新規試験法提案書

皮膚感作性試験代替法
human Cell Line Activation Test（h-CLAT）

平成29年 3 月

国立医薬品食品衛生研究所

新規試験法提案書

平成 29 年 3 月 21 日
No.　2016-03

皮膚感作性試験代替法　human Cell Line Activation Test（h-CLAT）に関する提案

平成 29 年 2 月 24 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： h-CLAT において陽性の結果が得られた場合、感作性物質と判定することは可能であるが、感作性強度分類や United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS)のサブカテゴリー分類は困難である。本試験法単独での皮膚感作性の判定は不十分であり、被験物質の特性を十分に理解した上で、Integrated Approaches to Testing and Assessment (IATA) を構成するその他の情報と組み合わせて適切に評価することが必要である。なお、本試験法の利用にあたっては、適用範囲を十分に配慮した上で使用されるべきである。

この提案書は、Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 442E; *In Vitro* Skin Sensitization: h-CLATおよび h-CLAT Validation Study Report, EURL ECVAM　Recommendation on the h-CLAT for skin sensitization 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　h-CLAT 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

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任期：平成 28 年 4 月 1 日～平成 30 年 3 月 31 日

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　　第二室）：事務局

JaCVAM statement on the human Cell Line Activation Test (h-CLAT) Skin Sensitization Test Method

At a meeting held on 24 February 2017 at the National Institute of Health Sciences (NIHS) in Tokyo, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

Proposal: Although it is possible to classify chemicals that yield positive results using the h-CLAT test method as sensitizers, it is not possible to assess accurately their sensitization strength nor their subcategorization under the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS). The h-CLAT test method is not suitable for predicting skin sensitization potential on its own; in order to make a suitable assessment, the results of h-CLAT testing must be used with a thorough understanding of the properties of each test chemical in combination with other information as part of an integrated approach to testing and assessment (IATA). Furthermore, thorough consideration must be given to the applicability domain when using this test.

This statement was prepared following a review of the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 442E "*In Vitro* Skin Sensitization: h-CLAT" as well as other documentation prepared by the Skin Sensitization Testing JaCVAM Editorial Committee based on the "h-CLAT Validation Study Report" and the "EURL ECVAM Recommendation on the h-CLAT for skin sensitization testing" to acknowledge that the results of a review and study by the JaCVAM Regulatory Acceptance Board have confirmed the usefulness of this assay.
Based on the above, we propose the h-CLAT skin sensitization test method as a useful means for safety assessment by regulatory agencies.

Yasuo Ohno
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

March 21, 2017

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. Noriyasu Imai (Japanese Society for Alternatives to Animal Experiments)
Mr. Tomoaki Inoue (Japanese Society of Immunotoxicology)
Mr. Yuji Ishii (Biological Safety Research Center: BSRC, NIHS)
Ms. Yumiko Iwase (Japan Pharmaceutical Manufacturers Association)
Mr. Takeshi Morita (Japanese Environmental Mutagen Society)
Mr. Shunji Nakai (Japan Chemical Industry Association)
Ms. Ruriko Nakamura (National Institute of Technology and Evaluation)
Mr. Akiyoshi Nishikawa (BSRC, NIHS)
Mr. Satoshi Numazawa (Japanese Society of Toxicology)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)

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

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)
Ms. Yoko Hirabayashi (Division of Toxicology, BSRC, NIHS)
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)
Ms. Mitsue Hirota (Pharmaceutical & Medical Devices Agency)
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)
Mr. Atsushi Kato (National Institute of Infectious Diseases)
Mr. Tetsuya Kusakabe (Ministry of Health, Labour and Welfare)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Mr. Taku Oohara (Ministry of Health, Labour and Welfare)
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 Toxicology, BSRC, NIHS)
Mr. Masaaki Tsukano (Ministry of Health, Labour and Welfare)
Mr. Hajime Kojima (Division of Risk Assessment, BSRC, NIHS): Secretary

皮膚感作性試験代替法

human Cell Line Activation Test（h-CLAT）

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評価会議報告書

human Cell Line Activation Test（h-CLAT）

JaCVAM 評価会議

平成 28 年（2016 年）12 月 20 日

JaCVAM 評価会議

大 野 泰 雄　（運営委員会推薦）：座長

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任期：平成 28 年 4 月 1 日～平成 30 年 3 月 31 日

human Cell Line Activation Test（h-CLAT）は、多くの皮膚感作性物質が樹状細胞を活性化することを利用し、樹状細胞のモデルのヒト単球系培養細胞 THP-1 細胞に被験物質を曝露したときの細胞表面分子 CD86 および CD54 の発現量を測定することにより、皮膚感作性の有無を判定する試験法である。

h-CLAT については、European Union Reference Laboratory for Alternatives to Animal Testing（EURL ECVAM）によるバリデーション研究が実施され[1]、EURL ECVAM Scientific Advisory Committee（ESAC）による第三者評価を受けた[2]。2016 年 7 月には、Organisation for Economic Co-operation and Development（OECD）試験法ガイドライン（Test Guideline: TG）442E として承認された[3]。JaCVAM 評価会議は、皮膚感作性試験資料編纂委員会により作成された「皮膚感作性試験評価報告書　human Cell Line Activation Test（h-CLAT）」[4]を用いて、本試験法の妥当性について検討した。

１．試験法の定義

名称：　human Cell Line Activation Test（h-CLAT）

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

試験法の概略：　本試験法では、樹状細胞のモデルとしてヒト単球系培養細胞である THP-1 細胞を用い、被験物質が THP-1 細胞を活性化する能力を評価する。THP-1 細胞に被験物質を添加して 24 時間培養した後、fluorescein isothiocyanate（FITC）で蛍光標識した CD86 および CD54 の特異抗体、および propidium iodide（PI）を用いて細胞を染色し、フローサイトメトリーにより細胞表面分子 CD86 及び CD54 の発現量、並びに細胞生存率を測定する。被験物質で処理したときの CD86 および CD54 それぞれの平均蛍光強度を、溶媒のみを添加したコントロールの蛍光強度と比較し、相対蛍光強度を算出する。

２．評価に用いた資料および評価内容の科学的妥当性

本試験法は、EURL ECVAM によるバリデーション研究[1]とそれに続く ESAC による第三者評価により、実験動物を用いた皮膚感作性試験の代替法として科学的に妥当であると報告されており[2]、2016 年 7 月に OECD 試験法ガイドラインとして承認されている[3]。JaCVAM 皮膚感作性試験資料編纂委員会では、現在まで公開されている情報[2,3,5-8]を基に本試験法の皮膚感作性試験代替法としての科学的妥当性について評価した。本試験法は、皮膚感作性発現機序における重要なステップである抗原提示細胞の活性化反応を指標にしたもので、活性化の際に認められる細胞表面分子の発現亢進を測定するという点で原理的にも妥当である。

３．本試験法の有用性と適用限界

本試験法は、ヒト単球系由来培養細胞株を用いる *in vitro* 試験法であり、3Rs の精神に合致している。また、1 試験あたりの費用は、マウスを用いる LLNA の 1/5 程度であり、試験期間も LLNA に比べて短期間であることから、本試験法は経済性および迅速性の面から有用といえる。しかしながら、本試験法は、細胞密度と培養時間を厳守することや公比 1.2 の濃度設定を必要とするため、別の皮膚感作発現イベントを指標とする Direct Peptide Reactivity Assay（OECD TG442C）や ARE-Nrf2 Luciferase Test Method（OECD TG442D）に比べて、操作が煩雑であり、制約が多い。また、陽性対照、陰性対照および媒体対照の測定を継続的に実施し、背景データを作成することが求められている。

EURL ECVAM によるバリデーション研究[1)]において、15 物質を用いて実施された施設内再現性は、73.3～86.7%であったが、試験時間の厳密な管理により再現性の向上が図れるとされている。一方、24 物質を用い 4 施設で実施された施設間再現性は 79.2%であった。一致しなかった物質は施設内再現性も悪かった。

EURL ECVAM[1)]、Takenouchi ら[7)]、および Nukada ら[8)]の結果を合わせた 142 物質（感作性物質：非感作性物質＝101：41、log Kow が 3.5 以上で陰性と判定された物質と混合物を除く）についての成績は、正確度 85%、感度 93%、特異度 66%であり、様々な化学物質に適用可能であることが示された。しかし、United Nations（UN）Globally Harmonized System of Classification and Labelling of Chemicals （GHS）分類における軽度～中等度感作性物質は偽陰性の判定が生じやすい傾向がある。また、試験溶媒に溶解しない物質および試験溶媒中で不安定な物質、揮発性物質、FITC や PI と同じ波長域に強い蛍光を有する物質、プロハプテンおよびプレハプテンのような活性化に代謝系を必要とする化学物質では正しく感作性が検出されない可能性があり、試験結果の解釈には注意が必要である。

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

社会的受け入れ性：

本試験法は、フローサイトメトリーに習熟した施設であれば実施できる試験法であり、蛍光標識された特異抗体も市販されている。また THP-1 細胞の入手は容易であり、短期間で安価に実施できる。生きた動物を用いないという点で、3Rs の精神に合致しており、社会的受け入れ性は高い。

行政上の利用性：

本試験法において陽性の結果が得られた場合、感作性物質と判定することは可能であるが、感作性強度分類や UN GSH のサブカテゴリー分類は困難である。本試験法単独での皮膚感作性の判定は不十分であり、被験物質の特性を十分に理解した上で、Integrated Approaches to Testing and Assessment（IATA）を構成するその他の情報と組み合わせて適切に評価することが必要である。なお、本試験法の利用にあたっては、適用範囲を十分に配慮した上で使用されるべきである。

参考文献

1) EURL ECVAM (2013). human Cell Line Activation Teat (h-CLAT) Validation Study Report. Accessible at: https://eurl-ecvam.jrc.ec.europa.eu/news/news_docs/h-clat-validation-study-report.
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皮膚感作性試験評価報告書

human Cell Line Activation Test（h-CLAT）

JaCVAM 皮膚感作性試験資料編纂委員会

平成 28 年 9 月 19 日

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）
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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（Quantitative Structure-Activity Relationship: QSAR）モデルや *in vitro* の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む化粧品の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。human Cell Line Activation Test（h-CLAT）は、多くの皮膚感作性物質が樹状細胞を活性化することを利用し、ヒト単球系培養細胞である THP-1 細胞を用い、活性化に伴い細胞表面上の発現量が変化する CD86 と CD54 を測定することにより皮膚感作性の有無を判定する試験法である。本報告書は、この h-CLAT について、European Union Reference Laboratory for Alternatives to Animal Testing（EURL ECVAM）により実施されたバリデーション報告書、評価（ピアレビュー）報告書および試験法開発者の投稿論文などを基に試験手順をまとめ、有用性と限界を評価したものである。

h-CLAT は、感作性発現機序における第三段階のイベントである樹状細胞が活性化する際の細胞表面分子の発現亢進を利用した *in vitro* 試験法であり、化学物質の感作性を判断する上で重要な情報を与えてくれる。

h-CLAT では、マウスを用いる Local Lymph Node Assay（LLNA）の 1/5 程度の経費で被験物質の皮膚感作性を判定することが可能と試算され、試験期間も LLNA に比べ短期であるため、経済性・迅速性の観点で有用性は高いと思われる。しかしながら、Direct Peptide Reactivity Assay（DPRA）や ARE-Nrf2 Luciferase Test Method に比べて、操作が煩雑であり、制約が多い。また、陽性対照、陰性対照および媒体対照の測定を継続的に実施し、ヒストリカルなデータベースを作成することが必要とされている。

本試験法の EURL ECVAM によるバリデーション試験において、15 物質を用いて実施された施設内再現性は 73.3～86.7%であり、全般的に高くなかった。ただし、Organisation for Economic Co-operation and Development（OECD）専門家会議において、試験に使用する THP-1 細胞の前培養条件および被験物質の曝露時間をより厳密に管理することにより、施設内再現性の向上が図れることが示され、OECD 試験法ガイドラインの試験手順にその内容が反映されていることから、施設内再現性の向上が期待できると考える。一方、24 物質を使い 4 施設で実施された施設間再現性は 79.2%であった。

OECD 試験法ガイドラインでは、142 物質の成績から正確度 85%、感度 93%、特異度 66%と記載されているが、log Kow が 3.5 以上で陰性と判定された物質を除いた上での数値である。したがって、特に log Kow が 3.5 以上の物質の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、本試験法のみで被験物質を皮膚感作性が陰性と判定することはできない。また、特異度が 66%であるため偽陽性と判定することも留意する必要がある。さらに、本試験法に用いる細胞の代謝能は限定的であるため、活性化に代謝系を必要とする化学物質では、正しくその皮膚感作性が検出されない可能性がある。

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

1. 緒言

皮膚感作性を評価することは化学物質の安全性評価において重要である。化学物質の皮膚での接触感作性のリスクを動物で予測する試験法としてモルモットを用いる皮膚感作性試験（Organisation for Economic Co-operation and Development: OECD Test Guideline: TG406）やマウスを用いる局所リンパ節試験（Local Lymph Node Assay(LLNA), 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）を使用せずに 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。

EU における欧州化学品規制（Registration, Evaluation, Authorization and Restriction of Chemicals: REACH）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（Quantitative Structure-Activity Relationship: QSAR）モデルや *in vitro* 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使用した化粧品の販売が禁止された（2013 年 3 月全面施行)。そのため、化学製品の皮膚感作性を判定する代替法の開発が強く求められている。

In chemico 試験法としてペプチド結合反応を利用した Direct Peptide Reactivity Assay（DPRA：OECD TG442C）および *In vitro* 試験法としてケラチノサイト細胞系の標的遺伝子を用いた ARE-Nrf2 Luciferase Test Method（OECD TG442D）が OECD から試験法ガイドラインとして公表されている。また、これ以外に、単球系細胞の活性化を利用した human Cell Line Activation Test（h-CLAT)、Myeloid U937 Skin Sensitization Test（U-SENS）および IL-8 Luc assay などの皮膚感作性試験の *in vitro* 法が提案されており、EURL ECVAM（the 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きた。

h-CLAT は、多くの皮膚感作性物質が樹状細胞を活性化することを利用し、ヒト単球系培養細胞である THP-1 細胞を用い、活性化に伴い細胞表面での発現量が変化する CD86 と CD54 を測定する試験法である。h-CLAT のバリデーション研究の結果については、EURL ECVAM による評価（ピアレビュー）が完了し [1)]、2016 年 7 月に OECD の試験法ガイドラインリストに追加された（TG442E）[7)]。

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

2. 試験法の原理

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

1)　化学物質とタンパク質のシステイン残基あるいはリジン残基との共有結合

2) ケラチノサイトにおける炎症性応答および Antioxidant/electrophile response element（ARE）-dependent pathway による遺伝子発現
3) 樹状細胞の活性化（特異的細胞表面マーカーの発現、ケモカインやサイトカインの産生）
4) リンパ節における T 細胞の増殖

h-CLAT は上記の第 3 の Key event に対応する試験法である。その基本的原理は、樹状細胞の活性化時に発現が増大する細胞表面分子の測定である。

樹状細胞の活性化時には、CD86、CD54 のような細胞表面分子の発現量が増大することが知られている。CD86 は、T 細胞に抗原提示する際に T 細胞表面の CD28 と相互作用することにより補助刺激分子として機能する。CD54 は、別名 Intercellular adhesion molecule-1（ICAM-1）という接着分子であり、T 細胞表面の Lymphocyte function-associated antigen 1（LFA-1）と結合する。

h-CLAT では、樹状細胞のモデルとしてヒト単球系培養細胞である THP-1 細胞を用い、化学物質が THP-1 細胞を活性化する能力を評価する。THP-1 細胞に検体である化学物質を添加して 24 時間培養した後、CD86 および CD54 の発現量を、蛍光標識した特異抗体を用いてフローサイトメトリーにより測定する。溶媒のみを添加したコントロールに対する相対蛍光強度を算出し、化学物質による樹状細胞の活性化の指標とする。

3．試験手順／判定

h-CLAT の試験手順は、用量設定試験と CD86/CD54 発現測定の 2 つに分けられる。

3-1．細胞調製および試薬

h-CLAT では、THP-1 細胞を使用する。細胞は American Type Culture Collection（ATCC）から入手（TIB-202™）することが望ましい。

