky A., Emter R. (2011). The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSens assay to predict skin sensitizers *in vitro*: results of a ring-study in five laboratories. Toxicol. *In Vitro* 25, 733-744.

13. Natsch A., Ryan C.A., Foertsch L., Emter R., Jaworska J., Gerberick G.F., Kern P. (2013). A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. Journal of Applied Toxicology, 33, 1337-1352.

14. EURL-ECVAM (2014). Recommendation on the KeratinoSens™ assay for skin sensitisation testing, 42 pp. Available at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations/recommendation-keratinosens-skin-sensitisation.

15. DB-ALM (INVITTOX) (2013) Protocol 155: KeratinoSens™, 17pp. Available: [http://ecvam-dbalm.jrc.ec.europa.eu/].

16. Natsch A., Emter R., Ellis G. (2009). Filling the concept with data: integrating data from different in vitro and in silico assays on skin sensitizers to explore the battery approach for animal-free skin sensitization testing. Toxicol Sci. 107, 106-121.

17. Ball N., Cagen S., Carrillo J.C., Certa H., Eigler D., Emter R., Faulhammer F., Garcia C., Graham C., Haux C., Kolle S.N., Kreiling R., Natsch A., Mehling A. (2011). Evaluating the sensitization potential of surfactants: integrating data from the local lymph node assay, guinea pig maximization test, and in vitro methods in a weight-of-evidence approach. Regul Toxicol Pharmacol. 60, 389-400.

18. Bauch C., Kolle S.N., Ramirez T., Eltze 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 potentials. Regul Toxicol Pharmacol. 63, 489-504.

19. Jaworska J., Dancik Y., Kern P., Gerberick F., Natsch A., 2013. Bayesian integrated testing strategy to assess skin sensitization potency: from theory to practice. J Appl Toxicol. 33, 1353-1364.

20. Andres E., Sa-Rocha V.M., Barrichello C., Haupt T., Ellis G., Natsch A. (2013). The sensitivity of the KeratinoSens™ assay to evaluate plant extracts: A pilot study. Toxicology *In Vitro* 27, 1220-1225.

21. 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.

22. Thorne N., Inglese J., Auld D.S. (2010). Illuminating Insights into Firefly Luciferase and Other Bioluminescent Reporters Used in Chemical Biology. Chemistry and Biology 17, 646-657.

23. OECD (2012). BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists. OECD Guidelines for Chemical Testing No. 457. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines].

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

25. Gildea L.A., Ryan C.A., Foertsch L.M., Kennedy J.M., Dearman R.J., Kimber I., Gerberick G.F. (2006). Identification of gene expression changes induced by chemical allergens in dendritic cells: opportunities for skin sensitization testing. J Invest Dermatol, 126, 1813-1822.

26. Ryan C.A., Gildea L.A., Hulette B.C., Dearman R.J., Kimber I. And Gerberick G.F. (2004). Gene expressing changes in peripheral blood-derived dendritic cells following exposure to a contact allergen. Toxicol. Lett. 150, 301-316.

27. Emter R., van der Veen J.W., Adamson G., Ezendam J., van Loveren H., Natsch A. (2013). Gene expression changes induced by skin sensitizers in the KeratinoSens™ cell line: Discriminating Nrf2-dependent and Nrf2-independent events. Toxicol In Vitro 27, 2225-2232.

28. OECD (2014, in preparation). Performance Standards for the assessment of proposed similar or modified in vitro skin sensitisation ARE-Nrf2 luciferase test methods in TG xxx. OECD Environment, Health and Safety publications, Series on Testing and Assessment N.XXX, OECD, Paris.

29. 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.

30. NAFTA (North American Free Trade Agreement) (2012). Technical Working Group on Pesticides – (Quantitative) Structure Activity Relationship ((Q)SAR) Guidance Document. 186 pp. Disponible à l'adresse suivante : http://www.epa.gov/oppfead1/international/naftatwg/guidance/qsar-guidance.pdf

ANNEX 1

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of "relevance." The term is often used interchangeably with "concordance", to mean the proportion of correct outcomes of a test method (29).

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 (2).

ARE: Antioxidant response element (also called EpRE, electrophile response element), is a response element found in the upstream promoter region of many cytoprotective and phase II genes. When activated by Nfr2, it mediates the transcriptional induction of these genes.

Coefficient of variation: a measure of variability that is calculated for a group of replicate data by dividing the standard deviation by the mean. It can be multiplied by 100 for expression as a percentage.

$EC_{1.5}$: Interpolated concentration for a 1.5 fold luciferase induction.

IC_{30}: Concentration effecting a reduction of cellular viability by 30%.

IC_{50}: Concentration effecting a reduction of cellular viability by 50%.

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.

I_{max}: Maximal induction factor of luciferase activity compared to the solvent (negative) control measured at any test chemical concentration.

Keap1: Kelch-like ECH-associated protein 1, is a sensor protein that can regulate the Nrf2 activity. Under un-induced conditions the Keap1 sensor protein targets the Nrf2 transcription factor for ubiquitinylation and proteolytic degradation in the proteasome. Covalent modification of the reactive cysteine residues of Keap 1 by small molecules can lead to dissociation of Nrf2 from Keap1 (8) (10) (11).

Mixture: A mixture or a solution composed of two or more substances in which they do not react (1).

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

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration ≥ 10% (w/w) and < 80% (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

Nrf2: nuclear factor (erythroid-derived 2)-like 2, is a transcription factor involved in the antioxidant response pathway. When Nrf2 is not ubiquitinylated, it builds up in the cytoplasm and translocates into the nucleus, where it combines to the ARE in the upstream promoter region of many cytoprotective genes, initiating their transcription (8) (10) (11).

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.

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 (29).

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 (29).

Reproducibility: The agreement among results obtained from testing the same substance using the same test protocol (see reliability) (29).

Sensitivity: The proportion of all positive / active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (29).

Solvent/vehicle control: A replicate containing all components of a test system except of the test chemical, but including the solvent that is used. It is used to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent.

Specificity: The proportion of all negative / inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (29).

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

deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition (1).

Test 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 (1).

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 (29).

ANNEX 2

PROFICIENCY SUBSTANCES

***In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method**

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly obtaining the expected KeratinoSens™ prediction for the 10 Proficiency Substances recommended in Table 1 and by obtaining the $EC_{1.5}$ and IC_{50} values that fall within the respective reference range for at least 8 out of the 10 proficiency substances. These Proficiency Substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were commercial availability, availability of high quality *in vivo* reference, and availability of high quality *in vitro* data from the KeratinoSens™ test method.

Table 1: Recommended substances for demonstrating technical proficiency with the KeratinoSens™ test method

Proficiency Substances	**CASRN**	**Physical Form**	***In Vivo* Prediction (1)**	**KeratinoSens™ Prediction (2)**	**$EC_{1.5}$ (µM) Reference Range (3)**	**IC_{50} (µM) Reference Range (3)**
Isopropanol	67-63-0	Liquid	Non-sensitiser	Negative	> 1000	> 1000
Salicylic acid	69-72-7	Solid	Non-sensitiser	Negative	> 1000	> 1000
Lactic acid	50-21-5	Liquid	Non-sensitiser	Negative	> 1000	> 1000
Glycerol	56-81-5	Liquid	Non-sensitiser	Negative	> 1000	> 1000
Cinnamyl alcohol	104-54-1	Solid	Sensitiser (weak)	Positive	25 - 175	> 1000
Ethylene glycol dimethacrylate	97-90-5	Liquid	Sensitiser (weak)	Positive	5 - 125	> 500
2-Mercaptobenzothiazole	149-30-4	Solid	Sensitiser (moderate)	Positive	25 - 250	> 500
Methyldibromo glutaronitrile	35691-65-7	Solid	Sensitiser (strong)	Positive	< 20	20 - 100
4-Methylaminophenol sulfate	55-55-0	Solid	Sensitiser (strong)	Positive	< 12.5	20 - 200
2,4-Dinitro-chlorobenzene	97-00-7	Solid	Sensitiser (extreme)	Positive	< 12.5	5 - 20

(1) The *in vivo* hazard (and potency) predictions are based on LLNA data (13). The *in vivo* potency is derived using the criteria proposed by ECETOC (24).

(2) A KeratinoSens™ prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 9 and 11 of the Test Guideline.

(3) Based on the historical observed values (12).

ANNEX 3

QUALITY CONTROL OF LUMINESCENCE MEASUREMENTS

Basic experiment for ensuring optimal luminescence measurements in the KeratinoSens™ assay

The following three parameters are critical to ensure obtaining reliable results with the luminometer:

- having a sufficient sensitivity giving a stable background in control wells;
- having no gradient over the plate due to long reading times; and
- having no light contamination in adjacent wells from strongly active wells.

Prior to testing it is recommended to ensure having appropriate luminescence measurements, by testing a control plate set-up as described below (triplicate analysis).

Plate setup of first training experiment

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
B	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
C	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
D	**EGDMA 0.98**	**EGDMA 1.95**	**EGDMA 3.9**	**EGDMA 7.8**	**EGDMA 15.6**	**EGDMA 31.25**	**EGDMA 62.5**	**EGDMA 125**	**EGDMA 250**	**EGDMA 500**	**EGDMA 1000**	**EGDMA 2000**
E	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
F	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
G	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
H	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	**CA 4**	**CA 8**	**CA 16**	**CA 32**	**CA 64**	**Blank**

EGDMA = Ethylene glycol dimethacrylate (CAS No.: 97-90-5) a strongly inducing compound
CA = Cinnamic aldehyde, positive reference (CAS No.: 104-55-2)

The quality control analysis should demonstrate:

- a clear dose-response in row D, with the I_{max} > 20 fold above background (in most cases I_{max} values between 100 and 300 are reached);
- no dose-response in row C and E (no induction value above 1.5 (ideally not above 1.3) due to possible light contamination especially next to strongly active wells in the EGDMA row;
- no statistically significant difference between the rows A, B, C, E, F and G. (i.e. no gradient over plate); and
- variability in any of the rows A, B, C, E, F and G and in the DMSO wells in row H should be below 20% (i.e. stable background).

European Commission

JRC SCIENTIFIC AND POLICY REPORTS

EURL ECVAM Recommendation on the KeratinoSens™ assay for skin sensitisation testing

January 2014

European Commission
Joint Research Centre
Institute for Health and Consumer Protection (IHCP)

Contact information
European Reference Laboratory for Alternatives to Animal Testing
Address: Joint Research Centre, Via Enrico Fermi 2749, 21027 Ispra (VA), Italy
E-mail: JRC-ECVAM-CONTACT@ec.europa.eu

http://ihcp.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

This publication is a Reference Report by the Joint Research Centre of the European Commission.