THP-1 細胞は、培養用培地（RPMI-1620 の基礎培地に 10% Fetal bovine serum, 0.05 mM 2-Mercaptoethanol, 100 unit/mL Penicillin, 100 μg/mL Streptomycin を加えたもの）にて、0.1~1.0×10^6 cells/mL の細胞濃度で播種し、2~3 日ごとに継代を行い、維持する。細胞濃度は 0.1~0.8×10^6 cells/mL を維持し、1.0×10^6 cells/mL を超えないようにする。また、ATCC から入手後の初回融解 2 週間後に、2,4-Dinitrochlorobenzene（DNCB, CAS No.97-00-7、純度≧99%以上）、Nickel sulfate（CAS NO. 10101-97-0、純度≧99%以上）および陰性対照として Lactic acid（CAS NO. 50-21-5、純度≧85%以上）を用いて、反応性の確認を行う。DNCB と Nickel sulfate では、CD86 と CD54 の両方で陽性となり、Lactic acid では、CD86 と CD54 のいずれでも陰性の結果となる。反応性試験で合格した細胞のみを本試験法で用いることができる。THP-1 細胞は、融解後 2 ヶ月まで継代させることができ、継代数が 30 を超えないようにすべきである。

試験実施前に、THP-1 細胞を 0.1 もしくは 0.2×10^6 cells/mL の細胞濃度で培養用フラスコに播種し、それぞれ 72 時間もしくは 48 時間培養する。実験日に、2×10^6 cells/mL の濃度で

新しい培養用培地に再懸濁した THP-1 細胞懸濁液を 24 穴平底プレートもしくは 96 穴平底プレートに、それぞれ 500 μL/well もしくは 80 μL/well で加える。

3-2. 用量設定試験

用量設定試験は、CV75（媒体対照と比較して 75%の細胞生存率を示す被験物質濃度）を決定するために実施する。CV75 は CD86 および CD54 発現量測定の濃度設定に利用される。

1) 被験物資および陰性対照の調製

被験物質および陰性対照は、用時調製する。h-CLAT では、被験物質は溶液もしくは安定な懸濁液に調製する。媒体は生理食塩水および基礎培地を第一候補、Dimethyl sulfoxide（DMSO, 純度≧99%以上）を第二候補とし、最終濃度は生理食塩水および培地の場合は 100 mg/mL、DMSO の場合は 500 mg/mL とする。100 mg/mL（生理食塩水および培地の場合）および 500 mg/mL（DMSO の場合）の保存溶液を用いて、以下の手順で希釈操作を行う。

－生理食塩水および基礎培地を使用する場合：それぞれの媒体を用いて公比 2 で希釈を行い、8 濃度の保存原液を作製する。これら保存原液はそれぞれ培養用培地でさらに 50 倍に希釈する（使用液）。もし最高濃度である 1,000 μg/mL で細胞毒性がない場合、最高濃度を上げて細胞毒性試験を再実施して決定するが、プレートにおける最終濃度は、5,000 μg/mL を超えてはいけない。

－DMSO を使用する場合：DMSO を用いて公比 2 で希釈を行い、8 濃度の保存原液を作製する。保存原液はその後、培養用培地でさらに 250 倍に希釈する（使用液）。たとえ細胞毒性がなくとも、プレート中の最終濃度は、1,000 μg/mL を超えてはいけない。

媒体対照には、被験物質が生理食塩水または基礎培地で溶液もしくは安定な懸濁液となる場合には培養用培地を用い、被験物質が DMSO で溶液もしくは安定な懸濁液となる場合は DMSO を用いて、そのプレートにおける最終濃度は 0.2%とする。それらは上記の使用液作製の手順と同じ希釈で行う。

2) 被験物質および陰性対照の曝露

1) で調製した被験物質を含む使用液および媒体対照は、細胞懸濁液と 1:1 で混合し（通常、最終濃度の範囲は、7.81~1,000 μg/mL）、24 穴平底プレートもしくは 96 穴平底プレートにて 37℃、5%CO_2 in air で 24 時間（±0.5 時間）培養する。揮発性の高い被験物質の場合は、ウェル間で被験物質のコンタミが生じる可能性があるので、プレートをシールするなどの処置を施すことが望ましい。

3) Propidium iodide（PI）染色

24 時間（±0.5 時間）の曝露後、細胞はサンプルチューブに移し、遠心により細胞を集める。上澄みを捨てた後、0.1%ウシ血清アルブミン（Bovine Serum Albumin: BSA）含有リン酸緩衝液 200μL（96 穴の場合）もしくは 600μL（24 穴の場合）を加え、再懸濁する。この細胞懸濁液 200μL は、96 穴の場合は 96 穴丸底型プレートに、24 穴の場合はマイクロチュー

ブに移し、96 穴では 200μL の 0.1% BSA 含有リン酸緩衝液で、24 穴では 600μL の 0.1%BSA 含有リン酸緩衝液 で 2 回以上洗浄する。最終的に、細胞は 0.1%BSA 含有リン酸緩衝液（例えば、400 μL）で再懸濁し、そこに 20 μL の PI 溶液（PI の最終濃度は 0.625 μg/mL）を加える。

4) 細胞毒性の測定と CV75 の算出

PI の取り込みは、フローサイトメーターで分析する。細胞毒性は、10,000 の生細胞（PI ネガティブ）がカウントできる条件で、以下の式により算出する。また、細胞生存率が低い場合には、死細胞数を含む細胞数を 30,000 とした条件にて、細胞生存率（cell viability）を算出する。

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

また、CV75（THP-1 細胞の 75%が生細胞である濃度）は、次式により算出する。

$$\text{Log CV75} = \frac{(75\text{-}c) \times \text{Log}(b) - (75\text{-}a) \times \log(d)}{a - c}$$

a：試験した濃度において生存率 75%を超える最小の生存率

c：試験した濃度において生存率 75%を超えない最大の生存率

b：a となる被験物質濃度

d：c となる被験物質濃度

3-3. CD86 および CD54 発現測定

1) 被験物質、陰性対照および陽性対照の調製

被験物質を溶解もしくは安定な懸濁液とさせる媒体には、生理食塩水、基礎培地および DMSO から適切なものを使用する。被験物質の最高濃度は用量設定試験にて算出された CV75 の 1.2 倍濃度とする。もし、CV75 が求められなかった場合（例えば、用量設定試験で十分な細胞毒性が認められなかった場合）には、被験物質の最大溶解もしくは安定な懸濁濃度を開始濃度とするが、プレートの最終濃度は、生理食塩水もしくは基礎培地では 5,000 μg /mL、DMSO の場合は 1,000 μg /mL を超えないようにする。その後、同じ媒体を用いて、公比 1.2 で希釈することで 8 濃度の保存原液（濃度幅：0.335×CV75～1.2×CV75：媒体が生理食塩液および基礎培地の場合はこの 100 倍の濃度、DMSO の場合はこの 500 倍の濃度）を作製する。保存原液はその後、培養用培地を用いて、媒体が生理食塩水および基礎培地の場合は 50 倍、媒体が DMSO の場合は 250 倍に希釈し、使用液を作製する。これら使用液は、最終的に細胞懸濁液でさらに 2 倍希釈され、曝露されることとなる。もし結果が、後述する試験成立の条件に合わない時は、正確な CV75 を得るために用量設定試験を再度実施してもよい。CD86 および CD54 発現測定では、24 well のみが使われることに注意する必要があ

る。

陰性対照/媒体対照は、用量設定試験と同様に調製する。陽性対照には DNCB を使用する。媒体に DMSO を使用し、上段に示すとおり、保存原液を作製する。DNCB は、CD86 および CD54 発現測定の陽性対照として、1 濃度（一般的には、プレート中の最終濃度が 4 μg/mL）が使われる。4 μg/mL とするために、DMSO を媒体にした 2 mg/mL の保存原液を培養用培地で 250 倍に希釈して、8 μg/mL の使用液を作製する。DNCB 濃度に関しては、各施設で決定された CV75 を陽性対照濃度として使用することも可能である。

陽性対照においてもプレートの最終濃度は、生理食塩水もしくは基礎培地では 5,000 μg/mL、DMSO の場合は 1,000 μg/mL を超えないようにし、陽性対照は 1 濃度であることから後述する被験物質の試験成立の条件のうち、最終項目のみ対象外となる。

2) 被験物質および陰性対照の曝露

被験物質の皮膚感作性の予測結果（陽性もしくは陰性）を得るためには、少なくとも 2 回の CD86 および CD54 発現測定を繰り返す必要がある。2 回の測定は、異なる日もしくは、同日のどちらでも良いが、それぞれの測定で保存原液, 被験物質の使用液および染色用抗体溶液は別々に調製されること、使用される細胞塊を分ける（例えば、細胞は異なるフラスコから集めるなど）ことが必要である。しかしながら、細胞は同じ継代数由来でも構わない。使用液として調製した被験物質および陰性対照は、それぞれ細胞懸濁液と 1:1 の割合で混合し、37℃、5%CO_2 in air、24 時間±0.5 時間、培養する。少なくとも 2 回の測定を実施するので、それぞれの測定では、測定内の被験物質の各濃度および陰性対照は、n=1 であっても問題ない。

3) 細胞の染色および分析

24 時間の曝露後、細胞液をサンプルチューブに移し、遠心により細胞を集め、1 mL の 0.1%BSA 含有リン酸緩衝液（pH 7.4）で 2 回洗浄する。洗浄後、600 μL の 0.1%BSA 含有リン酸緩衝液に 0.01%（w/v）の濃度でグロブリン（Cohn fraction II, III, Human; SIGMA, #2388-10G）を加えたものを加え、4℃で 15 分、インキュベートして、ブロックを行う。その後、細胞は、180 μL ずつ 3 つに等分し、丸底 96 穴プレートもしくはマイクロチューブに移す。

遠心後、細胞は、50 μL の Fluorescein isothiocyanate（FITC）標識抗 CD86 抗体、FITC 標識抗 CD54 抗体もしくは FITC 標識マウス IgG1 抗体（アイソタイプ）にて、4℃で 30 分間、染色する。h-CLAT DB-ALM プロトコール [2)]で記載されている抗体では、抗 CD86 抗体（BD-PharMingen #555657; Clone:Fun-1）では 3:25 の割合で抗体と 0.1%BSA 含有リン酸緩衝液を混合し、抗 CD54 抗体（DAKO, #F7143; Clone: 6.5B5）および IgG1（DAKO、#X0927）では 3:50 の割合で抗体と 0.1%BSA 含有リン酸緩衝液を混合する。

抗体染色後の細胞は 200μL の 0.1%BSA 含有リン酸緩衝液で 3 回洗浄後、400 μL の 0.1%BSA 含有リン酸緩衝液で再懸濁し、そこに PI 溶液（PI 濃度が 0.625 μg/mL であれば 20 μL）もしくはその他の細胞毒性マーカー液を添加する。CD86 および CD54 の発現、細胞生存率はフローサイトメーターを用いて測定する。

3-4 データおよび報告

1) データの評価

CD86 と CD54 の発現は、チャンネル FL-1 を有するフローサイトメーターで測定する。平均蛍光強度（Mean Fluorescence Intensity: MFI）を基に、以下の式から、陽性対照および被験物質で処理した細胞における CD86 および CD54 の相対蛍光強度（Relative Fluorescence Intensity: RFI）を求める。

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

アイソタイプコントロール（マウス IgG1 抗体で染色したもの）の細胞生存率は、3-2 4)の式を用いて計算する。

2) 予測方法

CD86 および CD54 発現測定では、それぞれの被験物質の予測（陽性もしくは陰性）を行うために、少なくとも 2 回の独立した測定を行う必要がある。以下の状況のうち 1 つが 2 回の測定のうち 2 回、もしくは 3 回の測定のうち 2 回で認められた場合、h-CLAT による予測は、陽性と判定する。

・細胞生存率 50%以上となるいずれかの濃度において、CD86 の相対蛍光強度が 150%以上となる場合

・細胞生存率 50%以上となるいずれかの濃度において、CD54 の相対蛍光強度が 200%以上となる場合

最初の 2 回の測定において、CD86 および/もしくは CD54 がともに陽性であれば、h-CLAT 予測は陽性と判定し、3 回目の測定は不要である。同様に最初の 2 回の測定で、CD86 および CD54 が共に陰性であれば、3 回目の測定は不要で h-CLAT 予測は陰性と判定する。一方、2 回の測定で二つのマーカーのうちいずれも連続して陽性を示さなかった場合、あるいは両者とも連続して陰性とならなかった場合、3 回目の測定が必要で、最終の予測は、3 回の測定の多数結果をもって決定する。

2 回の測定を行い、1 回目は CD86 が陽性、2 回目測定で CD54 が陽性となった場合、3 回目の測定が必要である。その結果、3 回目測定が陰性となった場合、h-CLAT 予測は陰性と判定する。一方、3 回目の測定で、どちらかのマーカー（CD86 もしくは CD54）が陽性、もしくは両マーカーが陽性となった場合、h-CLAT 予測は陽性と判定する。

次ページにフロー図（図 1）を示す。

3-5. 試験成立の条件

h-CLAT の場合、以下の条件が成立する必要がある。

・基礎培地対照および媒体対照の細胞生存率が、90%以上である。

・媒体対照における CD86 および CD54 の両方の相対蛍光強度値が陽性基準値（CD86 で

は 150%、CD54 では 200%）を超えない。溶媒対照の相対蛍光強度値は、3-4　1)に記載の式により計算される（その際、「MFI of chemical」は「MFI of solvent/vehicle」に、「MFI of solvent/vehicle」は「MFI of (medium) control」に読み換える必要がある。）

・基礎培地対照および媒体対照の両方において、アイソタイプに対する CD86 と CD54 の両方の平均蛍光強度の割合が 105%より大きくなる。

・陽性対照（DNCB）において、細胞生存率が 50%以上を示し、CD86 および CD54 の相対蛍光強度がいずれも陽性基準となる。

・被験物質において、それぞれの測定で試験された少なくとも 4 濃度において、細胞生存率が 50%を超える

最高濃度（1.2×CV75）での細胞生存率が 90%より小さい場合、陰性結果は受け入れられるが、1.2×CV75 の細胞生存率が 90%以上であった場合には、陰性結果は受け入れられない。そのような場合には、再度 CV75 を決定することで、濃度設定をやり直すことが望ましい。ただし、媒体に生理食塩水、基礎培地およびその他の培地を使用した 5,000 μg/mL、DMSO を使用した 1,000 μg/mL および最大溶解濃度が試験の最高濃度である場合、生存率が 90%以上であっても、陰性結果は許容される。

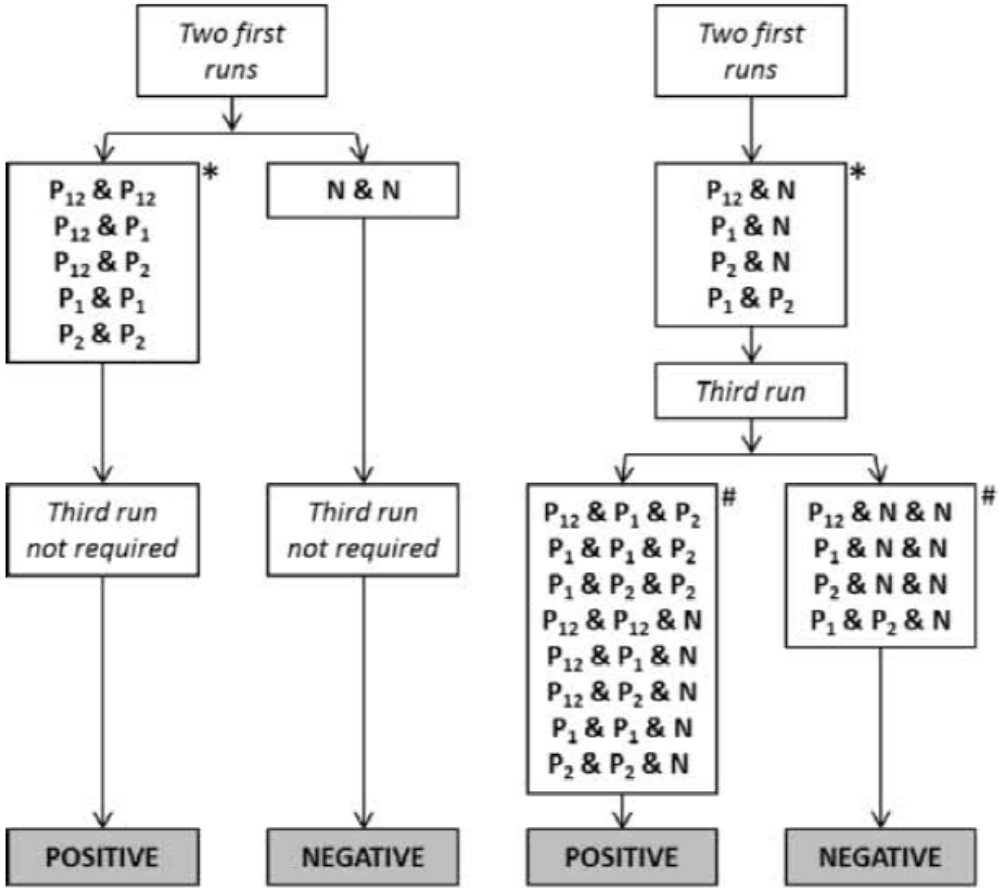

図 1　予測方法

P_1：CD86 のみが陽性の場合、P_2：CD54 のみが陽性の場合、P_{12}：CD86 と CD54 がともに陽性の場合、N：CD86 と CD54 がともに陰性の場合、*：最初の 2 回の測定結果の組み合わせ、#：最初の 2 回の測定結果に基づいて実施された 3 回目の測定結果の組み合わせ

4. 精度

EURL ECVAM により実施されたバリデーション試験[3]には 4 施設（Kao Corporation（主導施設）、Shiseido Quality Assessment Center（主導施設）、EURL ECVAM、Bioassay GmbH）が参加し、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

5 物質（3 感作性物質 : DNCB、Nickel Sulfate、 Phenylacetaldehyde および 2 非感作性物質 : Sodium lauryl sulfate、Lactic acid）のデータ取得を行い、主導施設である Kao と Shiseido から EURL ECVAM と Bioassay の 2 施設への技術移転性について評価が行われ、バリデーション報告書では、技術移転性に問題ないと記載されている。

ただし、OECD 試験法ガイドライン[7]では、本法を使用する前に試験施設において技能習熟用 10 物質および陽性対照、陰性対照、媒体対照を用いて反応性を確認し、技術が習熟していることを示すことを求めている。加えて、陽性対照、陰性対照および媒体対照の測定を継続的に実施し、ヒストリカルなデータベースの作成を求めている。

4-2. 施設内再現性（表 1）

盲検下で評価された 15 物質について、4 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、Kao : 86.7%（一致しなかった物質 : Benzylsalicylate、Methylsalicylate）、Shiseido : 80.0%（一致しなかった物質 : Kathon CG、Beryllium sulfate、Formaldehyde）、Bioassay : 73.3%（一致しなかった物質 : Formaldehyde、Benzylsalicylate、Methylsalicylate、Xylene）、ECVAM : 80.0%（一致しなかった物質 : Chlorpromazine HCl、Benzylcinnamate、Dimethylisophthalate）であった。

表 1　施設内再現性の評価成績

ID	Chemical name	CAS No	GHS potency category	LLNA potency	LLNA	Positve with EC 150 or EC200			
						Kao	Shiseido	Bioassay	ECVAM
10	Kathon CG	26172-55-4	1A	extreme	P	3/3	2/3	3/3	3/3
11	Beryllium sulfate	7787-56-6	1A	extreme	P	0/3	2/3	0/3	3/3
12	Formaldehyde	50-00-0	1A	strong	P	3/3	2/3	2/3	3/3
13	Chloramine T	149358-73-6	1A	strong	P	3/3	3/3	3/3	3/3
14	Chlorpromazine HCl	69-09-0	1A	strong	P	3/3	3/3	3/3	2/3
15	2-mercaptobenzothiazole	149-30-4	1A	moderate	P	3/3	3/3	3/3	3/3
16	Benzylsalicylate	118-58-1	1B	moderate	P	1/3	0/3	1/3	3/3
17	Benzylcinnamate	103-41-3	1B	weak	P	0/3	3/3	0/3	1/3
18	R(+) Limonene	5989-27-5	1B	weak	P	3/3	3/3	3/3	3/3
19	Methylsalicylate	119-36-8	1B	weak	N	2/3	0/3	1/3	3/3
20	Isopropanol	67-63-0	NC	NC	N	0/3	0/3	0/3	0/3
21	Dimethylisophtalate	1459-93-4	NC	NC	N	0/3	0/3	0/3	1/3
22	4-aminobenzoic acid	150-13-0	NC	NC	N	0/3	0/3	0/3	0/3
23	Nickel chloride	7718-54-9	1B	NC (false neg)	N	3/3	3/3	3/3	3/3
24	Xylene	1330-20-7	NC	weak (false pos)	P	0/3	0/3	1/3	3/3
			Number of concordancy			13/15	12/15	11/15	12/15
			Concordancy rate (%)			86.7	80.0	73.3	80.0

本試験では、達成基準を 85%に設定していたが、この基準を上回ったのは 4 施設中 1 施設のみであった。再現性の得られない物質は、UN GHS 1A 分類が 4 物質、1B 分類が 3 物質、分類不可物質が 2 物質の 9 物質であった。また、一つの施設でも偽陰性を示した物質は、UN GHS 1A 分類が 1 物質（Beryllium sulfate）、1B 分類が 3 物質（Benzylsalicylate、Benzylcinnamate、Methylsalicylate）の 4 物質であった。

ただし、OECD 専門家会議において、試験に使用する THP-1 細胞の前培養条件および被験物質の曝露時間をより厳密に管理することにより、施設内再現性の向上が図れることが示され（添付資料）、OECD 試験法ガイドライン [7]の試験手順にその内容が反映されている。

4-3. 施設間再現性（表 2）

盲検下で評価された 24 物質の 4 施設の施設間再現性（4 施設で同じ結果）は 79.2%（一致しなかった物質： Beryllium sulfate、Benzylsalicylate、Benzylcinnamate、Methylsalicylate、Xylene）であり、達成基準 80%を下回った。一致しなかった物質は施設内再現性の試験において一致しなかった 9 物質に含まれる。したがって、施設内再現性と同様に、本法では GHS 1A 分類の物質が適切に判定されない場合のあることが示された。

表 2　施設間再現性の評価成績

ID	Chemical name	CAS No	GHS potency category	LLNA potency	LLNA	Positve with EC 150 or EC200				Between 4 Laboratory Reproducibility
						Kao	Shiseido	Bioassay	ECVAM	
1	Benzoquinone	106-51-4	1A	extreme	P	1/1	1/1	1/1	1/1	Y
2	4-phenylenediamine	106-50-3	1A	strong	P	1/1	1/1	1/1	1/1	Y
3	Dihydroeugenol	2785-87-7	1B	moderate	P	1/1	1/1	1/1	1/1	Y
4	1-thioglycerol	96-27-5	1B	moderate	P	1/1	1/1	1/1	1/1	Y
5	Imidazolidinylurea	39236-46-9	1B	weak	P	1/1	1/1	1/1	1/1	Y
6	Methylmethacrylate	80-62-6	1B	weak	P	0/1	0/1	0/1	0/1	Y
7	Glycerol	56-81-5	NC	NC	N	0/1	0/1	0/1	0/1	Y
8	2,4-dichloronitrobenzene	611-06-3	NC	NC	N	1/1	1/1	1/1	1/1	Y
9	Benzyl alcohol	100-51-6	NC	NC	N	1/1	1/1	1/1	1/1	Y
10	Kathon CG	26172-55-4	1A	extreme	P	3/3	2/3	3/3	3/3	Y
11	Beryllium sulfate	7787-56-6	1A	extreme	P	0/3	2/3	0/3	3/3	N
12	Formaldehyde	50-00-0	1A	strong	P	3/3	2/3	2/3	3/3	Y
13	Chloramine T	149358-73-6	1A	strong	P	3/3	3/3	3/3	3/3	Y
14	Chlorpromazine HCl	69-09-0	1A	strong	P	3/3	3/3	3/3	2/3	Y
15	2-mercaptobenzothiazole	149-30-4	1A	moderate	P	3/3	3/3	3/3	3/3	Y
16	Benzylsalicylate	118-58-1	1B	moderate	P	1/3	0/3	1/3	3/3	N
17	Benzylcinnamate	103-41-3	1B	weak	P	0/3	3/3	0/3	1/3	N
18	R(+) Limonene	5989-27-5	1B	weak	P	3/3	3/3	3/3	3/3	Y
19	Methylsalicylate	119-36-8	1B	weak	N	2/3	0/3	1/3	3/3	N
20	Isopropanol	67-63-0	NC	NC	N	0/3	0/3	0/3	0/3	Y
21	Dimethylisophtalate	1459-93-4	NC	NC	N	0/3	0/3	0/3	1/3	Y
22	4-aminobenzoic acid	150-13-0	NC	NC	N	0/3	0/3	0/3	0/3	Y
23	Nickel chloride	7718-54-9	1B	NC (false neg)	N	3/3	3/3	3/3	3/3	Y
24	Xylene	1330-20-7	NC	weak (false pos)	P	0/3	0/3	1/3	3/3	N
						Number of concordancy				19/24
						Concordancy rate (%)				79.2

本試験は、2 段階で実施されている。1 段階目の ID No. 1-9 の物質による試験は 1 回のみの実施である。2 段階目の ID No. 10-24 の物質を用いた試験は 3 回実施し、2 回あるいは 3 回同じであった結果を採用している。その結果、n=3 で実施した 15 物質での施設間再現性は 66.7%となり、n=1 での評価を含めた 79.2%を下回る結果となった。これは施設内再現性の試験において、n=3 での繰り返しの際、3 回とも同じ結果を示さないケースが 60 回中 12 回（20.0%）含まれることが原因と考えられた。

5. 正確度（感度および特異度）

EURL ECVAMが24物質（感作性物質：非感作性物質＝16：8）で行ったバリデーション試験[2)]では、正確度76%、感度81%、特異度66%であった。この成績は、Ashikagaら[4)]による100物質（感作性物質：非感作性物質＝72：28）の成績正確度84%、感度88%、特異度75%）と比較して低いものであった。

Takenouchiら[5)]が水への溶解性が低い物質が偽陰性を示す影響を調べるために実施した37物質（感作性物質：非感作性物質＝30：7）の結果では、正確度68%、感度70%、特異度57%であり、30の感作性物質のうち9物質が陰性と判定された。この中の2物質は培養液中に沈殿が観察され、5物質では油滴が観察された。この9物質のlog Kowは、3.5以上であった。さらに、Nukadaら[6)]による106物質（感作性物質：非感作性物質＝75：31）の成績と合わせ合計143物質（感作性物質：非感作性物質＝105：38）で検討した結果、正確度は80%、感度は83%、特異度は71%であり、8物質が偽陰性を示した。このうちの5物質は3.5未満のlog Kowであり、そのうち2物質はプロハプテン/プレハプテンであった。また、11物質が偽陽性を示した。

OECD 試験法ガイドライン [7)]には、EURL ECVAM[3)]の 24 物質と、Takenouchi ら [5)]の 143 物質の結果を統合し、142 物質（感作性物質：非感作性物質＝101：41）の成績をもとに、正確度、感度、特異度を求め、それぞれ、85%、93%、66%と表記されている。この中で、両報告で重複する 10 物質については EURL ECVAM[3)]のデータを採用し、Takenouchi ら [5)]のデータからは、この 10 物質と log Kow が 3.5 以上の 14 物質と混合物 1 種を除く 118 物質のデータを採用している。一方、OECD 試験法ガイドライン [7)]の 142 物質に Takenouchi ら [5)]の論文の log Kow が 3.5 以上の 14 物質を加えた 156 物質（感作性物質：非感作性物質＝113：43）での正確度、感度、特異度は、それぞれ 83%、68%、79%となり、感度が低下する傾向がみられた。

6. 評価可能な物質の範囲

OECD 試験法ガイドライン [7)]に記述のある 142 物質による評価では、表 3 に示す通り、様々な化学物質の皮膚感作性の予測が可能であることが示されている。