JRC87551

EUR 26427 EN

ISBN 978-92-79-34795-5

ISSN 1831-9424

Doi 10.2788/52914

Luxembourg: Publications Office of the European Union, 2013

Printed in Ispra

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM RECOMMENDATION

on the KeratinoSens™ assay for skin sensitisation testing

January 2014

ACKNOWLEDGEMENTS

This Recommendation was prepared by the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), part of the Institute for Health and Consumer Protection (IHCP), Directorate-General Joint Research Centre (DG JRC) of the European Commission.

The Recommendation was drafted on the basis of the ESAC Opinion and ESAC Working Group Report outlining the detailed scientific peer review of the Givaudan-coordinated study on the KeratinoSens™ assay. The Recommendation further benefitted from comments and suggestions received from members of PARERE (EURL ECVAM's advisory body for Preliminary Assessment of Regulatory Relevance that brings together representatives of Member State regulatory bodies as well as EU agencies including ECHA, EFSA and EMA), and ESTAF (EURL ECVAM's Stakeholder Forum). Input was also provided by partner organisations of EURL ECVAM in the framework of the International Collaboration on Alternative Test Methods (ICATM), and by the general public.

Coordinator of the evaluation of the test submission was Silvia Casati. Coordinator of the ESAC Peer Review and EURL ECVAM Recommendation was Claudius Griesinger.

Comments or enquiries related to this EURL ECVAM Recommendation should be sent to:

EU Reference Laboratory for Alternatives to Animal Testing
European Commission DG Joint Research Centre,
Institute for Health and Consumer Protection,
Via E. Fermi 2749,
I-21027 Ispra (VA), Italy.
e-mail: JRC-ECVAM-CONTACT@ec.europa.eu

TABLE OF CONTENTS

BACKGROUND TO EURL ECVAM RECOMMENDATIONS

The aim of a EURL ECVAM Recommendation is to provide EURL ECVAM views on the validity of the test method in question, to advise on possible regulatory applicability, limitations and proper scientific use of the test method, and to suggest possible follow-up activities in view of addressing knowledge gaps.

During the development of its Recommendations, EURL ECVAM consults with its consultation body for Preliminary Assessment of Regulatory Relevance (PARERE) and its EURL ECVAM Stakeholder Forum (ESTAF). Moreover, EURL ECVAM consults with other Commission services and its international validation partner organisations of the International Cooperation on Alternative Test Methods (ICATM). Before finalising its recommendations, EURL ECVAM also invites comments from the general public and, if applicable, from the test method submitter.

EXECUTIVE SUMMARY

The KeratinoSens™ *in vitro* test method for skin sensitisation testing has been developed by Givaudan, a producer of fragrances and flavours. From 2009 to 2010 Givaudan coordinated a validation study on the KeratinoSens™ test method, focusing on its transferability and reproducibility. Following submission to EURL ECVAM of the study data as well as supplementary information, EURL ECVAM charged ESAC to review the KeratinoSens™ validation study which it finalised in December 2012. EURL ECVAM endorses the conclusions of the ESAC opinion (Annex I) on the Givaudan-coordinated study and makes the following recommendations.

(1) The Keap1-Nrf2-ARE pathway is considered a major regulator of cyto-protective responses to electrophile and oxidative stress by controlling the expression of detoxification, antioxidant and stress response enzymes and proteins. Since the majority of chemical skin sensitisers are electrophiles reacting with nucleophilic centres in skin proteins, the pathway is one relevant readout for skin sensitisation (OECD, 2012).

(2) Since activation of the Keap1-Nrf2-ARE pathway addresses only one single biological mechanism, it is likely that information from test methods based on this or similar pathways will not be sufficient to conclude on the skin sensitisation potential of chemicals. Therefore the KeratinoSens™ assay should not be considered a stand-alone full replacement method and data generated with the test method should always be considered in the context of integrated approaches, e.g. Weight-of-Evidence (WoE) or Integrated Testing Strategies (ITS), combining them with complementary information derived from *in vitro* assays addressing other key events of skin sensitisation (e.g. in chemico reactivity assays such as the Direct Peptide Reactivity Assay) as well as non-testing methods including read-across from chemical analogues.

(3) Based on the data generated in the study, the KeratinoSens™ test method proved to be transferable to laboratories experienced in cell culture and reproducible within- and between-laboratories (86% concordance in both cases).

(4) The Givaudan-coordinated validation study generated preliminary information on the test method's predictive capacity and it was found that the accuracy of the KeratinoSens™ to discriminate skin sensitisers from non-sensitisers was 90% (sensitivity 87%, specificity 100%; n=21)[1]. The accuracy calculated for an additional set of chemicals (77 sensitisers and 104 non-sensitisers) tested in-house by Givaudan was 75%. These figures are similar to those recently published by Natsch et al. (2013) based on in-house testing of about 145 chemicals (77% accuracy, 79% sensitivity, 72% specificity). Taken together, this information indicates the usefulness of the KeratinoSens™ assay to contribute to the identification of sensitisers and non-sensitisers.

(5) The KeratinoSens™ assay also provides concentration-response information that may contribute to the assessment of sensitising potency as recently proposed by Jaworska et al. (2013). Further work is required to determine to which extent KeratinoSens™ results relate to potency categories based on, preferentially, human data.

[1] N.B. The values presented here differ from those presented in the ESAC WG report. The Givaudan submission to EURL ECVAM contained data of the Givaudan-coordinated validation study plus data from in-house testing produced under non-validation conditions (e.g. no blind testing). While the ESAC WG calculated the predictive capacity on the basis of all data points irrespective of how they had been generated, the values presented above have been calculated a) on the basis of the validation study and b) on the basis of the additional non-validation data. This ensures a consistent approach with regard to the presentation of the predictive capacity of other skin sensitisation test methods summarised in EURL ECVAM Recommendations (e.g. DPRA), where the preliminary predictive capacity of the assays has been calculated on the basis of a small validation set and compared to the predictive capacity from additional information generated in-house by test submitters under non-validation conditions.

(6) To support the development of integrated approaches employing information from cyto-protective signalling pathways such as Keap1-Nrf2-ARE, the applicability of the KeratinoSens™ and its limitations should be further characterised. Based on the available data from the validation study and in-house testing of the submitter, the KeratinoSens™ assay seems applicable to a wide range of chemicals. Nevertheless, negative results should be interpreted with some caution due to (a) the cysteine-dependent mechanism of activation of the signalling pathway; (b) although some pro-haptens are reported to be correctly predicted, those requiring biotransformation by P450 enzymes are not detected; (c) while a variety of pre-haptens are reported to be detected, pre-haptens with a slow oxidation rate may go undetected unless oxidised before the actual experiment (Givaudan, 2011).

(7) The KeratinoSens™ test method can be considered as a valuable component of integrated approaches for skin sensitisation testing although further work is required to fully understand its limitations and to be specific about what complementary data would be desirable depending on the use case. Furthermore, its capacity to contribute to subcategorisation of sensitisers according to UN GHS (UN, 2007) and to potency assessment needs to be defined, the latter preferentially on the basis of human reference data.

(8) Respecting the provisions of Directive 2010/63/EU (EU, 2010) on the protection of animals used for scientific purposes, before embarking on animal experiments to identify substances with skin sensitisation potential, data from the KeratinoSens™ test method should be considered in combination with complementary information in order to reduce and possibly avoid animal testing. As provided for in Annex XI (point 1.2) of the REACH Regulation (EC, 2006), data from non-standard testing methods, such as the KeratinoSens™, may be used to adapt the standard information requirement in the context of Weight-of-Evidence (WoE) judgments.

1. Introduction

1) The assessment of skin sensitisation potential is an important component in the safety evaluation of substances and represents a standard information requirement of legislation on chemicals in the EU. These include: the Classification Labelling and Packaging of substances and mixtures (CLP) Regulation (EC, 2008a), the REACH Regulation, the Plant Protection Products (PPP) Regulation (EC, 2009a), the Biocides Directive (EC, 2012) and the Cosmetics Regulation (EC, 2009b). Determining skin sensitisation hazard in terms of GHS classification is actually sufficient to satisfy the majority of regulatory needs (EURL ECVAM, 2013). However, a more complete characterisation of the potency of a skin sensitiser with regard to both induction as well as elicitation of contact dermatitis is often required for a full risk assessment and the definition of appropriate risk management measures (e.g. setting of appropriate thresholds).

2) Currently only *in vivo* test methods are accepted by regulatory bodies for the generation of data satisfying regulatory requirements on skin sensitisation. For instance, in the frameworks of the Organisation for Economic Cooperation and Development (OECD) and the EU Test Methods Regulation (EC, 2008b), there are four accepted guidelines, describing: the Buehler Test and Guinea-pig Maximisation Test, TG406 (OECD, 1992; EU test method B.6), the Local Lymph Node Assay, TG429 (OECD, 2010a; EU test method B.42) and its non-radio-isotopic variants, the Local Lymph Node Assay: DA (TG 442A; OECD, 2010b) and the Local Lymph Node Assay: BrdU Elisa (TG 442B; OECD, 2010c).

3) The key mechanistic events underpinning the skin sensitisation process that leads to Allergic Contact Dermatitis (ACD) in humans have been identified and recently summarised in the OECD report on "The Adverse Outcome Pathway (AOP) for Skin Sensitisation Initiated by Covalent Binding to Proteins"(OECD, 2012). These key events include 1) the covalent binding of the chemical to the skin protein (haptenation), 2) the release of pro-inflammatory cytokines and the induction of cyto-protective pathways in keratinocytes 3) the maturation and mobilisation of dendritic cells (DC), immuno-competent cells in the skin, and 4) the antigen presentation to naïve T-cells and proliferation of memory T-cells. Considerable progress has been made in recent years towards the development of alternative non-animal methods that address these key mechanisms.

4) There is general agreement that it is unlikely that a single alternative method will be able to provide sufficient information to fully replace the use of animals for this endpoint (Adler et al., 2011). Instead it is held that information from different alternative testing and non-testing methods used in combination will need to be integrated to address this health endpoint (Jowsey et al., 2006; Adler et al., 2011). These methods should address different key events involved in skin sensitisation thus covering the mechanistic complexity of this endpoint. Against this background, activities are being pursued by academia, industry and the European Commission to evaluate mechanistically-based test methods that can contribute to skin sensitisation hazard identification and characterisation.

5) In May 2010, EURL ECVAM received a full submission reporting the experimental results generated by five laboratories participating in a Givaudan-coordinated study for the evaluation of the protocol transferability and the within- and between-laboratory reproducibility of the KeratinoSens™ *in vitro* assay. Following the evaluation of the submitted information, EURL ECVAM judged that the within-laboratory reproducibility (WLR) was not sufficiently addressed to progress the study into peer-review and requested Givaudan to generate additional experimental data on the WLR with eight coded chemicals provided by EURL ECVAM. In December 2010 EURL ECVAM received a revised full submission reporting the requested data plus results generated with an additional six

chemicals, not previously tested with the KeratinoSens™ method. The revised full submission was complemented with supplementary information which included an updated analysis of the KeratinoSens™ predictive capacity (PC) based on data from 47 chemicals in addition to the 67 chemicals originally considered for this purpose. Additional information on the PC of the method for 80 non-sensitising chemicals with LLNA reference data was provided by Givaudan in the phase of peer review. Besides the experimental data obtained with 21 coded chemicals in the Givaudan-coordinated study, most of the information on the PC of the KeratinoSens™ has been generated in-house by the test developer.