表 3

Chemical Name	LLNA		h-CLAT			Chemical Name	LLNA		h-CLAT		
	Potency Category	EC3 (%)	Results	CD86	CD54		Potency Category	EC3 (%)	Results	CD86	CD54
Benzo(a)pyrene	Extreme	0.00009	P	+	+	2-Ethylhexyl acrylate	Weak	10	P	+	+
Diphenylcyclopropenone	Extreme	0.003	P	-	+	Amyl cinnamic aldehyde	Weak	11	P	-	+
Oxazolone	Extreme	0.003	P	+	-	2,3-Butanedion	Weak	11	P	+	+
Dinitrofluorobenzene	Extreme	0.03	P	+	+	Citral	Weak	13	P	+	+
Tetrachlorosalicylanilide	Extreme	0.04	P	-	+	Eugenol	Weak	13	P	+	+
Bandrowskis base	Extreme	0.04	P	+	+	Oxalic acid	Weak	15	P	+	-
1-Benzoylacetone	Extreme	0.04	P	-	+	Lyral (CAS No. 31906-04-4)	Weak	17	P	+	+
4-Nitrobenzyl bromide	Extreme	0.05	P	+	+	4-Allylanisole	Weak	18	P	-	+
2,4-Dinitrochlorobenzene	Extreme	0.05	P	+	+	Lilial (CAS No. 80-54-6)	Weak	19	P	-	+
Potassium dichromate	Extreme	0.08	P	+	+	Pentachlorophenol	Weak	20	P	-	+
Beryllium sulfate	Extreme	0.001	N	-	-	Phenyl benzoate	Weak	20	P	+	+
Kathon CG (1.2% CMI)	Extreme	0.009	P	+	+	Cinnamic alcohol	Weak	21	P	+	+
Benzoquinone	Extreme	0.0099	P	+	+	α-iso-methylionone	Weak	21.8	P	-	+
Glutaraldehyde	Strong	0.1	P	+	+	Benzocaine	Weak	22	P	+	-
1,4-Dihydroquinone	Strong	0.11	P	+	-	Geraniol	Weak	26	P	+	-
Phthalic anhydride	Strong	0.16	N	-	-	5-Methyl-2,3-hexanedione	Weak	26	P	+	+
Maleic anhydride	Strong	0.16	P	-	+	2,2'-dihydroxyazobenzene	Weak	27.9	P	+	+
Hexyl salicylate	Strong	0.18	P	-	+	Ethyleneglycol dimethacrylate	Weak	28	P	-	+
Benzyl bromide	Strong	0.2	P	-	+	Penicillin G	Weak	30	P	-	+
Benzyl peroxide	Strong	0.22	N	-	-	Linalool	Weak	30	P	-	+
Lauryl gallate	Strong	0.3	P	+	+	Butyl glycidyl ether	Weak	31	N	-	-
Propyl gallate	Strong	0.32	P	-	+	Hydroxycitronellal	Weak	33	P	+	+
2-Aminophenol	Strong	0.4	P	+	-	Pyridine	Weak	72	P	+	-
2-Nitro-4-phenylenediamine	Strong	0.5	P	-	+	Aniline	Weak	89	P	+	+
Cobalt chloride	Strong	0.6	P	-	+	Nonanoic acid	Weak	21	P	+	-
Chloramine T (CAS No. 149358-73-6)	Strong	0.6	P	+	+	Benzylcinnamate	Weak	18.4	N	-	-
CD-3 (CAS No. 25646-71-3)	Strong	0.6	P	+	+	Imidazolidinylurea	Weak	24	P	+	+
Iodopropynyl butylcarbamate	Strong	0.9	P	-	+	R(+) Limonene	Weak	69	P	-	+
1,2-Dibromo-2,4-dicyanobutane	Strong	0.9	P	+	+	Methylmethacrylate	Weak	90	N	-	-
Chlorpromazine HCl	Strong	0.14	P	-	+	Furil (CAS No. 109-65-9)	Non-sensitizer	ND	P	+	+
4-Phenylenediamine	Strong	0.11	P	+	-	1-Butanol	Non-sensitizer	ND	N	-	-
Chloramine T (CAS No. 127-65-1)	Strong	0.4	P	+	+	1-Iodohexane	Non-sensitizer	ND	P	+	-
Formaldehyde	Strong	0.61	P	+	+	2-Acetylcyclohexanone	Non-sensitizer	ND	P	-	+
Isoeugenol	Moderate	1.2	N	-	-	2-Hydroxypropyl methacrylate	Non-sensitizer	ND	N	-	-
1-Naphtol	Moderate	1.3	P	+	+	4-Hydroxybenzoic acid	Non-sensitizer	ND	N	-	-
1-Phenyl-1,2-propanedione	Moderate	1.3	P	+	+	6-Methylcoumarin	Non-sensitizer	ND	N	-	-
2-Hydroxyethyl acrylate	Moderate	1.4	P	+	+	Acetanisole	Non-sensitizer	ND	N	-	-
Glyoxal	Moderate	1.4	P	-	+	Benzalchonium chloride	Non-sensitizer	ND	N	-	-
Bisphenol A-diglycidyl ether	Moderate	1.5	P	+	+	Benzaldehyde	Non-sensitizer	ND	P	+	+
Vinyl pyridine	Moderate	1.6	P	+	-	Benzoic acid	Non-sensitizer	ND	N	-	-
2-Methyl-2H-isothiazolone	Moderate	1.9	P	+	+	Chlorobenzene	Non-sensitizer	ND	P	+	-
3-Dimethylaminopropylamine	Moderate	2.2	P	+	+	Clofibrate	Non-sensitizer	ND	P	-	+
Ethylene diamine	Moderate	2.2	P	+	-	Coumarin	Non-sensitizer	ND	N	-	-
1,2-Benzisothiazoline-3-one	Moderate	2.3	P	-	+	Dextran	Non-sensitizer	ND	N	-	-
Methyl-2-nonynoate	Moderate	2.5	P	+	-	Diethylphthalate	Non-sensitizer	ND	P	-	+
Phenylacetaldehyde	Moderate	3.0	P	+	+	Dimethyl formamide	Non-sensitizer	ND	N	-	-
Cinamic aldehyde	Moderate	3.0	P	+	+	Ethyl benzoylacetate	Non-sensitizer	ND	N	-	-
3-Aminophenol	Moderate	3.2	P	-	+	Ethyl vanillin	Non-sensitizer	ND	N	-	-
Diethyl sulphate	Moderate	3.3	P	+	-	Furil (CAS No. 492-94-4)	Non-sensitizer	ND	N	-	-
3-Propylidenephthalide	Moderate	3.7	P	+	+	Kanamycin	Non-sensitizer	ND	N	-	-
Benzylidene acetone	Moderate	3.7	P	+	+	Lactic acid	Non-sensitizer	ND	N	-	-
2,4-Heptadienal	Moderate	4.0	P	+	+	Octanoic acid	Non-sensitizer	ND	P	-	+
5-Methyl-2-phenyl-2-hexanal	Moderate	4.4	P	-	+	Propyl paraben	Non-sensitizer	ND	P	+	+
Alpha-methyl cinnamic aldehyde	Moderate	4.5	P	+	+	Propylene glycol	Non-sensitizer	ND	N	-	-
Nickel sulfate	Moderate	4.8	P	+	+	Saccharin	Non-sensitizer	ND	N	-	-
Tetramethylthiuramdisulfide	Moderate	5.2	P	+	+	Salicylic acid	Non-sensitizer	ND	P	-	+
Trans-2-hexenal	Moderate	5.5	P	+	+	Streptomycin sulfate	Non-sensitizer	ND	N	-	-
Resorcinol	Moderate	5.5	P	-	+	Sulfanilamide	Non-sensitizer	ND	N	-	-
3,4-Dihydrocoumarin	Moderate	5.6	P	-	+	Tween 80	Non-sensitizer	ND	N	-	-
Diethyl maleate	Moderate	5.8	P	+	-	Vanillin	Non-sensitizer	ND	N	-	-
2-Methoxy-4-methyl-phenol	Moderate	5.8	P	+	+	Zinc sulfate	Non-sensitizer	ND	P	-	+
Diethylenetriamine	Moderate	5.8	N	-	-	Glycerol	Non-sensitizer	ND	N	–	–
2-Phenylpropionaldehyde	Moderate	6.3	P	+	+	2,4-Dichloronitrobenzene	Non-sensitizer	ND	P	+	+
4-Chloroaniline	Moderate	6.5	P	+	-	Benzyl alcohol	Non-sensitizer	ND	P	+	–
β-damascone	Moderate	6.7	P	+	+	Methylsalicylate	Non-sensitizer	ND	P	–	+
Perillaaldehyde	Moderate	8.1	P	+	-	Isopropanol	Non-sensitizer	ND	N	-	-
Trimellitic anhydride	Moderate	9.2	P	+	-	Dimethylisophthalate	Non-sensitizer	ND	N	-	-
2-Mercaptobenzothiazole	Moderate	1.7	P	–	+	4-Aminobenzoic acid	Non-sensitizer	ND	N	-	-
Benzylsalicylate	Moderate	2.9	N	–	–	Sodium lauryl sulfate	Others	14	N	-	-
1-Thioglycerol	Moderate	3.6	P	+	–	Nickel chloride	Others	ND	P	+	+
Dihydroeugenol	Moderate	6.8	P	+	+	Xylene	Others	95.8	N	–	–

: Takenouchiらの論文（2013）からの引用
: h-CLAT Validation Study Report (2012)からの引用

7. 有用性と限界

本法は細胞実験とフローサイトメトリーを組み合わせた試験法であり、細胞培養機器およびフローサイトメーターを保有し、その技術に習熟した施設であれば実施可能と思われる。また、本法は動物を用いない *in vitro* の手法であり、科学的目的

のために実施される動物実験に関し、「動物の愛護及び管理に関する法律」および 3R の精神と合致している。さらに h-CLAT および LLNA 実施の際に必要となる消耗品費は、LLNA では 1 アッセイ当たり約 10 万円であるのに対し、h-CLAT では約 2 万円であり 1/5 程度の経費で実施可能と試算された。試験期間も LLNA より短期間で実施可能であることから、試験法として迅速性・経済性の面から有用と思われる。

技術的限界として、液相での反応を必要とする試験系であるため、生理食塩液あるいは培地に 100 mg/mL、これらに所定の濃度で不溶の場合は、DMSO に 500 mg/mL の濃度で溶解あるいは安定的に分散する必要がある（ただし、科学的根拠があれば他の溶媒も使用可能とされている）。

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

物性に関する適用限界としては log Kow が 3.5 を超える物質は偽陰性を生じやすい傾向にあるが、該当物質による陽性の結果はある程度信頼できることが報告されている。

本細胞の代謝能は限定的であるため、プロハプテン（P450 等による代謝活性化を必要とする物質）やプレハプテン（酸化により活性化される物質）は偽陰性を生じる可能性がある。

バリデーション試験で評価した金属塩では United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals （GHS） 1A に分類される Beryllium sulfate が、偽陰性となったが、既報[4]によると Potassium dichromate、Cobalt chloride、Nickel sulfate は正しく評価されているため、金属塩を適用範囲外とする根拠はない。

本法による偽陰性の判定は、強度感作性物質（UN GHS 1A 分類）よりも軽度～中等度感作性物質（UN GHS 1B 分類）で生じやすい傾向にある。これらの結果から、陽性と判定された場合は感作性陽性と判断することは可能と考えるが、1 割程度は偽陽性の可能性があることに留意する必要がある。

また、本法は技術的には混合物にも応用可能とされているが、混合物での実験例は極めて限られている。

従って、現時点では以下の物質群への適用性は明確でないと考える。

－試験溶媒に溶解しない物質および試験溶媒中で不安定な物質

－プロハプテン、プレハプテン

－揮発性物質

－FITC や PI と同じ波長域に強い蛍光を有する物質

なお、バリデーション試験の成績からは、本法の感作性強度分類や UN GHS のサブカテゴリー分類への応用には適さないと考えられる。

8. 結論

h-CLAT は、感作性発現機序における第三段階のイベントである樹状細胞が活性化する際の細胞表面分子の発現亢進を利用した *in vitro* 試験法であり、化学物質の感作性を判断する上で重要な情報を与えてくれる。

マウスを用いる LLNA の 1/5 程度の経費で実施可能と試算され、試験期間も LLNA に比べ短期であるため、有用性は高いと思われる。しかしながら、細胞密度と培養時間を厳守すること、公比 1.2 の濃度設定が必要なため、DPRA や ARE-Nrf2 Luciferase Test Method に比べて、操作が煩雑であり、制約が多い。また、陽性対照、陰性対照および媒体対照の測定を継続的に実施し、ヒストリカルなデータベースを作成することが必要とされている。

本試験法のバリデーション試験において、15 物質を用いて実施された施設内再現性は、73.3～86.7%であり、全般的に高くなかった。ただし、OECD 専門家会議において、試験に使用する THP-1 細胞の前培養条件および被験物質の曝露時間をより厳密に管理することにより、施設内再現性の向上が図れることが示され、OECD 試験法ガイドラインの試験手順にその内容が反映されていることから、施設内再現性の向上が期待できると考える。一方、24 物質を用いて実施された施設間再現性は、79.2%であった。

OECD 試験法ガイドラインでは、142 物質の成績から正確度 85%、感度 93%、特異度 66%と記載されているが、log Kow が 3.5 以上で陰性と判定された物質を除いた上での数値である。また、OECD 試験法ガイドラインの 142 物質に log Kow が 3.5 以上の 14 物質を加えた 156 物質の正確度、感度、特異度は、それぞれ 83%、68%、79%となり、感度が低下する傾向がみられた。したがって、特に log Kow が 3.5 以上の物質の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と考える。また、特異度が 66%であるため偽陽性と判定することも留意する必要がある。

さらに、UN GHS 分類の物質で、軽度感作性物質では偽陰性が生じやすく、強度感作性物質ではその比率は低い。このことから、本試験法は、物質の感作性強度分類や UN GHS のサブカテゴリー分類への応用には適さないと考える。また、本試験法に用いる細胞の代謝能は限定的であるため、活性化に代謝系を必要とする化学物質では、正しくその感作性が検出されない可能性がある。

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

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2015.10.14

Supplemental explanation for the h-CLAT draft guideline

Kao and Shiseido

Potential Source of Variability in h-CLAT

1. Variability on cell seeding conditions in pre-culture
2. Variability on exposure time of test chemicals
3. Technical proficiency for laboratories that are not experienced with the h-CLAT

2

Cell Seeding Condition in Pre-culture

➢ **Original pre-culture condition**

Seeded at a density between
0.1×10^6 and 0.2×10^6 cells/mL

48 or 72 hours

Collect cells from culture flask, Prepare cell suspension at 2 x 10^6 cells/mL for the assay

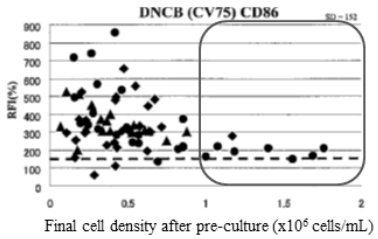
➢ **Potential variability**

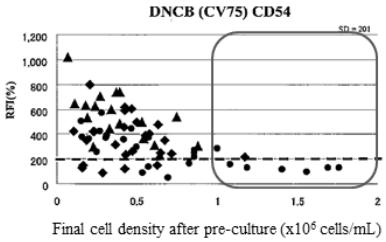
- In general, cell density may affect the state of cell differentiation, which, in turn, could be a source of variability in cell-based assays.
- In the h-CLAT, the cell density in the culture flask after pre-culture may affect the variability of CD86/CD54 expression induced by allergens (Mizuno et al, 2008).

3

Cell Seeding Condition in Pre-culture

➢ **Effect on final cell density in culture flask after pre-culture**
(Mizuno et al., 2008, AATEX)



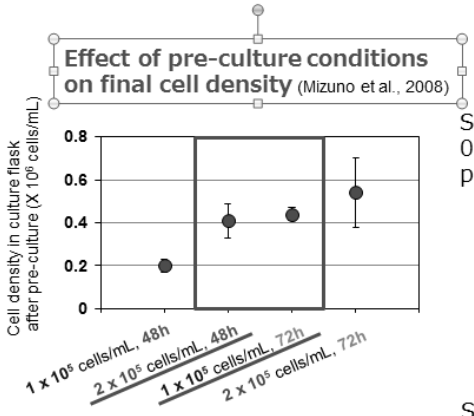


- CD86/CD54 expression levels induced by sensitizer are changed depending on the final cell density just after pre-culture.
- These results indicated that cell density should not exceed 1.0×10^6 cells/mL. (→ Already mentioned in the SOP)
- Variability of cell seeding condition in pre-culture could be a source of variability of h-CLAT.

4

Solution to Cell Seeding Condition in Pre-culture

To ensure high consistency within and between laboratories in the pre-culturing of THP-1 cells before testing



Seeded at a density between 0.1×10^6 and 0.2×10^6 cells/mL and pre-cultured for 48 or 72 hours

Seeded at a density of either 0.1×10^6 or 0.2×10^6 cells/mL, and pre-cultured for 72 or 48 hours, respectively

5

Solution to Cell Seeding Condition in Pre-culture

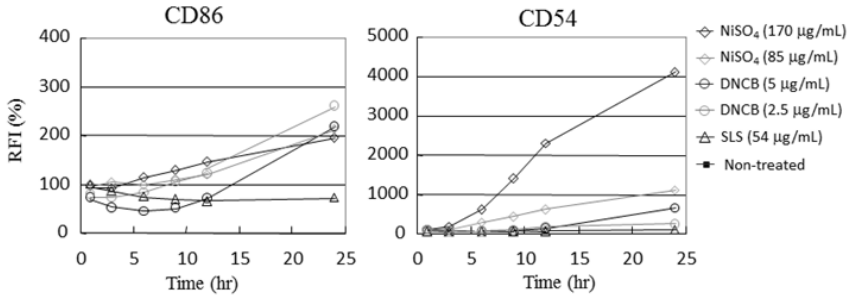
Revised pre-culture condition;

"For testing, **THP-1 cells are seeded at a density of either 0.1 × 10^6 cells/mL or 0.2 × 10^6 cells/mL, and pre-cultured in culture flasks for 72 or 48 hours, respectively**. It is important that the cell density in the culture flask just after the pre-culture period be as consistent as possible in each experiment (by using one of the two pre-culture conditions described above), because the cell density in the culture flask just after pre-culture could affect the variability of CD86/CD54 expression induced by allergens (26)."

6

Variability on Exposure Time of Test Chemicals

➢ **Time-dependency of CD86/54 expression induced by sensitizers**
(Miyazawa et al., 2008, J. Toxicol. Sci., Ashikaga et al., 2006, TIV)



- CD86/CD54 expression induced by a sensitizer would be changed depending on the exposure time.
- Exposure time of test chemicals as **24±0.5 hours** should be strictly controlled.

7

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Vitro Skin Sensitisation: human Cell Line Activation Test (h-CLAT)

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) describes the *in vitro* procedure called human Cell Line Activation test (h-CLAT), 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 current knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised in the form of an Adverse Outcome Pathway (AOP) (2), starting with the molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. In this instance, the molecular initiating event (i.e. the first key event) is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells (DC), typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation, which is indirectly assessed in the murine Local Lymph Node Assay (LLNA) (3).

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

4. More recently mechanistically-based *in chemico* (OECD TG 442C; Direct Peptide Reactivity Assay addressing the first key event of the skin sensitisation AOP) (7) and *in vitro* (OECD TG 442D; ARE-Nrf2 Luciferase Test Method addressing the second key event of the skin sensitisation AOP) (8) test methods have been adopted for contributing to the evaluation of the skin sensitisation hazard potential of chemicals. However, a combination of non-animal methods (*in silico*, *in chemico*, *in vitro*) within Integrated

This Guideline was adopted by the OECD Council by written procedure on 29 July 2016 [C(2016)103].

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)(9).

5. The h-CLAT method is proposed to address the third key event of the skin sensitisation AOP by quantifying changes in the expression of cell surface markers associated with the process of activation of monocytes and DC (i.e. CD86 and CD54), in the human monocytic leukaemia cell line THP-1, following exposure to sensitisers (10). The measured expression levels of CD86 and CD54 cell surface markers are then used for supporting the discrimination between skin sensitisers and non-sensitisers.

6. The h-CLAT method has been evaluated in a European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)-coordinated validation study and subsequent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC). Considering all available evidence and input from regulators and stakeholders, the h-CLAT was recommended by EURL ECVAM (11) to be used as part of an IATA to support the discrimination between sensitisers and non-sensitisers for the purpose of hazard classification and labelling. Examples of the use of h-CLAT data in combination with other information are reported in the literature (12) (13) (14) (15) (16) (17) (18) (19).

7. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS AND LIMITATIONS

8. Skin sensitisers have been reported to induce the expression of cell membrane markers associated with DC activation (2). Test methods such as the h-CLAT which measure markers of monocyte activation and may be related to DC activation (20) are therefore considered relevant for the assessment of the skin sensitisation potential of chemicals. However, since DC activation represents only one key event of the skin sensitisation AOP, information generated with test methods measuring markers of DC activation may not be sufficient on its own to conclude on the absence of skin sensitisation potential of chemicals. Therefore, data generated with the h-CLAT method should be considered in the context of integrated approaches, such as IATA, and combined 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.

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 Test Guideline cannot be used on its own, neither to sub-categorise skin sensitisers into subcategories 1A and 1B as defined by UN GHS (1), for authorities implementing these two optional subcategories, nor to predict potency for safety assessment decisions. However, depending on the regulatory framework, a positive result with the h-CLAT may be used on its own to classify a chemical into UN GHS category 1.

10. The h-CLAT method proved to be transferable to laboratories experienced in cell culture techniques and flow cytometry analysis. The level of reproducibility in predictions that can be expected from the test method is in the order of 80% within and between laboratories (11) (21). Results generated in the validation study (22) and other published studies (23) overall indicate that, compared with LLNA results, the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers is 85% (N=142) with a sensitivity of 93% (94/101) and a specificity of 66% (27/41) (based on a re-analysis by EURL ECVAM (21) considering all existing data and not considering negative results for chemicals with a Log Kow greater than 3.5 as described in paragraph 12). False negative predictions with the h-CLAT are more likely to concern chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS

subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A) (12) (22) (24). Taken together, this information indicates the usefulness of the h-CLAT method to contribute to the identification of skin sensitisation hazards. However, the accuracy values given here for the h-CLAT as a stand-alone test method are only indicative, since the test method should be considered in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraph 9 above. Furthermore, when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in humans.

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 h-CLAT to the testing of mono-constituent substances, multi-constituent substances and/or mixtures. On the basis of the data currently available, the h-CLAT method was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined in *in vivo* studies) and physicochemical properties (11) (23) (24). Limited information is currently available on the applicability of the h-CLAT method to multi-constituent substances/mixtures (24). 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[2]. Such considerations are not needed when there is a regulatory requirement for the 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.

12. The h-CLAT method is applicable to test chemicals soluble or that form a stable dispersion (i.e. a colloid or suspension in which the test chemical does not settle or separate from the solvent/vehicle into different phases) in an appropriate solvent/vehicle (see paragraph 21). Test chemicals with a Log Kow greater than 3.5 tend to produce false negative results (23). Therefore negative results with test chemicals with a Log Kow greater than 3.5 should not be considered. However, positive results obtained with test chemicals with a Log Kow greater than 3.5 could still be used to support the identification of the test chemical as a skin sensitiser. Furthermore, because of the limited metabolic capability of the cell line used (25) and because of the experimental conditions, pro-haptens (i.e. substances requiring enzymatic activation for example via P450 enzymes) and pre-haptens (i.e. substances activated by oxidation) in particular with a slow oxidation rate may also provide negative results in the h-CLAT (24). Fluorescent test chemicals can be assessed with the h-CLAT (26), nevertheless, strong fluorescent test chemicals emitting at the same wavelength as fluorescein isothiocyanate (FITC) or as propidium iodide (PI), will interfere with the flow cytometric detection and thus cannot be correctly evaluated using FITC-conjugated antibodies or PI. In such a case, other fluorochrome-tagged antibodies or other cytotoxicity markers, respectively, can be used as long as it can be shown they provide similar results as the FITC-tagged antibodies (see paragraph 31) or PI (see paragraph 25) e.g. by testing the proficiency substances in Annex II. In the light of the above, negative results should be interpreted in the context of the stated limitations and together with other information sources within the framework of IATA. In cases where there is evidence demonstrating the non-applicability of the h-CLAT method to other specific categories of test chemicals, it should not be used for those specific categories.

13. As described above, the h-CLAT method supports the discrimination between skin sensitisers from non-sensitisers. However, it may also potentially contribute to the assessment of sensitising potency (12) (13) (17) when used in integrated approaches such as IATA. Nevertheless, further work, preferably based

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

2 This sentence was proposed and agreed at the April 2014 WNT meeting.

on human data, is required to determine how h-CLAT results may possibly inform potency assessment.

PRINCIPLE OF THE TEST

14. The h-CLAT method is an *in vitro* assay that quantifies changes of cell surface marker expression (i.e. CD86 and CD54) on a human monocytic leukemia cell line, THP-1 cells, following 24 hours exposure to the test chemical. These surface molecules are typical markers of monocytic THP-1 activation and may mimic DC activation, which plays a critical role in T-cell priming. The changes of surface marker expression are measured by flow cytometry following cell staining with fluorochrome-tagged antibodies. Cytotoxicity measurement is also conducted concurrently to assess whether upregulation of surface marker expression occurs at sub-cytotoxic concentrations. The relative fluorescence intensity of surface markers compared to solvent/vehicle control are calculated and used in the prediction model (see paragraph 33), to support the discrimination between sensitisers and non-sensitisers

DEMONSTRATION OF PROFICIENCY

15. Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency, using the 10 Proficiency Substances listed in Annex II. Moreover, test method users should maintain an historical database of data generated with the reactivity checks (see paragraph 18) and with the positive and solvent/vehicle controls (see paragraphs 27-29), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

PROCEDURE

16. This Test Guideline is based on the h-CLAT DataBase service on ALternative Methods to animal experimentation (DB-ALM) protocol no. 158 (27) which represents the protocol used for the EURL ECVAM-coordinated validation study. It is recommended that this protocol is used when implementing and using the h-CLAT method in the laboratory. The following is a description of the main components and procedures for the h-CLAT method, which comprises two steps: *dose finding assay* and *CD86/CD54 expression measurement*.

Preparation of cells

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

18. THP-1 cells are cultured, at 37°C under 5% CO_2 and humidified atmosphere, in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, 100 units/mL penicillin and 100 µg/mL streptomycin. The use of penicillin and streptomycin in the culture medium can be avoided. However, in such a case users should verify that the absence of antibiotics in the culture medium has no impact on the results, for example by testing the proficiency substances listed in Annex II. In any case, in order to minimise the risk of contamination, good cell culture practices should be followed independently of the presence or not of antibiotics in the cell culture medium. THP-1 cells are routinely seeded every 2-3 days at the density of 0.1 to 0.2 × 10^6 cells/mL. They should be maintained at densities from 0.1 to 1.0 × 10^6 cells/mL. Prior to using them for testing, the cells should be qualified by conducting a

reactivity check. The reactivity check of the cells should be performed using the positive controls, 2,4-dinitrochlorobenzene (DNCB) (CAS n. 97-00-7, ≥ 99% purity) and nickel sulfate ($NiSO_4$) (CAS n. 10101-97-0, ≥ 99% purity) and the negative control, lactic acid (LA) (CAS n. 50-21-5, ≥ 85% purity), two weeks after thawing. Both DNCB and $NiSO_4$ should produce a positive response of both CD86 and CD54 cell surface markers, and LA should produce a negative response of both CD86 and CD54 cell surface markers. Only the cells which passed the reactivity check are to be used for the assay. Cells can be propagated up to two months after thawing. Passage number should not exceed 30. The reactivity check should be performed according to the procedures described in paragraphs 27-31.

19. For testing, THP-1 cells are seeded at a density of either 0.1×10^6 cells/mL or 0.2×10^6 cells/mL, and pre-cultured in culture flasks for 72 hours or for 48 hours, respectively. It is important that the cell density in the culture flask just after the pre-culture period be as consistent as possible in each experiment (by using one of the two pre-culture conditions described above), because the cell density in the culture flask just after pre-culture could affect the CD86/CD54 expression induced by allergens (28). On the day of testing, cells harvested from culture flask are resuspended with fresh culture medium at 2×10^6 cells/mL. Then, cells are distributed into a 24 well flat-bottom plate with 500 µL (1×10^6 cells/well) or a 96-well flat-bottom plate with 80 µL (1.6×10^5 cells/well).

Dose finding assay

20. A *dose finding assay* is performed to determine the CV75, being the test chemical concentration that results in 75% cell viability (CV) compared to the solvent/vehicle control. The CV75 value is used to determine the concentration of test chemicals for the *CD86/CD54 expression measurement* (see paragraphs 27-31).

Preparation of test chemicals and control substances

21. The test chemicals and control substances are prepared on the day of testing. For the h-CLAT method, test chemicals are dissolved or stably dispersed (see also paragraph 12) in saline or medium as first solvent/vehicle options or dimethyl sulfoxide (DMSO, ≥ 99% purity) as a second solvent/vehicle option if the test chemical is not soluble or does not form a stable dispersion in the previous two solvents/vehicles, to final concentrations of 100 mg/mL (in saline or medium) or 500 mg/mL (in DMSO). Other solvents/vehicles than those described above may be used if sufficient scientific rationale is provided. Stability of the test chemical in the final solvent/vehicle should be taken into account.

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

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

The working solutions are finally used for exposure by adding an equal volume of working solution to the volume of THP-1 cell suspension in the plate (see also paragraph 24) to achieve a further two-fold dilution (usually, the final range of concentrations in the plate is 7.81–1000 µg/mL).

23. The solvent/vehicle control used in the h-CLAT method is culture medium (for test chemicals solubilised or stably dispersed (see paragraph 12) either with medium or saline) or DMSO (for test chemicals solubilised or stably dispersed in DMSO) tested at a single final concentration in the plate of 0.2%. It undergoes the same dilution as described for the working solutions in paragraph 22.

Application of test chemicals and control substances

24. The culture medium or working solutions described in paragraphs 22 and 23 are mixed 1:1 (v/v) with the cell suspensions prepared in the 24-well or 96-well flat-bottom plate (see paragraph 19). The treated plates are then incubated for 24±0.5 hours at 37°C under 5% CO_2. Care should be taken to avoid evaporation of volatile test chemicals and cross-contamination between wells by test chemicals, e.g. by sealing the plate prior to the incubation with the test chemicals (29).

Propidium iodide (PI) staining

25. After 24±0.5 hours of exposure, cells are transferred into sample tubes and collected by centrifugation. The supernatants are discarded and the remaining cells are resuspended with 600 µL (in case of 96-well) or 1mL (in case of 24-well) of a phosphate buffered saline containing 0.1% bovine serum albumin (staining buffer). 200 µL of cell suspension is transferred into 96-well round-bottom plate (in case of 96-well) or micro tube (in case of 24-well) and washed twice with 200 µL (in case of 96-well) or 1mL (in case of 24-well) of staining buffer. Finally, cells are resuspended in staining buffer (e.g. 400 µL) and PI solution (e.g. 20 µL) is added (for example, final concentration of PI is 0.625 µg/mL). Other cytotoxicity markers, such as 7-Aminoactinomycin D (7-AAD), Trypan blue or others may be used if the alternative stains can be shown to provide similar results as PI, for example by testing the proficiency substances in Annex II.

Cytotoxicity measurement by flow cytometry and estimation of CV75 value

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

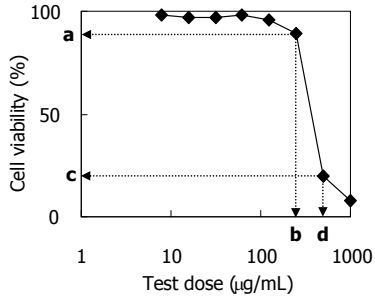
$$\textbf{Cell Viability} = \frac{\textbf{Number of living cells}}{\textbf{Total Number of acquired cells}} \times \textbf{100}$$

The CV75 value (see paragraph 20), i.e. a concentration showing 75% of THP-1 cell survival (25% cytotoxicity), is calculated by log-linear interpolation using the following equation:

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

Where:

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



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

CD86/CD54 expression measurement

Preparation of the test chemicals and control substances

27. The appropriate solvent/vehicle (saline, medium or DMSO; see paragraph 21) is used to dissolve or stably disperse the test chemicals. The test chemicals are first diluted to the concentration corresponding to 100-fold (for saline or medium) or 500-fold (for DMSO) of the 1.2 × CV75 determined in the *dose finding assay* (see paragraph 26). If the CV75 cannot be determined (i.e. if sufficient cytotoxicity is not observed in the *dose finding assay*), the highest soluble or stably dispersed concentration of test chemical prepared with each solvent/vehicle should be used as starting concentration. Please note that the final concentration in the plate should not exceed 5000 µg/mL (in case of saline or medium) or 1000 µg/mL (in case of DMSO). Then, 1.2-fold serial dilutions are made using the corresponding solvent/vehicle to obtain the stock solutions (eight concentrations ranging from 100×1.2 × CV75 to 100×0.335 × CV75 (for saline or medium) or from 500×1.2 × CV75 to 500×0.335 × CV75 (for DMSO)) to be tested in the h-CLAT method (see DB-ALM protocol N_O. 158 for an example of dosing scheme). The stock solutions are then further diluted 50-fold (for saline or medium) or 250-fold (for DMSO) into the culture medium (working solutions). These working solutions are finally used for exposure with a further final two-fold dilution factor in the plate. If the results do not meet the acceptance criteria described in the paragraphs 35 and 36 regarding cell viability, the *dose finding assay* may be repeated to determine a more precise CV75. Please note that only 24-well plates can be used for CD86/CD54 expression measurement.

28. The solvent/vehicle control is prepared as described in paragraph 23. The positive control used in the h-CLAT method is DNCB (see paragraph 18), for which stock solutions are prepared in DMSO and diluted as described for the stock solutions in paragraph 27. DNCB should be used as the positive control for *CD86/CD54 expression measurement* at a final single concentration in the plate (typically 4.0 µg/mL). To obtain a 4.0 µg/mL concentration of DNCB in the plate, a 2 mg/mL stock solution of DNCB in DMSO is prepared and further diluted 250-fold with culture medium to a 8 µg/mL working solution. Alternatively, the CV75 of DNCB, which is determined in each test facility, could be also used as the positive control concentration. Other suitable positive controls may be used if historical data are available to derive comparable run acceptance criteria. For positive controls, the final single concentration in the plate should not exceed 5000 µg/mL (in case of saline or medium) or 1000 µg/mL (in case of DMSO). The run acceptance criteria are the same as those described for the test chemical (see paragraph 35), except for the last acceptance criterion since the positive control is tested at a single concentration.

Application of test chemicals and control substances

29. For each test chemical and control substance, one experiment is needed to obtain a prediction. Each experiment consists of at least two independent runs for *CD86/CD54 expression measurement* (see paragraphs 33 and 34). Each independent run is performed on a different day or on the same day provided that for each run: a) independent fresh stock solutions and working solutions of the test chemical and antibody solutions are prepared and b) independently harvested cells are used (i.e. cells are collected from different culture flasks); however, cells may come from the same passage. Test chemicals and control substances prepared as working solutions (500 µL) are mixed with 500 µL of suspended cells ($1x10^6$ cells) at 1:1 ratio, and cells are incubated for 24±0.5 hours as described in paragraphs 27 and 28. In each run, a single replicate for each concentration of the test chemical and control substance is sufficient because a prediction is obtained from at least two independent runs.