6) On the basis of the revised submission EURL ECVAM requested the ECVAM Scientific Advisory Committee (ESAC) to provide an ESAC Opinion on the study and supportive information. The ESAC Working Group (WG) "Skin Sensitisation", charged with reviewing validation studies on skin sensitisation test methods, was requested to prepare a detailed WG report (EURL ECVAM, 2012a) on which basis ESAC adopted its Opinion (EURL ECVAM, 2012b; see Annex 1), endorsed on 17. 12. 2012.

2. Test Method definition

The important role of the transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2) in promoting the expression of genes coding for cyto-protective proteins (mainly phase 2 enzymes) following electrophilic or oxidative stress is extensively described in the literature (e.g. Baird & Dinkova-Kistova, 2011; Kensler et al., 2007). The activity of Nrf2 is considered to be primarily regulated by the cysteine-rich Keap1 sensor protein (Kelch-like ECH associated protein 1) although other signalling pathways are reported to be involved in its regulation (Baird & Dinkova-Kistova, 2011). Under un-induced conditions the Keap1 protein targets the Nrf2 transcription factor for ubiquitin-dependent proteasomal degradation (Itoh et al., 1999). It is proposed that covalent modification of the cysteine residues in the Keap-1 protein by electrophiles/oxidants leads to the dissociation of the Keap-1 protein from the Nrf2 transcription factor and induces the translocation of the Nrf2 from the cytoplasm to the nucleus where it promotes the activation of cyto-protective genes which have an antioxidant or electrophile response elements (AREs/EpREs) in their promoter sequence (Itoh et al., 1997; Suzuki et al., 2013).

7) Although direct covalent binding to certain Keap1 cysteine residues is considered to be one of the plausible mechanisms through which the Keap1-Nrf2-ARE pathway is activated, other types of modifications of the protein, like oxidation or glutathionylation, are reported to be responsible for its activation. In addition it is proposed that all electrophiles/oxidants may shift the redox balance of the cell through reaction with glutathione (GSH) which may in turn generate an oxidative burst able to modify Keap1 cysteines (Holland & Fishbein, 2010).

8) As reviewed by Natsch (2010) there is increasing evidence that ARE-regulated genes are induced in different cell types after challenge with skin sensitisers. The relevance of the Keap1-Nrf2-ARE regulatory pathway in the *in vivo* reaction to sensitisers was shown in studies with Nrf2 knockout mice (Kim et al., 2008; El Ali et al., 2013; van der Veen et al., 2013).

9) The KeratinoSens™ test method is a reporter gene assay which uses an immortalised adherent cell line derived from an expanded clone of HaCaT human keratinocytes transfected with a selectable plasmid. The plasmid contains the luciferase gene under the transcriptional control of the SV40 promoter fused with the ARE from the AKR1C2 gene which was identified as one of the genes up-regulated by contact sensitisers in dendritic cells (Gildea et al., 2006; Ryan et al., 2004). This allows

to quantitatively measure (by luminescence detection) luciferase gene induction, using well established light producing luciferase substrates, as an indicator of the activity of the Nrf2 transcription factor in cells following exposure to electrophilic chemicals.

10) At present, the KeratinoSens™ test method is designed for the identification of sensitisers/non sensitisers. Chemicals are classified as sensitisers if they induce a statistically significant induction of the luciferase gene above a given threshold in two out of three experiments performed on different days. This is established in parallel to cytotoxicity measurements to assess gene induction levels at sub-cytotoxic concentrations. Since cells are exposed to 12 concentrations of the test chemicals, the concentration needed for a statistically significant luciferase gene induction above the threshold (EC1.5 value) can be extrapolated from the dose response curve. In addition, the maximal fold induction of the luciferase gene over solvent control (I_{max}) is determined.

11) As a result of the Givaudan-coordinated study (Natsch et al., 2011) and additional information provided in the submission to EURL ECVAM, the standardised protocol was found to be transferable (to laboratories with cell culture technique experience) and reproducible within and between laboratories.

12) EURL ECVAM will disseminate a comprehensive description of the KeratinoSens™ method through its database on alternative methods (DB-ALM, at http://ecvam-dbalm.jrc.ec.europa.eu; protocol No. 155), together with all the necessary technical details (e.g. electronic data reporting formats) needed by an end-user laboratory to implement it in a reliable and self-sufficient manner.

3. Overall Performance of the KeratinoSens™ test method

Reference data

13) Reference classifications associated with the test chemicals were selected on the basis of a weight of evidence approach considering different data, i.e. the murine Local Lymph Node Assay (LLNA), the Guinea Pig Maximisation Test (GPMT) and, where available, human data. Reference chemicals from the LLNA performance standards (OECD, 2010a) were included in the chemical set. Additional details can be found in the submission (Givaudan, 2011).

    When interpreting the data of alternative methods, such as the KeratinoSens™ that have been largely developed and validated using animal reference data such as LLNA or GPMT, it should be kept in mind that the predictive relevance of these animal tests may not fully reflect the situation in the species of interest, i.e. humans. Notably, an evaluation of the LLNA in comparison to human data has shown an accuracy of about 72% (Anderson et al., 2011), i.e. there is a risk of false negative and false positive results. Moreover there is indication that the LLNA is deficient in detecting low to moderate sensitisers as well as metals and organometal compounds (EC, 2000).

Transferability

14) EURL ECVAM concludes that the KeratinoSens™ test method is transferable to laboratories sufficiently experienced in cell culture techniques. Since stable background levels of the luciferase gene are critical for the generation of reliable results, EURL ECVAM recommends that a number of training experiments, as described in the Standard Operating Procedure (SOP), be performed by

new laboratories to ensure optimal luminescence measurements before the test method is used for routine testing.

Reproducibility

15) For the set of coded chemicals tested during the validation study, the KeratinoSens™ protocol yielded concordant predictions within the Givaudan laboratory (86%; N=14) and between the five laboratories participating in the ring trial (86%, N=21).

Predictive Capacity

16) The accuracy of the test method in predicting the *in vivo* classification (sensitiser/non-sensitiser) determined on the basis of existing evidence from LLNA, GPMT, Buehler Test and human data for the 21 (coded) chemicals evaluated in the validation study was 90% (sensitivity 87%, specificity 100%). However, since the chemicals selected by Givaudan to be used in the validation study have already been used to develop and optimise the KeratinoSens™ prediction model, it is likely that these values reflect a best-case scenario. When calculating the predictive capacity on the basis of a larger set of data generated in-house by Givaudan, sensitivity and specificity are about 75% (n=77 sensitisers and 104 non-sensitisers). A recently published study correlating KeratinoSens™ data with classifications in the LLNA reported an accuracy of 77% (sensitivity 79% and specificity 72%) for a set of 145 chemicals (Natsch et al., 2013). Thus, it is plausible that these figures might reflect the actual performance of the test in discriminating between sensitisers and non-sensitisers.

4. Limitations

4.1 Technical limitations

17) **Solubility of test substances**: Chemicals which are not soluble in either water or DMSO, being these the two solvents prescribed by the SOP, cannot be tested in the KeratinoSens™. Chemicals with a calculated octanol/water partition coefficient (cLogP) up to 5 were reported by the test developer to be successfully tested with the method.

18) **Solvent effects:** As with many *in vitro*/*in chemico* assays, chemicals which are not stable in the prescribed solvents because of hydrolysis or other chemical reactions cannot be reliably tested.

4.2 Limitations with regard to applicability – negative results

19) As the key mechanism leading to the activation of the Keap1-Nrf2-ARE pathway appears to be the electrophilic reaction of stressors with nucleophilic thiols (cysteine sulfhydryl groups) of Keap-1 it is possible that skin sensitising chemicals with selective reactivity towards other nucleophiles may not be reliably identified by the KeratinoSens™ (e.g. amine reactive chemicals preferentially reacting with lysine residues), thereby leading to false negative results. However, there is scientific evidence that the pathway can be activated by other types of modification of Keap-1 cysteine residues, such as oxidation or conjugation with glutathione, and that, moreover, the Nrf2 transcription factor may be controlled by other signaling pathways. It is therefore plausible that sensitising chemicals not covalently modifying Keap-1 cysteine residues (e.g. amine-reactive chemicals) can nevertheless activate the Nrf2 pathway, leading to true positive responses in the KeratinoSens™ assay.

Complementary information from peptide reactivity assays may help addressing this uncertainty, in particular assays able to distinguish between cysteine and lysine reactivity.

20) While a number of pro-haptens requiring enzymatic oxidation or deamination are reported to be correctly classified by the KeratinoSens™, pro-haptens requiring P450 activation are reported not to be identified by the assay. According to the test developer, attempts to incorporate a metabolic system in the KeratinoSens™ assay have recently been published (Natsch & Haupt, 2013).

21) A variety of pre-haptens have been reported as correctly predicted by the assay (e.g. 1,4-phenylenediamine, hydroquinone and isoeugenol). However, some pre-haptens reported to have a slower rate of spontaneous oxidation (e.g. limonene) may require an oxidation step before the actual experiment.

22) Most of the misclassifications generated by the KeratinoSens™ concerns chemicals that are moderate and weak sensitisers *in vivo* (see ESAC WG report, page 31,), while the false negative rate for strong sensitisers is lower. This should be kept in mind when interpreting negative results.

4.3 Limitations with regard to applicability – positive results

23) Considering the pathway monitored (i.e. electrophilic / oxidative stress), chemicals that do not act as sensitisers but are nevertheless chemical stressors may lead to false positive results in the KeratinoSens™ test method. This could for example include reactive chemicals that cause dermal corrosion / irritation without, however, being skin sensitisers. Nevertheless, it was shown that irritating surfactants, which often are predicted positive in the LLNA, are negative in the KeratinoSens (Ball et al., 2011, Emter et al., 2010).

5. Suggested regulatory use

24) Due to the complexity of the mechanisms underlying skin sensitisation, it is likely that information from different methods (*in silico*, *in chemico*, *in vitro*) is needed to reduce or replace the need for animal testing, both for hazard identification and potency characterisation purposes.