Cell staining and analysis

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

31. After centrifugation, cells are stained with 50 µL of FITC-labelled anti-CD86, anti-CD54 or mouse IgG1 (isotype) antibodies at 4°C for 30 min. The antibodies described in the h-CLAT DB-ALM protocol no. 158 (27) should be used by diluting 3:25 (v/v, for CD86 (BD-PharMingen, #555657; Clone: Fun-1)) or 3:50 (v/v, for CD54 (DAKO, #F7143; Clone: 6.5B5) and IgG1 (DAKO, #X0927)) with staining buffer. These antibody dilution factors were defined by the test method developers as those providing the best signal-to-noise ratio. Based on the experience of the test method developers, the fluorescence intensity of the antibodies is usually consistent between different lots. However, users may consider titrating the antibodies in their own laboratory's conditions to define the best concentrations for use. Other fluorochrome-tagged anti-CD86 and/or anti-CD54 antibodies may be used if they can be shown to provide similar results as FITC-conjugated antibodies, for example by testing the proficiency substances in Annex II. It should be noted that changing the clone or supplier of the antibodies as described in the h-CLAT DB-ALM protocol no. 158 (27) may affect the results. After washing with 200 µL of staining buffer three times, cells are resuspended in staining buffer (e.g. 400 µL), and the PI solution (e.g. 20 µL to obtain a final concentration of 0.625 µg/mL) or another cytotoxicity marker's solution (see paragraph 25) is added. The expression levels of CD86 and CD54, and cell viability are analysed using flow cytometry.

DATA AND REPORTING

Data evaluation

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

$$\mathbf{RFI} = \frac{\mathbf{MFI\ of\ chemical\text{-}treated\ cells - MFI\ of\ chemical\text{-}treated\ isotype\ control\ cells}}{\mathbf{MFI\ of\ solvent/vehicle\text{-}treated\ ctrl\ cells - MFI\ of\ solvent/vehicle\text{-}treated\ isotype\ ctrl\ cells}} \mathbf{x100}$$

The cell viability from the isotype control (ctrl) cells (which are stained with mouse IgG1 (isotype) antibodies) is also calculated according to the equation described in paragraph 26.

Prediction model

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

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

Based on the above, if the first two runs are both positive for CD86 and/or are both positive for CD54, the h-CLAT prediction is considered POSITIVE and a third run does not need to be conducted. Similarly, if the first two runs are negative for both markers, the h-CLAT prediction is considered NEGATIVE (with due consideration of the provisions of paragraph 36) without the need for a third run. If however, the first two runs are not concordant for at least one of the markers (CD54 or CD86), a third run is needed and the final prediction will be based on the majority result of the three individual runs (i.e. 2 out of 3). In this respect, it should be noted that if two independent runs are conducted and one is only positive for CD86 (hereinafter referred to as P_1) and the other is only positive for CD54 (hereinafter referred to as P_2), a third run is required. If this third run is negative for both markers (hereinafter referred to as N), the h-CLAT prediction is considered NEGATIVE. On the other hand, if the third run is positive for either marker (P_1 or P_2) or for both markers (hereinafter referred to as P_{12}), the h-CLAT prediction is considered POSITIVE.

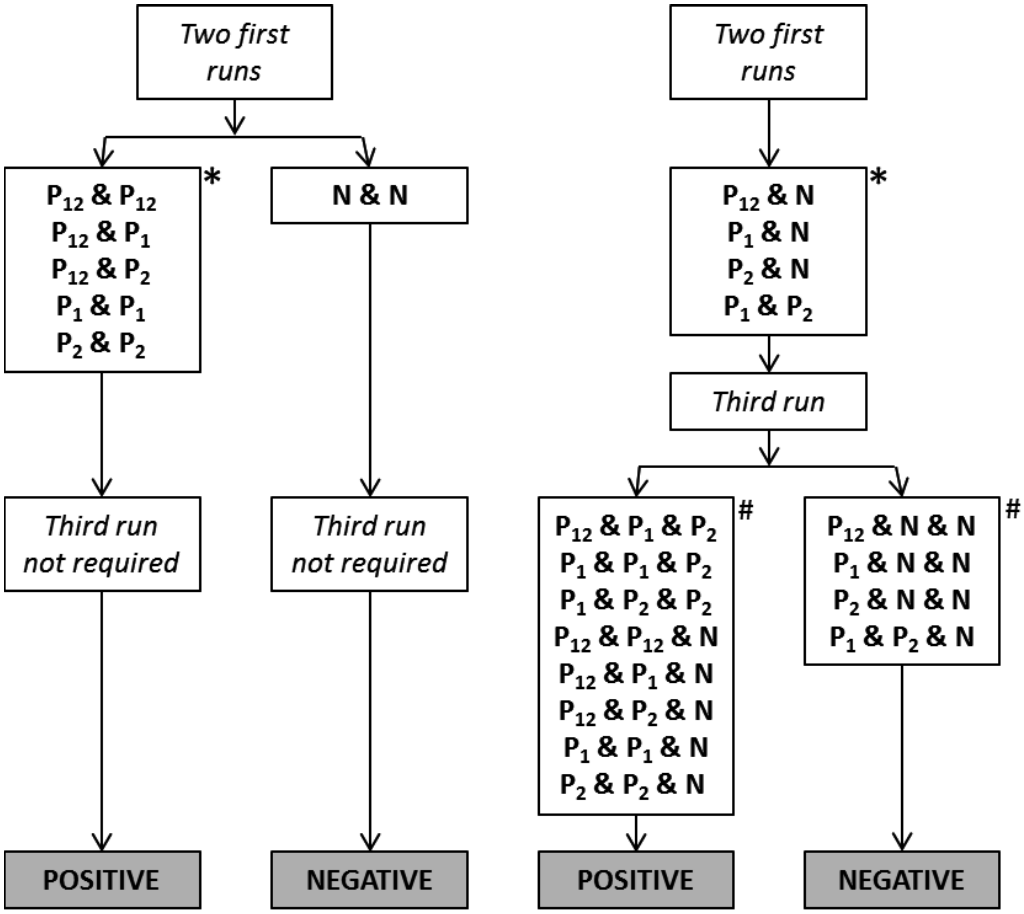


Figure 1: Prediction model used in the h-CLAT test method. An h-CLAT prediction should be considered in the framework of an IATA and in accordance with the provision of paragraphs 9, 11 and 12. P_1: run with only CD86 positive; P_2; run with only CD54 positive; P_{12}: run with both CD86 and CD54 positive; N: run with neither CD86 nor CD54 positive. *The boxes show the relevant combinations of results from the first two runs, independently of the order in which they may be obtained. #The boxes show the relevant combinations of results from the three runs on the basis of the results obtained in the first two runs shown in the box above, but do not reflect the order in which they may be obtained.

34. For the test chemicals predicted as POSITIVE with the h-CLAT, optionally, two Effective Concentrations (EC) values, the EC150 for CD86 and EC200 for CD54, i.e. the concentration at which the test chemicals induced a RFI of 150 or 200, may be determined. These EC values potentially could contribute to the assessment of sensitising potency (3) when used in integrated approaches such as IATA (12) (13) (14) (15) (16). They can be calculated by the following equations:

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

$$\mathbf{EC200\ (for\ CD54) = B_{concentration} + [(200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{concentration} - B_{concentration})]}$$

where

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

B_{RFI} is the RFI at the highest concentration with RFI < 150 (CD86) or 200 (CD54)

For the purpose of more precisely deriving the EC150 and EC200 values, three independent runs for *CD86/CD54 expression measurement* may be required. The final EC150 and EC200 values are then determined as the median value of the ECs calculated from the three independent runs. When only two of three independent runs meet the criteria for positivity (see paragraph 33), the higher EC150 or EC200 of the two calculated values is adopted.

Acceptance criteria

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

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

- In the solvent/vehicle control, RFI values of both CD86 and CD54 should not exceed the positive criteria (CD86 RFI ≥ 150% and CD54 RFI ≥ 200%). RFI values of the solvent/vehicle control are calculated by using the formula described in paragraph 32 ("MFI of chemical" should be replaced with "MFI of solvent/vehicle", and "MFI of solvent/vehicle" should be replaced with "MFI of (medium) control").

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

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

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

36. Negative results are acceptable only for test chemicals exhibiting a cell viability of less than 90% at the highest concentration tested (i.e. 1.2 × CV75 according to the serial dilution scheme described in paragraph 27). If the cell viability at 1.2 × CV75 is equal or above 90% the negative result should be discarded. In such a case it is recommended to try to refine the dose selection by repeating the CV75 determination. It should be noted that when 5000 µg/mL in saline (or medium or other solvents/vehicles), 1000 µg/mL in DMSO or the highest soluble concentration is used as the maximal test concentration of a test chemical, a negative result is acceptable even if the cell viability is above 90%.

Test report

37. The test report should include the following information.

Test chemical

- Mono-constituent substance

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

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

- Multi-constituent substance, UVCB and mixture:

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

Controls

- Positive control

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

- Negative and solvent/vehicle control

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

- Storage conditions and stability to the extent available;
- Justification for choice of solvent/vehicle for each test chemical.

Test method conditions

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

Test acceptance criteria

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

Test procedure

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

Results

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

Discussion of the results

- Discussion of the results obtained with the h-CLAT method;

- Consideration of the test method results within the context of an IATA, if other relevant information is available.

Conclusions

LITERATURE

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ANNEX I

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with concordance to mean the proportion of correct outcomes of a test method (30).

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).

CV75: The estimated concentration showing 75% cell viability.

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

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

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

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

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

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

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

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

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

Positive control: A replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Pre-haptens: chemicals which become sensitisers through abiotic transformation

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

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

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (30).

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 (30).

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

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (30).

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

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

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (30).

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

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

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (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 (30).

ANNEX II

PROFICIENCY SUBSTANCES

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly obtaining the expected h-CLAT prediction for the 10 substances recommended in Table 1 and by obtaining CV75, EC150 and EC200 values that fall within the respective reference range for at least 8 out of the 10 proficiency substances. Proficiency substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were that the substances are commercially available, and that high-quality *in vivo* reference data as well as high quality *in vitro* data generated with the h-CLAT method are available. Also, published reference data are available for the h-CLAT method (11) (23).

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

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

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

[1] The *in vivo* hazard and (potency) prediction is based on LLNA data (11) (23). The *in vivo* potency is derived using the criteria proposed by ECETOC (31).

[2] Based on historical observed values (22) (32).

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

European Commission

JRC SCIENCE AND POLICY REPORT

EURL ECVAM Recommendation on the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing

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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 summarised in the OECD report on: "The Adverse Outcome Pathway (AOP) for Skin Sensitisation Initiated by Covalent Binding to Proteins". Within this AOP the activation of dendritic cells (DC), typically assessed by expression of cell surface markers, chemokines and cytokines, is considered to be a key event. Therefore, test methods able to provide information on the ability of a chemical to up-regulate markers of DC activation may contribute to skin sensitisation hazard assessment. The human Cell Line Activation Test (h-CLAT) measures the upregulation of the CD86 and CD54 markers of DC activation in THP-1 cells, a human monocytic leukemia cell line. The test method has undergone a validation study addressing the test method's transferability and within- and between-laboratory reproducibility. Following independent peer-review by the EURL ECVAM's Scientific Advisory Committee (ESAC) and having considered input from regulators, stakeholders, international partners and the general public, EURL ECVAM concluded that the h-CLAT test method should prove valuable within Integrated Approaches to Testing and Assessment (IATA) for hazard assessment. The h-CLAT may also be able to contribute to the assessment of sensitising potency, however it is recognised that further efforts are required to explore how h-CLAT data may contribute to potency assessment.

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

EURL ECVAM RECOMMENDATION

on the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing

February 2015

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 advisory 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.

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.

Executive Summary

The human Cell Line Activation Test (h-CLAT) for skin sensitisation testing was developed by Kao Corporation and Shiseido (Japan). With a view to facilitating its use as a component of integrated approaches to assessing the skin sensitisation potential of chemicals, EURL ECVAM coordinated a validation study to assess the reliability of the h-CLAT method and to gain some preliminary insight into its predictive capacity. On completion of the study, EURL ECVAM requested ESAC to conduct a scientific peer review of the validation study report and the resulting ESAC opinion was delivered in May 2014. Following consideration of the ESAC opinion EURL ECVAM makes the following observations and recommendations:

1) The h-CLAT addresses one of the key events of the skin sensitisation Adverse Outcome Pathway (AOP) by measuring markers of dendritic cell (DC) activation in THP-1 cells, a human monocytic leukemia cell line. Therefore information generated by the h-CLAT is considered relevant for the assessment of the skin sensitisation potential of chemicals.

2) The validation study demonstrated that the h-CLAT test method is transferable to laboratories experienced in cell culture techniques and flow cytometry analysis. The within-laboratory and between-laboratory reproducibly, as characterised on the basis of concordant classifications of the chemicals employed (n=15 and n=24 respectively), were both in the order of 80%.

3) The accuracy of h-CLAT to discriminate sensitisers from non-sensitisers was calculated to be 76% (sensitivity 81% and specificity 66%) with the chemicals tested (n=24). However this result is only an approximation since the validation study was clearly not designed to fully assess the predictive capacity of the h-CLAT as a stand-alone method. Published information actually reports a higher accuracy (80%) for a larger set of chemicals (n=143; Takenouchi et al., 2013).

4) Based on the outcome of the validation study and reports from the scientific literature, data generated with the h-CLAT method should prove valuable as part of Integrated Approaches to Testing and Assessment (IATA) together with complementary information (e.g. *in chemico* or other *in vitro* data, QSAR or read-across predictions).

5) Besides providing information that contributes to the assessment of the skin sensitisation potential of chemicals, the h-CLAT assay also generates concentration-response information that may contribute to the assessment of potency. Nevertheless, additional work is still required to determine to which extent h-CLAT results can contribute to potency prediction.

6) The h-CLAT method should be further assessed with respect to its response to chemicals that need to be activated (e.g. through biotransformation or auto-oxidation) before eliciting their sensitisation effect, and to its applicability to chemical mixtures and polymers.

7) EURL ECVAM fully supports the development of an OECD Test Guideline for the h-CLAT.

8) Respecting the provisions of Directive 2010/63/EU (EU, 2010) on the protection of animals used for scientific purposes, h-CLAT data should be considered before embarking on animal experiments for assessing skin sensitisation potential. As described in Annex XI of the REACH Regulation (EC, 2006), h-CLAT data may be used to adapt the standard information requirement in the context of Weight-of-Evidence (WoE) judgments (point 1.2) or by the use of *in vitro* methods (point 1.4).

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 Biocidal Products Regulation (EU, 2012) and the Cosmetics Regulation (EC, 2009b). Determining skin sensitisation hazard according to the Globally Harmonised System to Classification and Labelling (GHS) is actually sufficient to satisfy the majority of regulatory needs (EURL ECVAM, 2013a). 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 classification of mixtures, appropriate risk management measures (e.g. setting of appropriate exposure levels) and eventually a full risk assessment.

2) Traditionally, skin sensitisation hazard assessment has involved the use of laboratory animals. In the framework 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). Following the ESAC peer review of the validation studies and the publication of the EURL ECVAM Recommendations on the *in chemico* Direct Peptide Reactivity Assay (DPRA) (EURL ECVAM, 2013b) and the *in vitro* KeratinoSens™ test method (EURL ECVAM, 2014a), OECD Test Guidelines on these two assays have been developed and are in the acceptance process.

3) The key biological events underpinning the skin sensitisation process are well established and have been summarised in the OECD report on "The Adverse Outcome Pathway (AOP) for Skin Sensitisation Initiated by Covalent Binding to Proteins" (OECD, 2012a, 2012b). These key events include 1) the covalent binding of the chemical to skin proteins (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. Following the ESAC peer review of the validation studies and the publication of the EURL ECVAM Recommendations on the *in chemico* Direct Peptide Reactivity Assay (DPRA) (EURL ECVAM 2013b), and the *in vitro* KeratinoSens™ test method (EURL ECVAM 2014a), OECD Test Guidelines on these two non-animal methods have been recently adopted (TG 442C, OECD 2015a and TG 442D, OECD 2015b).

4) There is general agreement that it is unlikely that alternative (non-animal) methods designed to address a single key event of the skin sensitisation pathway will be able to provide sufficient information to fully replace the use of animals for this endpoint (Adler et al., 2011). Instead, what is likely needed is some combination of information from complementary alternative methods (Jowsey et al., 2006; Adler et al., 2011). 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 2008, EURL ECVAM received a joint submission by Kao Corporation and Shiseido (Japan) on the h-CLAT test method that described the extensive work performed to develop and optimise the method. This included the results of multiple laboratories studies (Sakaguchi et al., 2006, 2010; Ashikaga et al., 2006, 2008; Kosaka et al., 2008; Sono et al., 2008; Mizuno et al., 2008) and h-CLAT data for 100 chemicals. As a consequence, in the period between November 2009 and November 2012, EURL ECVAM coordinated a validation study on the h-CLAT (EURL ECVAM 2013c). The study was designed to generate information according to the modular approach to validation (Hartung et al., 2004) with the primary objective of fully assessing the reliability of the h-CLAT (i.e. its transferability and within and between laboratory reproducibility). Only as a secondary study objective, the experimental data generated were used to perform a preliminary evaluation of the ability of the h-CLAT to discriminate between skin sensitising and non-sensitising chemicals, as defined by the United Nations (UN) Globally Harmonised System (GHS) to classification and labelling (UN GHS, 2013). Assessment of the preliminary predictive capacity of the h-CLAT was performed as a step towards determining the potential contribution of the method within integrated approaches to skin sensitisation hazard assessment. In addition, where possible, the experimental data were used to derive preliminary considerations on the ability of the test method to sub-categorise sensitising chemicals, e.g. into sub-categories 1A and 1B as defined by the UN GHS.

6) Following completion of the study and finalisation of the Validation Study Report (EURL ECVAM, 2013c), EURL ECVAM requested the ECVAM Scientific Advisory Committee (ESAC) to provide an ESAC Opinion on the study. The ESAC Working Group (WG) "Skin Sensitisation" prepared a detailed WG report (EURL ECVAM, 2013d) which formed the basis of the ESAC Opinion (EURL ECVAM, 2014b; see Annex 1), endorsed by members at an ESAC meeting in March 2014 and formally delivered to EURL ECVAM in May 2014.

2. Test Method definition

7) The important role played by Dendritic Cells (DC) in the initiation of adaptive immune responses is well established, including the cutaneous immune response to chemical allergens. DC can recognise and internalise antigens such as haptenated proteins, transport them via the lymphatic system to the regional lymph nodes and present them via major histocompatibility complex (MHC) molecules to naïve T lymphocytes to induce differentiation and proliferation of specific memory T-cells. Recognition and processing of the antigen by DC requires the local production of various danger signals including inflammatory mediators resulting from the activation of the innate immunity of the skin (Ainscough et al., 2013; Kaplan et al., 2012; Vocanson et al., 2009). Once activated, DC migrate from the skin to the lymph nodes and undergo a process of maturation characterised by phenotypic and functional changes resulting in the loss of their capability to process antigens and in the acquisition of the functionality of antigen presentation. The maturation process involves the decrease of phagocytic activity, increased expression of MHC molecules on the cell surface, changes in cytokines and chemokines secretion and up-regulation of several co-stimulatory (e.g. CD80, CD86, and CD40) and intercellular adhesion molecules (e.g. CD11a, and ICAM-1/CD54) (Quah and O'Neill, 2005; Vocanson et al., 2009; Kimber et al., 2011).

   Some of these biomarkers have been considered in the development of cell-based assays for assessing the skin sensitisation potential of chemicals (Aiba et al., 1997; Casati et al., 2005; dos Santos et al., 2009; Vandebriel et a.l., 2010). It is recognised that the mechanisms that lead *in vitro* to the augmentation of these membrane markers by sensitising chemicals

may only partially reflect the complexity of the mechanisms inducing DC maturation in integral biological models such as rodents and humans (Kimber et al., 2011; 2013). However, assays based on human myeloid cell lines and measuring markers known to be over-expressed during DC maturation *in vivo*, are considered to provide mechanistic information that can contribute to the *in vitro* assessment of the skin sensitisation potential of chemicals (OECD 2012a; 2012b; Adler et al., 2011; Vanderbriel et al., 2010; van Helden et al., 2008) .

8) In the h-CLAT method the modulation of the CD86 and CD54 membrane markers in THP-1 cells (Tsuchiya et al., 1980), a human monocytic leukemia cell line used as a surrogate model for DC (van Helden et al., 2008), is measured by flow cytometry following 24 hours of exposure to eight serial concentrations of test chemical selected on the basis of a pre-determined CV75 (i.e. the concentration of test chemical that allows 75% of cell survival). The h-CLAT test method is designed to discriminate between sensitising and non-sensitising chemicals whereby chemicals are classified as sensitisers if the relative fluorescence intensity (RFI) of either CD86 and/or CD54 exceeds a defined threshold (i.e. RFI CD86≥150 and RFI CD54≥200; Sakaguchi et al., 2009) compared to the vehicle control wells at any tested concentration, in at least two out of three independent measurements (i.e. repetitions). Cell viability is measured concurrently by Propidium Iodide (PI) staining and RFI values are considered for the prediction only if cell viability is above 50%.

9) Since the THP-1 cells are exposed to 8 serial concentrations of test chemicals, for positive chemicals it is generally possible to calculate from the concentration-response curve an Estimated Concentration (EC)[1] value for the CD86 and the CD54 representing the concentration of test chemical needed to induce an RFI equal to the respective threshold values, i.e. CD86 EC150 and CD54 EC200. Proposals have been made on how to use these values for potency prediction (Nukada et al., 2011, 2013), nevertheless additional work is still required to determine how h-CLAT data may inform potency assessment.

10) As a result of the validation study a revised and more detailed Standard Operating Procedure (SOP) was defined (EURL ECVAM, 2013c) which EURL ECVAM will disseminate, together with a comprehensive description of the h-CLAT method through its database on alternative methods (DB-ALM, see http://ecvam-dbalm.jrc.ec.europa.eu; protocol No. 158). The SOP contains all the necessary technical details (including electronic data reporting templates) needed by an end-user laboratory to implement it in a reliable and self-sufficient manner. In addition, EURL ECVAM intends to make available an online video tutorial with practical demonstration of how to perform the most critical steps of the h-CLAT SOP.

3. Overall performance of the h-CLAT test method

Reference data

11) A key criterion employed for selecting the validation test chemicals was availability of high quality *in vivo* data from the murine LLNA and GPMT or Buehler test, with concordant classification from these assays. In addition, chemicals with available human data and/or which are known to produce misleading responses in the animal tests (e.g. Nickel chloride and Xylene which produce false negative and false positive responses in the LLNA test,

[1] Estimated Concentrations are not to be confused with Effect Concentrations which are also usually abbreviated "EC".

respectively) were considered in the selection. The set of chemicals used in the study comprised one third of non-sensitisers and two thirds of sensitisers, with a balanced representation of potency classes (weak, moderate, strong and extreme) for the sensitisers. Also included in the reference set were: chemicals from the LLNA performance standards (OECD 2010a), two well characterised pre-haptens (i.e. chemicals requiring abiotic activation to exert their sensitisation potential), 4-Phenylendiamine and R(+)-Limonene, a well-known pro-hapten (i.e. a chemical requiring metabolic activation to act as sensitiser), Dihydroeugenol, to challenge the potential of THP-1 cells to metabolically activate inert substances, and two metal salts, Berillium sulphate and Nickel chloride. Additional details on chemical selection can be found in the Validation Study Report (EURL ECVAM, 2013c).

12) When interpreting the data from alternative non-animal methods such as the h-CLAT that have been largely developed and validated using animal reference data such as LLNA or GPMT, it should be kept in mind that the animal tests are not fully reflective of the human situation. Notably, an evaluation of the LLNA in comparison to human data has shown an accuracy of about 72% (Anderson et al., 2011) indicating an appreciable risk of both false negative and false positive predictions for humans. 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

13) EURL ECVAM concludes that the h-CLAT test method is transferable to laboratories sufficiently experienced in cell culture techniques and flow cytometry analysis and that have received proper training. The h-CLAT procedure is composed of several tasks which need to be performed sequentially, i.e., the qualification of the cell batch, the determination of an accurate CV75 value, the cell staining and measurement of CD86 and CD54 expression by flow cytometry and the data analysis and interpretation. EURL ECVAM recommends therefore that a step-wise approach similar to the one implemented in the transferability phase of the validation study is used when implementing the method before the test is performed for routine testing.

Reproducibility

14) The between laboratory reproducibility, assessed in the validation study by testing a set of 24 coded chemicals and determining concordant predictions of sensitiser versus non-sensitiser, met the expected value of 80% set a priori by the Validation Management Team (VMT). The overall within laboratory reproducibility (calculated from 15 of the 24 chemicals tested) was found to be 80%, which was lower than the expected value of 85% set by the VMT.

15) ESAC raised concerns in relation to within laboratory reproducibility since the VMT target value was not met. EURL ECVAM acknowledges this but notes that the VMT targets were derived from quite limited historical data on between laboratory reproducibility only, generated under non-blinded conditions (Sakaguchi et al., 2010). EURL ECVAM believes that these VMT target values should not be interpreted as 'cut-off' validation criteria since what can be considered as acceptable in terms of reproducibility typically depends on the context of use, such as within an IATA. However, as with data from any other experimental method, the reproducibility of h-CLAT needs to be taken into account when it is applied in any decision-making context. In this respect, it is worth noting that the reproducibility of the h-CLAT for discriminating between skin sensitisers and non-sensitisers appears to be

comparable to that of the LLNA (i.e. 70-80%, as calculated from the data available in the NICEATM database, see: http://ntp.niehs.nih.gov).

16) The ESAC peer review of the h-CLAT study included valuable expert discussion of various statistical approaches to assess within and between laboratory reproducibility. As a follow-up, EURL ECVAM proposes to re-analyse the data from the validation study with a view to exploring the merits of various statistical methods for describing the reproducibility of a test method that produces a classification-based prediction.

17) As indicated by the ESAC, further fine-tuning of the h-CLAT testing protocol and additional characterisation of the test system (THP-1 cells) may lead to an improved performance of the test method including the level of reproducibility that can be achieved. Nevertheless, the validation of the h-CLAT did not highlight any specific feature of the test method that would require additional optimisation in the short term to substantially improve its performance.

Predictive Capacity

18) Full evaluation of the predictive capacity of the h-CLAT was not within the scope of the EURL ECVAM study since the test method is not proposed as a stand-alone full replacement method. Nevertheless, the accuracy of the h-CLAT in predicting the *in vivo* classification (sensitiser/non-sensitiser) determined on the basis of concordant results in the LLNA, guinea pig tests and where available human data (see paragraph 11), was determined as 76% (sensitivity 81% and specificity 66%) (EURL ECVAM, 2013c). A recently published study that reported data on 143 chemicals (Takenouchi et al., 2013) suggested an accuracy of 80% in predicting LLNA classifications indicating that the actual performance of the h-CLAT test in discriminating between sensitisers and non-sensitisers may thus be actually higher. The accuracy of the h-CLAT in predicting human skin sensitising potential is indicated in the scientific literature to be 83% (sensitivity 88%, specificity 67%) for a set of 66 chemicals for which human patch test data and case reports are available (Nukada et al., 2011) and for a smaller set of chemicals (n=23; sensitivity 81%, specificity 86%) (Bauch et al., 2011).

4. Limitations

4.1 Technical limitations

19) **Solubility of test substances**: The test chemicals should be dissolved in a solvent compatible with the cell culture conditions. Therefore, chemicals which are not soluble in either medium, saline or DMSO, these being the solvents prescribed by the SOP, cannot be tested in the h-CLAT assay.

20) **Test substance stability:** As with many *in vitro* and *in chemico* assays, chemicals which are not stable in the prescribed solvents because of hydrolysis or other chemical reactions cannot be reliably tested.

21) **Maximum testable concentration:** In order to prevent osmotic stress of the cells, the maximum concentration of test substance should not exceed 5000 µg/mL.

22) **Interference with flow cytometry analysis:** Since the h-CLAT uses a fluorescein isothiocyanate (FITC)-labelled antibody, strong fluorescent test chemicals emitting at the same wavelength as FITC may interfere with the flow cytometry light-signal acquisition. To circumvent the problem, antibodies labelled with alternative fluorescent dyes may be used

provided that it can be shown that equivalent results to those obtained with the FITC-labelled antibodies are obtained. Also, flow cytometry analysis cannot be conducted correctly in the case of excessive cytotoxicity due to artefacts arising from diffuse labelling of cytoplasmic structures.

4.2 Limitations with regard to applicability

23) A recently published analysis of h-CLAT data suggests that chemicals with an octanol-water partition coefficient (log Kow) value lower than 3.5 can be tested in the assay and provide accurate predictions, whereas chemicals with a log Kow greater than 3.5 tend to produce false negative results (Takenouchi et al., 2013). For this reason, it was suggested that positive h-CLAT predictions obtained with chemicals with a log Kow greater than 3.5 are likely to be trustworthy whereas a negative prediction should be considered inconclusive.

24) As for many other assays based on an individual cellular model, the metabolic capacity (biotransformation) of the h-CLAT only partially represents the skin metabolism in vivo (Hennen et al, 2011; Chipinda et al., 2011; Fabian et al., 2013). Therefore, pro-haptens such as Isoeugenol may not be correctly identified by the assay. Nevertheless, putative pro-haptens such as 2-Aminophenol, Eugenol, 1-Naphtol and 2-Methoxy-4-methylphenol (Gerberick et al., 2009) have been reported in the h-CLAT submission to EURL ECVAM as being correctly predicted by the assay. In addition, Dihydroeugenol, a well characterised pro-hapten, was correctly classified as a sensitiser by all of the laboratories participating in the validation study (EURL ECVAM, 2013c).

25) Some pre-haptens are reported to be false negative in the h-CLAT (e.g. Abietic Acid) whereas others are reported as being correctly predicted by the assay (e.g. Geraniol and Linalool) (Ashikaga et al., 2010). The two pre-haptens evaluated in the EURL ECVAM validation study, 1,4-Phenylendiamine and R(+)Limonene, were correctly detected as potential sensitisers by all of the laboratories.

26) Most of the misclassifications generated by the h-CLAT in the EURL ECVAM study (EURL ECVAM 2013c), and in other published studies (Ashikaga et al., 2010) concerns chemicals that are weak sensitisers *in vivo* while the false negative rate for strong sensitisers is much lower. This should be kept in mind when interpreting negative results.

5. Suggested regulatory use

27) 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.

28) Based on the validation study results and other available information, the h-CLAT test method appears to be effective in providing information on the ability of a chemical to enhance the expression of the CD54 and/or CD86 cell membrane markers in THP-1 cells. Such markers are considered useful readouts for the identification of skin sensitising chemicals (OECD 2012a; 2012b). In addition, evidence in the literature clearly indicates the predictive value of h-CLAT data when combined with complementary information (Bauch et al., 2012; Nukada et al., 2013; Hirota et al., 2013; Tsujita-Inoue et al., 2014; van der Veen et al., 2014). Therefore results from the h-CLAT assay can be used within an IATA to determine the sensitisation potential of chemicals.

29) Taking into consideration the concentration-response information generated by the assay, it is plausible that h-CLAT may potentially contribute within an IATA to the characterisation of skin sensitisation potency. The extent of additional evidence needed to complement a h-CLAT result will depend on the intended application (e.g. hazard identification or potency assessment) and context (availability and quality of other information). Examples of the use of h-CLAT data in integrated non-animal approaches for hazard and potency assessment have been published in scientific literature (Bauch et al., 2012; Nukada et al., 2013; Hirota et al., 2013; Tsujita-Inoue et al., 2014; van der Veen et al., 2014).

30) Negative h-CLAT results should be interpreted with care, taking into due consideration (1) the limited capacity of the assay to metabolise (biotransform) pro-haptens, (2) the fact that some pre-haptens may not be sufficiently oxidised under the h-CLAT experimental conditions, and (3) the high rate of false negative predictions obtained with chemicals with a log Kow greater than 3.5.

31) Employed within an appropriate IATA, the h-CLAT assay 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

32) When applying the h-CLAT method, EURL ECVAM recommends that the revised protocol available at EURL ECVAM's DB-ALM service (http://ecvam-dbalm.jrc.ec.europa.eu, protocol No. 158) be used.

33) EURL ECVAM will undertake additional statistical analysis of the validation study results to better describe and understand aspects of reproducibility of this method.

34) Further testing to assess the performance of the h-CLAT method should include emphasis on assessing pre-and pro-haptens. In addition, its applicability to chemical mixtures and polymers (Jung YS et al., 2011) should be further investigated.

35) Predictive capacity of the assay for the discrimination between sensitisers and non-sensitisers should be further evaluated in the context of its inclusion within IATA. 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.