25) Based on the validation study results and other available information, the KeratinoSens™ appears to be a reliable test method that provides information on the ability of a chemical to activate the Nrf2 electrophilic and oxidative-stress response signalling pathway which has been shown to be a relevant pathway in the induction of skin sensitisation as demonstrated by studies in Nrf2-knockout mice (Kim et al., 2008; El Ali et al., 2013; van der Veen et al., 2013). Therefore, Nrf2–dependent luciferase induction measurements in the KeratinoSens™ assay when combined with information from other non-animal methods in the context of a Weight-of-Evidence (WoE) approach or Integrated Testing Strategy (ITS) may provide useful information about the sensitisation potential of chemicals. Taking into consideration the dose-response information generated by the assay, it is plausible that KeratinoSens™ data may also contribute to characterisation of skin sensitisation potency within integrated approaches. The extent of information needed to complement a KeratinoSens™ result will depend on the intended application (e.g. hazard identification, classification or potency assessment) and context (availability and quality of other information). An example of the use of KeratinoSens™ data in a WoE approach for hazard assessment is published in the scientific literature (Ball et al., 2011).

26) Notably, due to the nature of the pathway monitored (i.e. general electrophilic and oxidative stress), KeratinoSens™ provides information on reactivity of chemicals that elicit protective stress responses in exposed cells. Such data may be relevant for other health endpoints such as, for example, dermal irritation and cancer (Reuter et al., 2010, Kansanen et al., 2013).

27) As outlined in more detail in section 4.2, negative KeratinoSens™ results should be interpreted with care, taking into due consideration the possibility of false negatives due to (1) possible selective reactivity of the chemical with amino acids other than cysteine, (2) the limited metabolic capacity of the assay leading to possible misclassification of pro-haptens (especially those requiring biotransformation by P450 enzymes), (3) the uncertain capacity to identify pre-haptens, (4) the uncertain capacity to correctly identify moderate and especially weak sensitisers.

28) Chemicals able to activate the Keap1-Nrf2-ARE pathway by other mechanisms than covalent binding to the Keap-1 cysteine residues may give false positive results in the KeratinoSens™ (see section 4.3).

29) Employed within an integrated approach, the KeratinoSens™ may be useful to satisfy information requirements for Cosmetics (Regulation EC/1223/2009), Chemicals (Regulation EC/1907/2006), Biocides (Regulation EC/528/2012) and Plant Protection Products (Regulation EC/1107/2009).

6. Follow-up activities recommended by EURL ECVAM

(1) In view of further prospective testing with the KeratinoSens™ method, EURL ECVAM recommends that the revised protocol available at EURL ECVAM's DB-ALM service (http://ecvam-dbalm.jrc.ec.europa.eu) be used: [DB-ALM protocol on KeratinoSens™ No. 155].

(2) Further testing should investigate possible limitations of the assay that relate to the cellular pathway chosen and the need for abiotic or biotic activation of some sensitisers (i.e. pre- and

pro-haptens). Moreover, since there is at present limited information on the applicability of the KeratinoSens™ to chemical mixtures including plant extracts (Andres et al., 2013), additional data may be helpful.

(3) Predictive capacity, applicability and limitations of the assay should be further evaluated in the context of its use as part of integrated approaches to testing and assessment. When doing so, the limitations of available reference data e.g. from LLNA (EC, 2000) with regard to reproducibility and relevance to the human situation should be however kept in mind. In particular, the capability of the method to detect accurately weak and moderate skin sensitisers should be further investigated.

(4) Further attention should be given to: (a) an evaluation of the possible contribution of KeratinoSens™ data to sub-categorisation of sensitisers according to GHS (i.e. sub category 1A and 1B); (b) an evaluation of whether and how the dose-response information generated by the assay could contribute to potency assessment allowing quantitative risk assessment. For such evaluation, the use of human reference data will be particularly useful.

(5) Considering the limitations of the assay, integrated approaches using Nrf2–dependent luciferase induction measurements should also make use of other information sources, in particular peptide reactivity assays able to distinguish between cysteine and lysine reactivity. In addition, *in silico* methods (expert systems and QSAR models) may prove useful. *In silico* methods that explicitly incorporate metabolic considerations (e.g. TIMES-SS: Patlewicz et al., 2007) may help to identify pre- and pro-haptens. Analogues which have a similarly predicted mechanism of action, based on protein binding, can be found using the OECD QSAR Toolbox (www.qsartoolbox.org). The Toolbox also includes a specific profiler for the KeratinoSens™ assay. A variety of proposals concerning the use of KeratinoSens™ data in combination with other information sources have been published and may support further work (Natsch et al., 2009; Bauch et al., 2012; Jaworska et al., 2013).

(6) EURL ECVAM supports the development of an OECD Test Guideline for the KeratinoSens™. As this test may be best employed in combination with complementary methods, it should be considered in the current initiative being undertaken at OECD to develop a guidance document on Integrated Approaches for Testing and Assessment (IATA) for skin sensitization.

(7) Since the assay is amenable for automation, the development of an automated version of the protocol is recommended.

(8) As the assay addresses a key signaling pathway of cyto-protective responses following electrophilic and oxidative stress, the relevance of the test system for assessing other toxicological endpoints should be considered.

7. PROPRIETARY ASPECTS

The 'KeratinoSens' name is a trade mark of the test method developer (Givaudan SA, Switzerland). EURL ECVAM has received confirmation from Givaudan that the Keratinosens™ test method will be made available to third parties subject to specific conditions including a one-time transfer fee.

REFERENCES

Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J, Zuang V, Andersen KE, Angers-Loustau A, Aptula A, Bal-Price A, Benfenati E, Bernauer U, Bessems J, Bois FY, Boobis A, Brandon E, Bremer S, Broschard T, Casati S, Coecke S, Corvi R, Cronin M, Daston G, Dekant W, Felter S, Grignard E, Gundert-Remy U, Heinonen T, Kimber I, Kleinjans J, Komulainen H, Kreiling R, Kreysa J, Leite SB, Loizou G, Maxwell G, Mazzatorta P, Munn S, Pfuhler S, Phrakonkham P, Piersma A, Poth A, Prieto P, Repetto G, Rogiers V, Schoeters G, Schwarz M, Serafimova R, Tähti H, Testai E, van Delft J, van Loveren H, Vinken M, Worth A, Zaldivar JM (2011) Alternative (non-animal) methods for cosmetics testing: current status and future prospects. Arch. Toxicol., 85:367-485.

Anderson SE, Siegel PD, Meade BJ (2011) The LLNA: A Brief Review of Recent Advances and Limitations. J. Allergy (Cairo), 424203.

Andres E, Sa-Rocha VM, Barrichello C, Haupt T, Ellis G, Natsch A (2013) The sensitivity of the KeratinoSens™ assay to evaluate plant extracts: A pilot study. Tox. In Vitro, 27:1220-1225.

Baird L, Dinkova-Kostova AT (2011) The cytoprotective role of the Keap1-Nrf2 pathway. Arch. Toxicol., 85:241-272.

Ball N, Cagen S, Carrillo JC, Certa H, Eigler D, Emter R, Faulhammer F, Garcia C, Graham C, Haux C, Kolle SN, Kreiling R, Natsch A, Mehling A (2011) Evaluating the sensitization potential of surfactants: integrating data from the local lymph node assay, guinea pig maximization test, and in vitro methods in a weight-of-evidence approach. Regul. Toxicol.Pharm., 60:389-400.

Bauch C, Kolle SN, Ramirez T, Eltze 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 potential. Regul. Toxicol. Pharm., 63:489-504.

EC, 2000: Opinion on the Murine Local Lymph Node Assay (LLNA) adopted by the SCCNFP during the 12th plenary meeting of 3 May 2000. http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/sccnfp_opinions_97_04/sccp_out114_en.htm. Last accessed July 2013.

EC, 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal No L 396, p. 0001-1355.

EC, 2008a. Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Official Journal No L 353, p. 0001-1355.

EC, 2008b. Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official Journal L 142 , 31/05/2008 P. 0001 – 0739.

EC, 2009a. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC Official Journal No L 309, p. 01-50.

EC, 2009b. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Official Journal No L 342, p. 01-59.

El Ali Z, Gerbeix C, Hemon P, Esser PR, Martin SF, Pallardy M, Kerdine-Römer S (2013) Allergic skin inflammation induced by chemical sensitizers is controlled by the transcription factor Nrf2. Toxicol. Sci., 134(1):39-48.

Emter R, Ellis G, Natsch A (2010) Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. Toxicol. Appl. Pharm., 245:281-290.

EU, 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal L 276, 20/10/2010 P. 0033 – 0079.

EU, 2012. Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal No L167, p.01-123.

EURL ECVAM (2012a) ESAC Working Group Peer Review Consensus Report on a Givaudan-coordinated study transferability and reliability of the KeratinoSens assay for skin sensitisation testing. Online (XXXX):http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations

EURL ECVAM (2012b) ESAC Opinion on a Givaudan-coordinated study concerning transferability and reliability of the KeratinoSens assay for skin sensitisation testing. Online (XXXX):http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations

EURL ECVAM (2013). EURL ECVAM Strategy for Replacement of Animal Testing for Skin Sensitisation Hazard Identification and Classification. JRC Scientific and Policy Reports. Publication Office of the European Union. http://publications.jrc.ec.europa.eu/repository/handle/111111111/27708

Gildea LA, Ryan CA, Foertsch LM, Kennedy JM, Dearman RJ, Kimber I, Gerberick GF (2006) Identification of gene expression changes induced by chemical allergens in dendritic cells: opportunities for skin sensitization testing. J. Invest. Dermatol., 126:1813-1822.

Givaudan (2011). Test method submission to EURL ECVAM: KeratinoSens™ assay for the testing of skin sensitizers.

Holland R, Fishbein JC (2010) Chemistry of the cysteine sensors in Kelch-like ECH-associated protein 1. Antioxid. Redox Signal., 17:1749-1761.

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun., 236(2):313-322.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev., 13:76-86.

Jaworska J, Dancik Y, Kern P, Gerberick F, Natsch A (2013) Bayesian integrated testing strategy to assess skin sensitization potency: from theory to practice. J. Appl. Toxicol. Epub ahead of print.

Jowsey IR, Basketter DA, Westmoreland C, Kimber I (2006) A future approach to measuring relative skin sensitising potency: a proposal. J. Appl. Toxicol., 26:341-350.

Kansanen E, Kuosmanen SM, Leinonen H, Levonen AL (2013) The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. Redox Biol., 1(1):45-49.

Kensler TW, Wakabayashi N, Biswal S (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu. Rev. Pharmacol. Toxicol., 47:89-116.

Kim HJ, Barajas B, Wang M, Nel AE (2008) Nrf2 activation by sulforaphane restores the age-related decrease of T(H)1 immunity: role of dendritic cells. J. Allergy Clin. Immunol., 121:1255-1261.

Natsch A, Emter R, Ellis G (2009) Filling the concept with data: integrating data from different in vitro and in silico assays on skin sensitizers to explore the battery approach for animal-free skin sensitization testing. Toxicol. Sci., 107:106-121.

Natsch A (2010) The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers-functional relevance and a hypothesis on innate reactions to skin sensitizers. Toxicol. Sci., 113:284-292.

Natsch A, Bauch C, Foertsch L, Gerberick F, Norman K, Hilberer A, Inglis H, Landsiedel R, Onken S, Reuter H, Shepky A, Emter R (2011) The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSens™ assay to predict skin sensitizers in vitro: results of a ring-study in five laboratories. Toxicol. In Vitro., 25:733-44.

Natsch A, Ryan CA, Foertsch L, Emter R, Jaworska J, Gerberick F, Kern P (2013) A dataset on 145 chemicals tested in alternative assays for skin sensitisation undergoing prevalidation. J. Appl. Toxicol. Epub, ahead of print.

Natsch A, Haupt T (2013). Utility of rat liver S9 fractions to study skin-sensitizing prohaptens in a modified KeratinoSens assay. Toxicol. Sci., 135(2):356-368.

OECD (1992) OECD Guideline for the Testing of Chemicals No. 406: Skin Sensitisation. Paris, France: Organisation for Economic Cooperation and Development. Online at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

OECD (2010a) OECD Guideline for the Testing of Chemicals No. 429: Skin Sensitisation: Local Lymph Node Assay. Paris, France: Organisation for Economic Cooperation and Development. Online at:http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4 health-effects_20745788

OECD (2010b) OECD Guideline for the Testing of Chemicals No. 442a Skin Sensitisation: Local Lymph Node Assay DA. Paris, France: Organisation for Economic Cooperation and Development. Online at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4 health-effects_20745788

OECD (2010c) OECD Guideline for the Testing of Chemicals No. 442b: Skin Sensitisation: Local Lymph Node Assay BrdU-ELISA. Paris, France: Organisation for Economic Cooperation and Development. Online at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

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. Online at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2012)10/PART1&docLanguage=En

Patlewicz G, Dimitrov SD, Low LK, Kern PS, Dimitrova GD, Comber MIH, Aptula AO, Phillips RD, Niemela J, Madsen C, Wedebye EB, Roberts DW, Bailey PT, Mekenyan OG (2007) TIMES-SS – A promising tool for the assessment of skin sensitization hazard. A characterisation with respect to the OECD Validation Principles for (Q)SARs. Reg. Toxicol. Pharmacol., 48:225–239.

Ryan CA, Gildea LA, Hulette BC, Dearman RJ, Kimber I, Gerberick GF (2004) Gene expression changes in peripheral blood-derived dendritic cells following exposure to a contact allergen. Toxicol. Lett., 150(3): 301-316.

Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB (2010) Oxidative stress, inflammation, and cancer: how are they linked? Free Radic. Biol. Med., 49(11):1603-1616.

Suzuki T, Motohashi H, Yamamoto M (2013) Toward clinical application of the Keap1-Nrf2 pathway. Trends Pharmacol. Sci., 34:340-346.

UN, United Nations N (2011). Globally Harmonised System of Classification and Labelling of Chemicals (GHS). Part 3: Health and Environmental Hazards. New York, NY, USA, and Geneva, Switzerland. United Nations Economic Commission for Europe. Online at:http://www.unece.org/trans/danger/publi/ghs/ghs_rev04/04files_e.html

van der Veen JW, Gremmer ER, Vermeulen JP, van Loveren H, Ezendam J (2013) Induction of skin sensitization is augmented in Nrf2-deficient mice. Arch. Toxicol., 87(4):763-766.

Annex 1 ESAC OPINION

Opinion of the EURL ECVAM Scientific Advisory Committee (ESAC) on a Givaudan-coordinated study on the transferability and reliability of the KeratinoSens assay for skin sensitisation testing.

Ispra, 07.11.2012

Summary of the ESAC Opinion

The ESAC was asked to provide an opinion on a Givaudan-led study assessing the transferability and reproducibility (within- and between-laboratories) of the KeratinoSens (primary objective of the study) in view of its possible future use as part of a non-animal testing strategy for skin sensitization. The ESAC was also asked to provide an opinion on the predictive capacity of the test method.
A wealth of information about the test chemicals, and the assessment of with laboratory reproducibility (WLR), transferability, between laboratory reproducibility (BLR) and predictive capacity of the test were presented. Also the applicability domain of the test was addressed in detail. The evaluation by the ESAC WG was complicated by the lack of detail in the body of the report and the excessive reliance on annexes.
On the basis of the submitted and additionally requested information, the ESAC came to the following conclusions:

Test chemicals:
The 114 selected chemicals represented a sufficient number of materials, reasonable structural diversity and a variety of sensitising potency classes. Pre- and pro-haptens were included. Therefore, the selection of chemicals was considered sufficient to gain information on the applicability domain and limitations of the test method.
The small number of non-sensitizers (N=4) in the list of additional chemicals (N=47) considered eligible for assessing the predictive capacity of the test was supplemented with 80 chemicals with negative LLNA data.

WLR (14 chemicals/1 laboratory):
The ESAC considers the level of concordance acceptable and in agreement with target values (85%) for WLR performance.

Transferability (7 chemicals/4 laboratories):
Concordant predictions between the lead laboratory and the 4 naive laboratories were obtained, demonstrating that the test method can be transferred to naive laboratories that are experienced with cell culture techniques.

BLR (21 chemicals/5 laboratories):
The predictions were concordant for the large majority of chemicals, demonstrating an acceptable level of between laboratory reproducibility.

Predictive capacity:
The conclusions regarding the predictivity are sound. A positive point is that reference information from several *in vivo* tests were used for comparison as opposed to a single assay outcome. Since approximately 1 in 5 sensitizers are likely to be missed, the test method should

be considered in the future as part of an integrated testing strategy and not as a standalone assay.

Applicability domain:
In principle, the applicability domain of this method is limited to cysteine reactive chemicals. However, the evidence indicates that the applicability domain is wider, so it would be advisable to assess this further by testing additional chemicals. Specific amine reactivity and metabolic activation are among the key issues that need to be addressed.

1. Mandate of the ESAC

The opinion of ESAC should support ECVAM with respect to the evaluation of the validity status of the test method and possible necessary further work required to characterize the test method's performance (predictive capacity, applicability and limitations of the test method). Moreover, based on the evaluation of the data submitted, the ESAC should provide advice on the potential usefulness of the KeratinoSens test method within a testing strategy for skin sensitization testing.

2. Detailed opinion of the ESAC

Following a request from ECVAM to ESAC for peer review of and scientific advice on an ECVAM-coordinated prevalidation study concerning the KeratinoSens assay, an ESAC Working Group (ESAC WG) was set up by ESAC. The ESAC WG was charged with conducting a detailed scientific peer review the ECVAM study concerning the transferability and reliability of the KeratinoSens assay.
The ESAC WG had been set up by the ESAC during its meeting on March 2011 (ESAC 34). Basis for the scientific review was the ECVAM request to ESAC concerning a scientific review (ESAC request ER2011-04).
The date for the opinion was set to be 4-5 October 2011 (ESAC 35). However, unclarities and inconsistencies in the report required clarification by the test submitter. Two WG requests were sent: 16.12.2011 and 08.02.2012. These extra steps resulted in substantial additional information that had to be reviewed and caused a 1 year delay.
The ESAC WG conducted the peer review from December 2011 to April 2012. Two face-to-face meetings were organized (December 2011, and February 2012), followed by two telephone conferences (February and April 2012) and finalized by written procedure.
The WG was a presented a wealth of information about the test chemicals, and the assessment of WLR, transferability, BLR and predictive capacity of the test. Also the applicability domain of the test was addressed in detail.
The data and the flow of events would have been more transparent if the report had followed the EURL-ECVAM guidance and reporting template more closely. It would have been very helpful if the test submitters had formulated their own conclusions/opinions when referring to any of the numerous attachments that had followed the report. By plane referral to the attachments, the WG had to figure out itself what was meant and how data had to be interpreted.
The WG identified a number of unclarities and inconsistencies which added hurtles to the evaluation of the report, without explanations being provided.
Issues that needed clarification:

- It was not clear why the applied statistical approach was chosen for the evaluation of the test results.
- The test design was not clear.

Inconsistencies:

- Data analysis apparently moved from a test result oriented (e.g. Imax, EC1.5) to a prediction (S/NS) oriented approach.
- Test acceptance criteria changed over time without explanation as to why this was introduced.
- Acceptance criteria were not consistently applied.
- Chemicals that were used for test development and refinement were inappropriately included in the assessment of the BLR and the predicitive capacity.
- The WG addressed these issues by requesting additional information and re-analysis of the data from the test submitter (See Annexes).

The provided information did not provide any clarity about the statistical approach applied in the study. The WG decide not to go into further discussion, and to focus on the outcome of the prediction model (S/NS).
The test design was sufficiently clarified, and the data were re-analysed on the basis of the various identified test acceptance criteria. This allowed the WG to properly assess reproducibility, transferability and predictive capacity.
The WG attempted to recalculate the predictive capacity of the KeratinoSens based upon the chemicals that had not been included in test development and refinement. Since the number of well-characterized non-sensitizers (i.e. chemicals with negative LLNA outcome) among the eligible chemicals was considered too low, the WG requested data on more negative compounds.

On the basis of the submitted and upon request acquired information the WG came to the following conclusion:

Test chemicals:
The 114 selected chemicals were representing a sufficient number of materials, reasonable structural diversity and a variety of sensitising potency classes. Pre- and pro-haptens were included. Therefore, the selection of chemicals was considered sufficient to gain information on the applicability domain and limitation of the test method.
The number of test items was considered sufficient to draw conclusions about the transferability (N=7) and reproducibility (N=21) of the test.
The small number of non-sensitizers (N=4) in the extended list of chemicals (N=47) for assessing the predictive capacity of the test was considered too low. The 67 chemicals used for development, refinement and evaluation of the test were not taken into consideration for assessing the predictive capacity by the WG.

WLR (14 chemicals, 1 laboratory):
Including all available data concordant results were obtained for 12/14 chemicals (85.7%). The WG endorsed the conclusion of the VMG that the test is reproducible with laboratories. WG considered this concordance in agreement with target values (85%) for WLR performance standards as published in international accepted guidelines (e.g. Performance standards of TG439 in vitro skin irritation).
The ESAC WG agreed that the re-analysis that was resubmitted upon request (see section 6.1) was satisfying with regard to answering the question to which extent non-qualified test results might have influenced the WLR analysis**.** The impact on WLR was felt to be negligible as even under the most stringent criteria (set 2 in Annex 4, p60) only 3 individual laboratory predictions had not qualified.

Transferability (7 chemicals, 4 laboratories):
The conclusion on transferability was justified on the basis of concordant predictions (S/NS) between the lead laboratory and the naive laboratories. The WG endorses the conclusion that the test method can be transferred to naive laboratories that are experienced with cell culture techniques.
Concerns were raised about the reliability of luciferase measurements for transferability. Differences in brand of luminometer or substrate were demonstrated by the test submitters not to affect the liability of luminescence measurement. Based on this fact, it seems obvious to the WG that the observed variation in luminescence measurements between laboratories is due to lack of experience, stressing the necessity of operating a number of training experiments in the naïve laboratory before the test method can be used to identify skin sensitizers.
Regarding dose-response curve or EC1.5 (Attachment 8a & 8b), certain variability among the laboratories was observed to cinnamic aldehyde and ethylene glycol dimethacrylate. But, no further explanation was given whether these variabilities originated from the chemicals' own physico-chemical characteristics or luminescence measurement issues.

BLR (21 chemicals, 5 laboratories):
The S/NS prediction gave congruent results for the majority of chemicals (85.7 – 90.5%), taking into consideration the explanations give for the outliers, also between laboratories. (See section 6.1).
The test acceptance criteria provided to the participating laboratories during the ring trial had not been applied consequently when analysing the data. The reason for this inconsistency was that the criteria were found too stringent. In contrast to WLR and transferability assessment, these nonqualified data had an effect on the concordance of predictions (Annex 4, p62 (C. 2)). There were no provisions made for re-testing in case of nonqualified predictions.

Predictive capacity:
The conclusions regarding the predictivity are sound given the overall value of 76.6%, the key here is that weight of evidence data were used for comparison as opposed to a single assay outcome.
The WG was impressed by the wealth of information that was provided by the test submitter on the 114 chemicals assessed in this study. Based upon the 114 chemicals included in the study, the predicitive capacity of the KeratinoSens was 78%. However, the 114 chemicals included the 67 chemicals of the Silver list. Including chemicals that were used for development, refinement and evaluation of a test system might induce a bias in the assessment of the predictive capacity and was therefore considered by the WG as inappropriate.
Considering only the new chemicals (43 sensitizers and 4 non-sensitizers), the calculations showed that the predictive capacity (69%) was considerably lower than the 78% presented by the submitter. It was noted that the number of new qualified non-sensitizers used in this study was considered insufficient (N = 4).
The submitters were requested to submit additional data on chemicals with negative LLNA reference data. Such data were provided for an additional 80 chemicals. Compiling all the data provided by the submitters, the KeratinoSens revealed a sensitivity, specificity and accuracy of 79.3%, 79.8% and 79.5%. Omission of the seven reactive, peptide alkylating chemicals, for which the LLNA data were not trusted despite absence of human data, the remaining chemicals resulted in a sensitivity, specificity and accuracy of 79.3%, 84.5% and 81.7% (Annex 4, p64 (C8)).
The WG observed a poor performance of the test on weak sensitizers. Based on the predictions using the 114 chemicals, 41% of the weak and 86% of the very weak sensitizers were missed (Table 4). Furthermore, the frequency false negative results were found to increase with decreasing potency of the test chemical. This limitation is not clearly indicated in the submission.

Applicability domain:
The applicability domain was well described in the section 1.6 of KeratinoSens report. The authors stated a variety of chemical classes which were expected to be successfully tested in the KeratinoSens assay. These limitations were mainly limited to the issues of solubility or stability in vehicle (e.g. interactions with vehicle, such as hydrolysis).
The WG discussed this issue (See section 2.2) and came to the conclusion that there is indirect evidence that the applicability domain of test may extent to chemicals that not (only) react with the cysteine residues of Keap1. Alternative mechanisms may lead to Nrf2 activation.
Study design allowed testing of some of the limitations of the applicability domain.

Readiness for standardized use:
The WG considered the test method sufficiently mature for classification and labelling of chemicals (relevant to Regulation EC N° 1271/2008).

Negative results have to be considered with care as weak sensitizers (and possibly also moderate sensitizers at the lower end of the scala) will be probably missed (see section 9). Unless this issue gets solved, the KeratinoSens has to be seen as a brick in an integrated testing strategy of weight-of-evidence approach. The consideration of the chemistry /reactivity must be included either by combination with a peptide reactivity test or predictive chemistry assessment. This reactivity assessment should include consideration concerning activating mechanism(s). The KeratinoSens was considered useful for screening purposes, to identify molecular initiators and to gain mechanistic information on the role of e.g. oxidative stress in sensitization.

Identified gaps:
Weak and low-moderate sensitizers, as well as pro-haptens were performing poorly.
When considering cytotoxicity, more emphasis could have been paid to GSH status of the cells and their GSH regenerating capacity. This system may have an impact the inherent chemical reactivity whether directly conjugating to GSH or oxidising it (ref.).
The data do not support the expectation that this test can be used as a stand-alone (preliminary, waiting for PC and reproducibility assessment).
It appears that the correlation between *in vivo* and *in vitro* data needs further improvement as there was a relative high variation among the *in vitro* scores of chemicals belonging to the same potency class (Natsch et al., 2009).

Recommendations:
The test method can be used for S/NS identification of chemicals. Therefore, the test was considered ready for the next steps in the ECVAM process. A Validation study should however include more well-defined non-sensitizing compounds. Furthermore, a consistent use of acceptance criteria nr 3 should be assured.
Since the test revealed issues around weak and low moderate sensitizers, negative results cannot rule out a sensitization potential. This problem should be clearly flagged and/or addressed to be solved.
At SOP level, the test submitters were recommended to modify the 96-well plate design, which currently is prone to bias.
Integration of this assay with other predictive tests as they emerge needs to be based on the better defined applicability domain.
Eventual combination of the KeratinoSens assay with a reactivity based approach needs to include unambiguous identification of reactivity and any specificity associated with it.
Training should be considered.

3. Informative background to the Mandate and Opinion

Skin sensitization is the toxicological endpoint associated with substances that have the intrinsic ability to cause Allergic Contact Dermatitis, ACD in humans. ACD represents the most common manifestation of immunotoxicity in humans, i.e. adverse effects of xenobiotics involving the immune system. The identification of the skin sensitization potential represents an important component of
the safety assessment of any new substance and especially those intended for topical use (e.g. cosmetics). Current regulatory predictive tests for skin sensitization rely on the use of animals, these
include:
a) the traditional guinea pig tests: *Buehler Test* and *Guinea-pig Maximization Test* (OECD TG 406, Ref.1),

b) the *Local Lymph Node Assay* (LLNA, OECD TG 429, Ref.2) and its recently OECD adopted non-radioactive variants (OECD TG 422A, Ref.3 and OECD TG 422B, Ref.4).
Despite the progress that has been made in the development of alternative methods for skin sensitization hazard identification, there are currently no validated methods available. In addition none of the tests under development/evaluation is able to fully characterize the relative potency of sensitizing substances and therefore, none of these assays is considered a stand-alone method, capable of fully replacing current animal procedures.
The current view therefore is to combine different test methods in order to address different key mechanisms of skin sensitization, these includes: skin bioavailability, haptenation (the protein binding of chemicals which triggers immunological responses), epidermal inflammation, dendritic cell activation and migration, T cell proliferation. Before these test methods can be routinely used, their capacity to produce reproducible results needs to be demonstrated as a first step.

There is evidence in the literature showing that the Nrf2-Keap1-ARE regulatory pathway is induced by electrophilic chemicals. Since a considerable proportion of chemicals that lead to skin sensitization have these properties, the Nrf2-Keap1-ARE regulatory pathway is considered one of the most relevant pathways for the identification of potential skin sensitizers (recently reviewed by Natsch A, Ref.5). This knowledge was exploited by Givaudan to develop the KeratinoSens assay which uses an adherent cell line derived from an expanded clone of HaCaT keratinocytes transfected with a selectable plasmid. The plasmid contains the luciferase gene under the transcription control of the SV40 promoter fused with the ARE (antioxidant response element) from the AKR1C2 gene.

Using well-established light-producing luciferase substrates, the activity of ARE-binding transcription factors in the cells in response to exposure with soluble chemicals can be easily measured. Luciferase induction is the read-out of the KeratinoSens test method and the concentration at which the induction is 50% above the background level (EC1.5) is established in parallel to the IC50 value to classify chemicals as having skin sensitization potential.

The test method submitter proposes this method to be used in future as part of an integrated approach for the full replacement of the animal tests or as a stand-alone method for skin sensitisation hazard identification. In relation to the ability of the test method to differentiate between sensitizing and non-sensitizing chemicals, the test method submitter reported an accuracy of 85.1% (sensitivity 86.4%, specificity 82.6%) with respect to *in vivo* data for a set of 67 chemicals tested in-house.

The KeratinoSens test method has been evaluated in a ring study involving 5 laboratories including Givaudan who acted as the study coordinator. The transfer of the protocol was evaluated with a set of 7 chemicals. 21 additional chemicals (15 sensitizers and 6 non sensitizers) have been tested coded to generate information on the test method reliability and predictive capacity. The laboratories consistently classified 18 of the 21 coded chemicals. The accuracy of the in vitro classification with respect to the *in vivo* classification is reported to vary between 85.4% and 96.7% for the different laboratories. Following the formal submission of the KeratinoSens assay to ECVAM, Givaudan was asked to generate additional information on the within-laboratory reproducibility. In order to achieve this, ECVAM supplied Givaudan with 8 coded chemicals which have not been tested before with the KeratinoSens test method. Data for these additional chemicals were generated at the Givaudan laboratories and were submitted to ECVAM middle of March 2011.

With respect to the modular approach of validation (Hartung et al., 2004, Ref.6) the study provides information on module 1) test definition, module 2) within laboratory reproducibility, module 3) transferability and module 4) between laboratory reproducibility. Information for module 5), predictive capacity, is only partially fulfilled.

4. References

1. OECD, Organization for Economic Cooperation and Development (1992) Skin Sensitization Guidelines for Testing of Chemicals No. 406, Paris.
2. OECD, Organization for Economic Cooperation and Development (2002) The Local Lymph Node Assay. Guidelines for Testing of Chemicals No. 429, Paris.
3. OECD, Organization for Economic Cooperation and Development (2010a) Skin Sensitization: Local Lymph Node Assay: DA, Guidelines for Testing of Chemicals No. 442A, Paris.
4. OECD, Organization for Economic Cooperation and Development (2010b) Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA, Guidelines for Testing of Chemicals No. 442B, Paris.
5. Natsch A. The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers-functional relevance and a hypothesis on innate reactions to skin sensitizers. Toxicological Sciences 2010 113(2):284-92.
6. Hartung, T., Bremer, S., Casati, S., Coecke, S., Corvi, R., Fortaner, S., Gribaldo, L., Halder, M., Hoffmann, S., Roi A.J., Prieto, P., Sabbioni, E., Scott, L., Worth, A. and Zuang. V. (2004) A Modular Approach to the ECVAM Principles on Test Validity. ATLA 32, 467

ANNEX 2 EURL ECVAM request for ESAC advice

EURL ECVAM request to ESAC for scientific advice on the Givaudan-coordinated study on the transferability and reliability of the KeratinoSens assay for skin sensitisation testing

Title page information	
Abbreviated title of ESAC request	ESAC peer review of and ESAC opinion on the Givaudan-led study on the KeratinoSens test method.
ESAC REQUEST Nr.	2011-04
Template used for preparing request	EP 2.01
Date of finalising request	2011-03-07
Date of submitting request to ESAC	2011-03-09
Request discussed through	ESAC 34. 22-23 March 2011 and ESAC 35. 4-5 October 2011 (mandate adopted: objective/questions of review and ESAC WG)
Opinion expected at (date)	ESAC 36. 20-21 March 2012
File name of this request	**ER2011-04_KeratinoSens_ESACadopted.doc**

1. TYPE OF REQUEST

Request Type		**Identify request ("YES")**
R1 ESAC Peer Review **of a Prevalidation Study or Validation Study**		**YES**
If R1)applies please specify further:		
►Prevalidation Study	**YES** The KeratinoSens assay for skin sensitisation testing has been evaluated in a ring study involving five laboratories and led by Givaudan, a producer of fragrances and flavours. The study has been designed to generate information on the test method's transferability and reproducibility to allow recommendations to be made on these two aspects in view of the future use of this test method in an integrated approach for the full replacement of the currently used regulatory animal tests. In addition the data generated in this study will inform possible future evaluations of the test method's predictive capacity.	
►Prospective Validation Study	**No**	
►Retrospective Validation Study	**No**	
►Validation Study based on Performance Standards	**No**	
R2 Scientific Advice on a test method submitted to ECVAM for validation **(e.g. the test method's biological relevance etc.)**		**No**
R3 Other Scientific Advice **(e.g. on test methods, their use; on technical issues such as cell culturing, stem cells etc.)**		**No**

2. TITLE OF STUDY OR PROJECT FOR WHICH SCIENTIFIC ADVICE OF THE ESAC IS REQUESTED

Givaudan study on the KeratinoSens assay for skin sensitisation testing.

3. BRIEF DESCRIPTION OF THE STUDY OR PROJECT

1) Background to skin sensitization and current predictive tests

Skin sensitisation is the toxicological endpoint associated with substances that have the intrinsic ability to cause Allergic Contact Dermatitis, ACD in humans. ACD represents the most common manifestation of immunotoxicity in humans, i.e. adverse effects of xenobiotics involving the immune system. The identification of the skin sensitization potential represents an important component of the safety assessment of any new substance and especially those intended for topical use (e.g. cosmetics). Current regulatory predictive tests for skin sensitization rely on the use of animals, these include:

a) the traditional guinea pig tests: *Buehler Test* and *Guinea-pig Maximisation Test* (OECD TG 406, Ref.1),

b) the *Local Lymph Node Assay* (LLNA, OECD TG 429, Ref.2) and its recently OECD adopted non-radioactive variants (OECD TG 422A, Ref.3 and OECD TG 422B, Ref.4).

Despite the progress that has been made in the development of alternative methods for skin sensitisation hazard identification, there are currently no validated methods available. In addition none of the tests under development/evaluation is able to fully characterise the relative potency of sensitising substances and therefore, none of these assays is considered a stand-alone method, capable of fully replacing current animal procedures.

The current view therefore is to combine different test methods in order to address different key mechanisms of skin sensitisation, these includes: skin bioavailability, haptenation (the protein binding of chemicals which triggers immunological responses), epidermal inflammation, dendritic cell activation and migration, T cell proliferation. Before these test methods can be routinely used, their capacity to produce reproducible results needs to be demonstrated as a first step.

2) Background to the KeratinoSens

There is evidence in the literature showing that the Nrf2-Keap1-ARE regulatory pathway is induced by electrophilic chemicals. Since a considerable proportion of chemicals that lead to skin sensitisation have these properties, the Nrf2-Keap1-ARE regulatory pathway is considered one of the most relevant pathways for the identification of potential skin sensitisers (recently reviewed by Natsch A, Ref.5). This knowledge was exploited by Givaudan to develop the KeratinoSens assay which uses an adherent cell line derived from an expanded clone of HaCaT keratinocytes transfected with a selectable plasmid. The plasmid contains the luciferase gene under the transcription control of the SV40 promoter fused with the ARE (antioxidant response element) from the AKR1C2 gene. Using well established light-producing luciferase substrates, the activity of ARE-binding transcription factors in the cells in response to exposure with soluble chemicals can be easily measured. Luciferase induction is the read-out of the KeratinoSens test method and the concentration at which the induction is 50% above the background level (EC1.5) is established in parallel to the IC50 value to classify chemicals as having skin sensitisation potential.

The test method submitter proposes this method to be used in future as part of an integrated approach for the full replacement of the animal tests or as a stand alone method for skin sensitisation hazard identification.

In relation to the ability of the test method to differentiate between sensitising and non-sensitising chemicals, the test method submitter reported an accuracy of 85.1% (sensitivity 86.4%, specificity 82.6%) with respect to in vivo data for a set of 67 chemicals tested in house.

3) Background to the KeratinoSens ring study

The KeratinoSens test method has been evaluated in a ring study involving 5 laboratories including Givaudan who acted as the study coordinator. The transfer of the protocol was evaluated with a set of 7 chemicals. 21 additional chemicals (15 sensitisers and 6 non sensitisers) have been tested coded to generate information on the test method reliability and predictive capacity. The laboratories consistently classified 18 of the 21 coded chemicals. The accuracy of the in vitro classification with respect to the in vivo classification is reported to vary between 85.4% and 96.7% for the different laboratories.

Following the formal submission of the KeratinoSens assay to ECVAM, Givaudan was asked to generate additional information on the within-laboratory reproducibility. In order to achieve this, ECVAM supplied Givaudan with 8 coded chemicals which have not been tested before with the KeratinoSens test method. Data for these additional chemicals are being generated at the Givaudan laboratories and are expected to be submitted to ECVAM middle of March 2011.

With respect to the modular approach of validation (Hartung et al., 2004, Ref.6) the study provides information on module 1) test definition, module 2) within laboratory reproducibility, module 3) transferability and module 4) between laboratory reproducibility. Information for module 5), predictive capacity, is only partially fulfilled.

References

OECD, Organisation for Economic Cooperation and Development (1992) Skin Sensitisation Guidelines for Testing of Chemicals No. 406, Paris.

OECD, Organisation for Economic Cooperation and Development (2002) The Local Lymph Node Assay. Guidelines for Testing of Chemicals No. 429, Paris.

OECD, Organisation for Economic Cooperation and Development (2010a) Skin Sensitization: Local Lymph Node Assay: DA, Guidelines for Testing of Chemicals No. 442A, Paris.

OECD, Organisation for Economic Cooperation and Development (2010b) Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA, Guidelines for Testing of Chemicals No. 442B, Paris.

Natsch A. The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers--functional relevance and a hypothesis on innate reactions to skin sensitizers. Toxicological Sciences 2010 113(2):284-92.

Hartung, T., Bremer, S., Casati, S., Coecke, S., Corvi, R., Fortaner, S., Gribaldo, L., Halder, M., Hoffmann, S., Roi A.J., Prieto, P., Sabbioni, E., Scott, L., Worth, A. and Zuang. V. (2004) A Modular Approach to the ECVAM Principles on Test Validity. ATLA 32, 467-72.

4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

Objective *Why does ECVAM require advice on the current issue?*	The opinion of ESAC on the present Prevalidation study of the KeratinoSens test method should support ECVAM with respect to the evaluation of the validity status of the test method at present and with regard to possible necessary further work required to fully characterise the test method's performance (reproducibility, predictive capacity, applicability, limitations of the test method). Moreover, based on the evaluation of the data submitted, the ESAC should provide advice on the potential usefulness of the KeratinoSens test method within a testing strategy for skin sensitisation testing and the proper scientific use of the test method within such a testing strategy (e.g. with respect to its specific applicability and limitations). It is explicitly noted that the ESAC is not requested to suggest the precise placing of the submitted method in a hypothetical ITS, but rather to provide advice on the characteristics of the method relevant for its subsequent integration into an ITS at a later point in time (i.e. when other buildings blocks of such an ITS are known).

4.2 QUESTION(S) TO BE ADDRESSED

Questions *What are the questions and issues that should be addressed in view of achieving the objective of the advice?*	1) **DESIGN & CONDUCT OF STUDY:** The ESAC is requested to review whether the submitted prevalidation study was conducted appropriately in view of the objective of the study (see attachment 17e of the submission). The study objective was to assess (1) the reproducibility of the KeratinoSens method in one (the lead) laboratory (n=14 substances plus further 28 substances which were however not tested in a sufficient number of runs), (2) its transferability to other laboratories (n=7), (3) its reproducibility in other laboratories (BLR) when test items were tested repeatedly, but in deviation from the complete procedure as conducted by the lead laboratory in the intra-laboratory study (n=21). (4) Furthermore, the study aimed at assessing, in a preliminary manner, the predictive capacity of the test method based on the testing of published reference chemicals in the lead laboratory (n=114; this included 67 chemicals used for test development/optimisation and development of the prediction model) and during the ring trial to assess transferability (n=7) and BLR (n=21). When reviewing the design and conduct of the study, the following issues should be addressed in particular: • Clarity of the test definition (module 1) • Clarity of the definition of the study objective (see attachment 17e of the Prevalidation study report). • Appropriateness of the study design in view of study objective, *inter alia:* - Is the **number of chemicals** sufficient for the purposes of the study? - Are the **reference data** used for assessing in particular the predictive capacity appropriate and of good quality? - Was the identification of **chemicals** conducted in an appropriate manner (i.e. presence or absence of selection criteria, justification etc.)? - Is the **adverse effect range of the selected chemicals** appropriate for the purpose of the study - In case of **gaps** (chemical class etc.) – are these justified? - Is the **number of laboratories** sufficient? Appropriateness of the **study execution** (e.g. were there pre-defined acceptance criteria, were these respected? How were exceptions / deviations handled? Were provisions specified for retesting? Were the number of repetitions sufficient? etc.) Appropriateness of the **statistical analysis** used for analysing WLR, transferabilitiy, BLR and (preliminary) predictive capacity. 2) **CONCLUSIONS OF STUDY:** The ESAC is requested to assess whether the conclusions, as presented in the Test Submission Template (TST), Annex 17e, are substantiated by the information generated during prevalidation and are plausible with respect to existing information and current views (e.g. literature).

	In particular: • Do the data on the basis of these chemicals provide sufficient information on **applicability and possible limitations** of the test method, in particular in view of its potential use within an ITS for sensitisation? • Are the conclusions on **reproducibility** (WLR and BLR) as well as transferability justified and plausible? • Are the conclusions on **predictive capacity** justified and plausible with respect to existing information • Are there **possible gaps between study design and study conclusions** which remain to be addressed in view of the suggested conclusions / use (see also point 3)? 3) **SUGGESTED USE OF THE TEST METHOD:** The ESAC is requested (a) to evaluate, on the basis of the data submitted in the Prevalidation study, the possible use of the validated method (also within a strategy) to identify skin sensitisers, (b) to make additional recommendations (as required) on the proper scientific use of the test method within such a strategy taking specific aspects of this method into account (e.g. applicability, limitations etc.) and (c) to identify possible further information required (i.e. are there gaps) to be able to conclude on the plausibility of the suggested use (including within an ITS).

4.3 TIMELINES

Timelines concerning this request *When does ECVAM require the advice?*	**Timeline**	**Indication**
	Finalised ESAC Opinion required by:	ESAC 36, 20-21 March 2012
	Request to be presented to ESAC by written procedure (e.g. due to urgency) prior to the next ESAC	NO
	Request to be presented to ESAC at ESAC plenary meeting	ESAC 34, 22-23 March 2011 and ESAC 35, 4-5 October 2011 (mandate)

5. ECVAM PROPOSALS ON HOW TO ADDRESS THE REQUEST WITHIN ESAC

5.1 ECVAM PROPOSAL REGARDING REQUEST-RELATED STRUCTURES REQUIRED

Specific structures required within ESAC to address the request *Does the advice require an ESAC working group, an ESAC rapporteur etc.?*	**Structure(s) required**	**Required according to ECVAM? (YES/NO)**
	S1 ESAC Rapporteur	NO
	S2 ESAC Working Group	**YES**
	S3 Invited Experts	NO
	Ad S3: If yes – list names and affiliations of suggested experts to be invited and specify whether these are member of the EEP	
	If other than above (S1-S3):	NO

5.2 DELIVERABLES AS PROPOSED BY ECVAM

Deliverables *What deliverables (other than the ESAC opinion) are required for addressing the request?*	**Title of deliverable other than ESAC opinion**	**Required? (YES/NO)**
	D1 ESAC Rapporteur Report and draft opinion	NO
	D2 ESAC Peer Review Report and draft opinion	**YES**
	If other than above (D1-D2):	

6. LIST OF DOCUMENTS TO BE MADE AVAILABLE TO THE ESAC

Count	Description of document	Available (YES/NO)	File name
1	Validation study report (external validation) of Givaudan-coordinated KeratinoSens study based on the ECVAM Test Submission Template following	YES	ER2011-03_Ring_study_KeratinoSens.pdf

	ECVAM's Modular approach.		
2	Review paper on biological relevance of Nrf2-Keap1-ARE toxicity pathway for sensitisation testing	YES	ER2011-03_Toxicol. Sci.-2010-Natsch.pdf

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

During its 34th meeting on March 22-23 the ESAC plenary unanimously decided to establish an ESAC Working Group Sensitisation charged with the detailed scientific review of four test methods for skin sensitisation.

7.2 TITLE OF THE ESAC WORKING GROUP

Full title:
ESAC Working Group on Skin Sensitisation Test Methods
Abbreviated title:
ESAC WG Sensitisation

7.3 MANDATE OF THE ESAC WG

The EWG is requested to conduct a scientific review of the relevant studies concerning four skin sensitisation test methods (DPRA, MUSST, h-CLAT, KeratinoSens). The review needs to address the questions put forward to ESAC by ECVAM.

The review should focus on the appropriateness of design and conduct of the study in view of the study objective and should provide an appraisal to which extent the conclusions of the Validation Management Team (VMT) / test method submitter are substantiated by the information generated during the study and how the information generated relates to the scientific background available.

7.4 DELIVERABLE OF THE ESAC WG

The ESAC WG is requested to deliver to the chair of the ESAC and the ESAC Secretariat a detailed **ESAC Working Group Report** outlining its analyses and conclusions**.** A reporting template has been appended (Appendix 1) intended to facilitate the drafting of the report.
The conclusions drawn in the report should be based preferably on consensus. If no consensus can be achieved, the report should clearly outline the differences in the appraisals and provide appropriate scientific justifications.

7.5 PROPOSED TIMELINES OF THE ESAC WG

The ESAC Coordinator has proposed timelines* which should be agreed upon during the first Teleconference (Item 1 in the table):

Item	Proposed date/time	Action	Deliverable
1	**7. September 2011 (Wednesday)** 13:30 CET	**Kick-off teleconference** Discussion of a) the submission b) the mandate put forward by the Secretariat c) the working procedure (ESAC WG template)	Feedback on the mandate.
2	**14. October 2011 (Friday)**	**Deadline** for submitting first comments within ESAC WG template	Draft observations of each ESAC WG member in the ESAC WG

			template (to be compiled by ECVAM)
3	**24-26 October 2011 (Monday to Wednesday)**	ESAC WG meeting in Ispra. Discussion of contentious items. Drafting of the report.	Draft report
4	Further teleconferences and work progress to be agreed during meeting (Nr. 3).	Progressing of draft report	Draft report
5	**15. February 2012 (Wednesday)**	Final report to be delivered to ESAC Coordinator/Secretariat.	Final report

7.6 QUESTIONS WHICH SHOULD BE ADDRESSED BY THE ESAC WG

The ESAC WG is requested to address the **three questions posed to the ESAC** which have been broken down further in more **specific questions** by Secretariat (see section 4.2) and were discussed with the ESAC WG and approved by the ESAC.

When preparing the final ESAC WG report to address these questions, the ESAC WG is requested to use a pre-defined reporting template. This template (see appendix 1) follows ECVAM's modular approach and addresses to which extent the standard information requirements have been addressed by the study. In addition, the template allows for addressing the specific questions outlined in section 4.2. The Secretariat will provide guidance if necessary.

APPENDIX 1 REPORTING STRUCTURE FOR THE *ESAC WG REPORT*

The following suggested structure follows the ECVAM information requirements ("modules") for scientific review following validation and allows at the same time for the description of the analysis and conclusions concerning more specific questions. A template has been created on the basis of the structure below and this template will be made available to the ESAC.

The template can be used for various types of validation studies (e.g. prospective full studies, retrospective studies, performance-based studies and prevalidation studies). Depending on the study type and the objective of the study, not all sections may be applicable. However, for reasons of consistency and to clearly identify which information requirements have not been sufficiently addressed by a specific study, this template is uniformly used for the evaluation of validation studies.

1. Data collection

1.1 Information / data sources used

1.2 Search strategy

1.3 Selection criteria applied to the available information

2. Study objective and design

2.1 Clarity of the definition of the study objective

2.2 Analysis of the scientific rationale provided

2.3 Analysis of the regulatory rationale provided

2.4 Appropriateness of the study design

2.5 Appropriateness of the statistical evaluation

3. Test definition (Module 1)

3.1 Quality and completeness of the overall test definition

3.2 Quality of the background provided concerning the purpose of the test method

3.3 Quality of the documentation and completeness of (a) standardised protocols (SOPs) and (b) prediction models

4. Data quality

4.1 Overall quality of the evaluated data

4.2 Sufficiency of the evaluated data in view of the study objective

4.3 Quality of the reference data for evaluating reliability and relevance[2]

5. Test materials

5.1 Sufficiency of the number of evaluated test items in view of the study objective

5.2 Representativeness of the test items with respect to applicability

6. Within-laboratory reproducibility (Module 2)

6.1 Assessment of repeatability and reproducibility in the same laboratory

6.2 Conclusion on within-laboratory reproducibility as assessed by the study

7. Transferability (Module 3)

7.1 Quality of design and analysis of the transfer phase

7.2 Conclusion on transferability to a second laboratory/other laboratories as assessed by the study

8. Between-laboratory reproducibility (Module 4)

8.1 Assessment of reproducibility in different laboratories

8.2 Conclusion on reproducibility as assessed by the study

9. Predictive capacity (Module 5)

9.1 Adequacy of the assessment of the predictive capacity in view of the purpose

9.2 Overall relevance (biological relevance and accuracy) of the test method in view of the purpose

[2] OECD guidance document Nr. 34 on validation defines relevance as follows: "Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It sis the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of accuracy (concordance) of a test method."

10. Applicability domain (Module 6)
10.1 Appropriateness of study design to conclude on applicability domain, limitations and exclusions
10.2 Quality of the description of applicability domain, limitations, exclusions
11. Performance standards (Module 7)
11.1 Adequacy of the proposed Essential Test Method Components
11.2 Adequacy of the Reference Chemicals
11.3. Adequacy of the defined Accuracy Values
12. Readiness for standardised use
12.1 Assessment of the readiness for regulatory purposes
12.2. Assessment of the readiness for other uses (in house screening etc.)
12.3 Critical aspects impacting on standardized use
12.4 Gap analysis
13. Other considerations
14. Conclusions on the study
14.1 Summary of the results and conclusions of the study
14.2 Extent to which conclusions are justified by the study results alone
14.3 Extent to which conclusions are plausible in the context of existing information
15. Recommendations
15.1 General recommendations concerning the study
15.2 Recommendations concerning the test method (test system, protocol, prediction model)
16. References
17. Annexes

END OF EURL ECVAM RECOMMENDATION

European Commission
EUR 26427 – Joint Research Centre – Institute for Health and Consumer Protection

Title: EUR 26427 - EURL ECVAM Recommendation on the KeratinoSensTM assay for skin sensitisation testing

Luxembourg: Publications Office of the European Union

2013 – 39 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1018-5593 (print), ISSN 1831-9424 (online)

ISBN 978-92-79-34795-5

doi: 10.2788/52914

Abstract

Identification of the skin sensitisation hazard of chemicals has traditionally relied on the use of animals. Progress in the development of alternative methods has been prompted by the increasing knowledge of the key biological mechanisms underlying this human health effect, as documented by the OECD's recent report summarising the key biological events leading to skin sensitisation ("Adverse Outcome Pathway" (AOP) for skin sensitisation). Within this AOP the activation of cellular signalling pathways, such as the Keap1-Nrf2- antioxidant/electrophile response element (ARE)-dependent pathway, known to play a relevant role in keratinocytes' responses to skin sensitisers, is postulated to be a key event. Therefore, test methods able to provide information on the ability of a chemical to activate this or other relevant pathways in keratinocytes, may contribute to skin sensitisation hazard and safety assessment. The KeratinoSensTM test method measures ARE-Nrf2 activation through a luciferase reporter gene. The test method has undergone a validation study addressing mainly the test method's transferability and within- and between-laboratory reproducibility. Following independent scientific peer review by EURL ECVAM's Scientific Advisory Committee (ESAC) and having considered the input from regulators, stakeholders, international partners and the general public, EURL ECVAM concluded that the KeratinoSensTM may prove a useful component of integrated approaches such as Weight of Evidence (WoE) or Integrated Testing Strategies (ITS) for skin sensitisation hazard assessment. In addition to this, the KeratinoSensTM may also be able to contribute to the assessment of sensitising potency, e.g. by supporting sub-categorisation of sensitisers according to UN GHS. However it is recognised that further efforts are required to explore how KeratinoSensTM data may contribute to potency assessment.

LB-NA-26427-EN-N

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new standards, methods and tools, and sharing and transferring its know-how to the Member States and international community.

Key policy areas include: environment and climate change; energy and transport; agriculture and food security; health and consumer protection; information society and digital agenda; safety and security including nuclear; all supported through a cross-cutting and multi-disciplinary approach.

Publications Office