36) Integrated approaches using the h-CLAT method should also make use of other information sources, in particular from testing and non-testing methods (e.g. chemoinformatics, read-across and QSAR models). In silico methods that incorporate metabolic considerations (e.g. TIMES-SS: Patlewicz et al., 2007) may also help to identify pre- and pro-haptens. Analogues which have a similarly predicted mechanism of action, e.g. based on protein binding, can be found using the OECD QSAR Toolbox (www.qsartoolbox.org). The Toolbox also includes a specific profiler based on the h-CLAT assay. A variety of proposals concerning the use of h-CLAT data in combination with other information sources to discriminate between sensitising and non-sensitising chemicals have been published (Bauch et al., 2012; Nukada et al., 2013; van der Veen et al., 2014) and may support further work.

37) The possible contribution of h-CLAT CD86 EC150 and CD54 EC200 values derived from the concentration-response curve to support sub-categorisation of sensitisers according to GHS (i.e. sub category 1A and 1B) and to contribute to potency assessment should be evaluated in the context of integrated approaches. Examples are published in the scientific literature

on how these values can contribute to both purposes (Ashikaga et al., 2010; Hirota et al., 2013; Nukada et al., 2013; Tsujita-Inoue et al., 2014). For such evaluation, the use of human reference data (Basketter et al., 2014) will be particularly useful.

38) To reduce the cost and time needed for deriving a h-CLAT prediction for the purpose of skin sensitisation hazard identification, consideration should be given to adapting the h-CLAT SOP to eliminate the need for a third run in case of consistent and unequivocal predictions in the first two runs.

39) EURL ECVAM supports the development of an OECD Test Guideline for the h-CLAT. 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 IATA for skin sensitisation.

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ANNEX 1 ESAC OPINION

on the ECVAM-led study of the human Cell Line Activation Test (h-CLAT)

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Ispra, 11 March 2014

Summary of the ESAC Opinion

The ESAC was requested to provide a scientific opinion on an EURL-ECVAM led validation study assessing mainly the transferability and reproducibility (within- and between-laboratories) of the h-CLAT test method (primary objective of the study) in view of its possible future use as part of a non-animal testing strategy for skin sensitization. The study had also been designed to provide *preliminary* information on a) the predictive capacity of the test method and b) its potential use for contributing to sub categorisation of sensitizing chemicals.

Overall, the conclusions made by the ESAC based on the ESAC WG report correspond well with the conclusions drawn by the Validation Management Group overseeing the study and as described in the Validation Study Report, indicating that, generally, the conclusions are supported by the results shown in the report (see Section 15.1).

The ESAC disagreed, however, with the VMG conclusion concerning the Within Laboratory Reproducibility (WLR).

- Acceptance criteria were determined at the start of the study by the VMG. WLR was assessed using 15 chemicals in three independent experiments. The average reproducibility of 80% (KAO (86.7%), Shiseido (80.0%), EURL-ECVAM (80.0%) and Bioassay (73.3%)) did not meet the 85% reproducibility target set by the VMG. Actually, only one out of four participants met this target. Despite missing the expected performance level, the VMG nevertheless concluded that the h-CLAT is a reproducible method within laboratories. This conclusion was

partly based on the premise that a subset of chemicals consistently drove the discrepancies in reproducibility, and that some of these problem chemicals might fall outside the applicability domain. While the chemical limitations of the test are appreciated, the ESAC is concerned that there may be other inherent characteristics or critical aspects of the h-CLAT test method, which could be important sources of variability (e.g. the time course for expression of the cell surface markers; the state of cell differentiation be a source of variability). The other reason given by the VMG to support their conclusion is that the h-CLAT assay is intended to be used as part of an ITS. Generally, the ESAC does not support this reasoning: a low reproducibility, i.e. high variability will cause problems when using a test method in practice and this is independent of whether it is used as a stand-alone test method or within integrated approaches. Other information sources within an integrated approach will not be able to remedy the intrinsic variability of one information source. With regard to the h-CLAT assay, the ESAC Working Group is concerned that the poor reproducibility of the assay may actually create difficulties with respect to the interpretation of data generated as part of an ITS, as results are likely to be conflicting.

- The data were considered strong enough to support transferability of the test to properly equipped, trained and staffed laboratories with the appropriate analytical capabilities.
- Five of the 24 chemicals produced a discordant classification by the laboratories resulting in an average BLR reproducibility of 81.3%, meeting the target (80%).
- For S/NS classification, values (accuracy: 76%; sensitivity: 81.3%; specificity: 65.6%) are, overall, lower than the values (84%, 87%, 75%, respectively) resulting from the historical data on 100 chemicals (Ashikaga et al., 2010), which were provided to EURL-ECVAM as part of the test submission. Due to this discrepancy the ESAC concludes that the number of substances and the information available for these substances in the peer reviewed publication was insufficient for allowing more than a purely preliminary indication on the predictive capacity in terms of S/NS.
- For sub-categorization, the data generated and statistically assigned cut-offs propose a maximum accuracy of 58% accuracy, which is in contrast to previously published data (N=100) that reported an accuracy of 72% (Ashikaga et al. 2010). The ESAC does not understand why the VMT considers the values obtained in the validation study as promising. Our conclusion is that the number of substances and the information available for these substances was insufficient for allowing more than a purely preliminary indication on the predictive capacity in terms of potency classification.
- The number of chemicals did not allow us to draw conclusions about the applicability domain of the test (which, notably, was not one of the study objectives). Empirically the applicability domain seems to exclude pro-haptens, auto-fluorescent compounds, chemicals with limited water solubility/stability, metal salts and volatile compounds. However, pre-/pro-haptens were reported as correctly identified.

The predictive capacity, applicability domain and limitations of the test are not, in our view, yet fully defined. The submitted study does not provide strong evidence supporting the usefulness of the h-CLAT for GHS sub-categorisation of sensitizers. However, recent studies substantiate the preliminary data of the VSR (Nukada et al., 2012; Nukada et al., 2013).

- The ESAC recommends that the sources of the unsatisfactory WLR (below the 85% target) be identified and addressed;

- better defining (1) the predictive capacity and (2) the applicability domain of the h-CLAT (to eliminate the uncertainty currently associated with a negative result) either through further testing (i.e. prospective validation) or through retrospective analysis of existing information (retrospective validation: data grouping / meta-analysis);
- to adapt the SOP to reduce resource costs by eliminating the need for a third evaluation run in case where the first two runs are consistent;
- to reassess the amended SOP version 7 using existing/historical results with the purpose to re-evaluate the predictive capacity of this test method.
- that further studies be conducted to determine the potential of the test method to properly sub-categories chemicals with skin sensitisation potential.

Recently, Nukada et al. (2013) reported a data integration strategy including the h-CLAT, the Direct Peptide Reactivity Assay (DPRA) and the knowledge-based expert system 'DEREK' for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. Using a tiered strategy of h-CLAT and DPRA an accuracy of 86% and 73% for the potential and potency prediction, respectively, was obtained. Further studies are needed to identify the best integrated testing strategy or strategies able to address the different regulatory goals and risk assessments (hazard identification, classification, potency assessment, etc.) in reliable and relevant a manner.

1. Mandate of the ESAC

The opinion of ESAC should support ECVAM with respect to the development of recommendations regarding the reliability (transferability, within and between laboratory reproducibility) of the h-CLAT and the potential regulatory use of the test method.

1. Study design – transferability, reliability and relevance

- The ESAC was requested to review whether the validation study was conducted appropriately in view of the objective of the study:
  - Reproducibility of the h-CLAT method within laboratories (WLR);
  - Transferability;
  - Reproducibility between laboratories (BLR);
  - Predictive capacity of the test method.

- With respect to the design and conduct of the study, the following issues were to be addressed:
  - Clarity of the test definition (module 1)
  - Clarity of the definition of the study objective
  - Appropriateness of the study design in view of study objective
  - Appropriateness of the study execution:
  - Appropriateness of the statistical analysis used for analysing WLR, transferability, BLR and (preliminary) predictive capacity.

2. Conclusions of the study

- The ESAC was requested to assess the justification and plausibility of
  - Reproducibility (WLR and BLR) and transferability;
  - Preliminary predictive capacity;
  - Possible gaps between study design and study conclusions which remain to be addressed in view of the suggested conclusions/use;
  - Applicability and possible limitations of the test method, in particular in view of its potential use within an ITS for sensitisation testing and assessment.

3. The ESAC is requested (a) to evaluate, on the basis of the data submitted in the validation study, the possible use of the test method (also within a strategy) to identify skin sensitizers, (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).

2. Detailed opinion of the ESAC

The ESAC was asked to provide an opinion on a EURL-ECVAM-coordinated study assessing the transferability and reproducibility (within- and between-laboratories) of the h-CLAT (primary objective of the study) in view of its possible future use as part of a non-animal testing strategy for skin sensitization. The study had also been used to provide *preliminary* information on a) the predictive capacity of the test method and b) its potential use for contributing to sub-categorisation of sensitizing chemicals.

1) Study design – transferability, reliability and relevance.

- The Test Definition of the h-CLAT assay would benefit from a more detailed rationale behind the selection of the THP-1 cell line, and CD86 and CD54 membrane markers; in particular as to why both of the markers are required. Furthermore, their biological and mechanistic relevance to the human situation is not sufficiently explained. There is ample evidence showing that CD86 and CD54 are generally up-regulated in response to challenges that cause cell damage, inflammation and cytotoxicity. There is a need to explain what special features of the test or the prediction model are making the test specific for sensitization.
- The WLR was assessed at the level of concordance with a binary prediction (S/NS). An average reproducibility of 80% did not meet the 85% reproducibility target set by the VMG. Actually, only one out of the four participating laboratories met this target. The definition of the reproducibility target (85%) set by the VMG was based on the performance of methods previously evaluated at EURL-ECVAM. The expected performance of the test (WLR) is derived from the BLR calculated from the test submissions. The ESAC does not consider 85% to be an unreasonably high target. Furthermore, the explanation offered according to which it was a small number of compounds with special properties that caused problems with reproducibility was not

further substantiated. Indeed, it was noted that the problem concerned 9 out of 15 chemicals and none of the problem chemicals gave issues in all 4 laboratories.

- The ESAC is concerned, in the absence of evidence, that low WLR was caused by the characteristics of the chemicals tested and that there may be inherent characteristics of the h-CLAT, which could be important sources of WLR variability The low reproducibility of the test, raised also the concern that the h-CLAT as potential ITS building block with poor reproducibility might actually create more difficulties in interpreting data as part of an ITS due to conflicting results.
- The training and transfer phases of the validation study were well planned and executed. All the stages appear well documented. Some key issues have been identified during the process of transfer to the naïve laboratories and effort has been put into identifying and solving these issues. These changes were taken up in SOP version 5 (used for transfer) and resulted in SOP versions 6 and 7. It is clear from the transfer data that adopting this method in a laboratory requires sufficient experience in flow cytometry and cell culture.
- The BLR was assessed in terms of concordance in predictions. Two BLR values were generated by testing 24 chemicals, one comparing the consistency of the two naïve labs with the first lead lab and the second comparing them with the second lead lab. ESAC agreed with the VMG's conclusions with respect to the acceptability of the BLR because of the marginal difference between the lowest BLR (79.2%) and expected performance of 80%. The chemicals that drove discrepancies in the BLR study were the same as those driving discrepancies in the WLR study. The ESAC notes, in the absence of a defined applicability domain, that some of these test chemicals may have physicochemical properties making them incompatible with this test method.
- The ESAC recognizes the fact that this study was not designed to address the predictive capacity of the h-CLAT due to the low number of chemicals. This also applies to the sub-categorization. Three chemicals (methyl methacrylate, DCNB and benzyl alcohol) were consistently and reproducibly wrongly classified.
- The project was described and designed in clearly recognizable and well described phases including Test Definition (Module 1), Transferability (Module 3), Within Laboratory Reproducibility (WLR) (Module 2), Between Laboratory Reproducibility (BLR) (Module 4). The data were also used for a preliminary evaluation of Predictive Capacity (Module 5).
- Overall, the chosen statistical approach was considered appropriate. The 'expected proportion' of concordant classifications (between laboratories) was calculated to be 90% on the basis of available data on between-laboratory reproducibility as submitted to ECVAM (see Appendix 2 of VSR, page 5). However, it was not clear why a power of 75% rather than the more conventional 80% or 90% power had been applied. This power allows for detecting 25% changes in each direction and, as a consequence, leads to a lower limit of the confidence interval of 65 % (90%-25%).

2) Conclusions of the study

- Overall, the study design, including the chemicals and their associated reference data, were considered appropriate for the purpose of addressing the first objective of the study: Assessing the WLR (N=15) and BLR (N=24) of the h-CLAT.
- Overall, the conclusions made by the ESAC correspond well with the conclusions drawn by the VMG as described in the VSR, tending for confirm that these conclusions are supported by the results shown in the report.

- The ESAC disagreed, however, with the VMG conclusion concerning the WLR. An average reproducibility with the validation study test chemicals of 80% (not meeting the 85% VMG reproducibility target) set by the VMG. Actually, only one out of four participating laboratories met this target. The validation study did not fully establish the reasons for the WLR performance figures obtained.
- The conclusion on the BLR is considered reasonable in light of the marginal difference between the lowest BLR (79.2%) and expected performance of 80%. *See above*
- The accuracy values for S/NS classification (76%) and sub-categorization (57%) are lower than those reported earlier (84% (S/NS) and 72% (sub-categorization)) and based on historical data on 100 chemicals (Ashikaga et al. 2010. ATLA 38; 275-284) which were submitted to EURL-ECVAM as part of the test submission. The ESAC believes this may be explained in part by the smaller number of chemicals used for the validation study, some of which were not part of the historical data set. A separate communication from the test developers suggests that the historical data may have contained proportionately fewer difficult chemicals (e.g. the three chemicals consistently wrongly classified in the validation study are not part of the 100 chemical set). Assessment/description of the applicability domain was not the objective of this study. Consequently, the small number of chemicals used in the validation study, which was set to satisfy the primary goal of the study, is not sufficient on its own to draw robust conclusions on the applicability domain.

3) Possible use of the test method, i.e. to identify (also within a strategy) skin sensitizers, and additional recommendations (as required) on the proper scientific use of the test method within such a strategy.

- As yet no applicability domain has been described for this method. Deciding whether or not a chemical falls within the applicability domain of the test will be a challenge with regard to pro-haptens, metal (salts), chemicals with limited solubility/stability in water, volatile compounds and auto-fluorescent compounds.
- Regarding potency class, the data obtained did not support the use of the h-CLAT as a stand-alone assay for potency classification. This is in agreement with the statement of the VMG that the assay should be further evaluated for its capacity to "contribute" to a potency classification.

<u>Recommendations:</u>

- The ESAC considered that the target value for WLR was a realistic and justified one and were therefore concerned that three of the four laboratories failed to meet this target. The ESAC recommends that the sources of variability be identified (e.g. the time course for expression of the cell surface markers; the state of cell differentiation be a source of variability), and that solutions be provided. Poor reproducibility may create difficulties in interpreting data as part of an ITS due to conflicting results. A review of the existing 100 chemical/24 chemical datasets might identify properties of chemicals for which this test is not an appropriate method for investigating skin sensitisation potential.
- The ESAC recommends better explaining, clarifying or defining (1) the predictive capacity, the ability of the cell system and biomarkers to selectively identify skin sensitisation, and the sources of variability; and (2) the applicability domain of the h-CLAT to reduce the frequency to inconsistent results, either through further testing (i.e. prospective

validation) or through retrospective analysis of existing information (retrospective validation: data grouping / meta-analysis).

- For greater efficiency, the SOP could be adapted by eliminating the need for a third evaluation run in case where the first two runs are consistent as a third inconsistent run does not change the outcome.
- Based on the ESAC assessment of the validation study data, the available limited evidence does not support the use of the test method for GHS sub-classification of sensitizers: that was not, however, a primary objective of the validation study. Additional information and evidence are required when further consideration is given to the use of the test method for this purpose (see for example Nukada et al., 2012; 2013). Nukada et al. (2013) reported a data integration strategy including HCLAT, DPRA and DEREK for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. Using a tiered system of h-CLAT and DPRA an accuracy of 86% and 73% for the potential and potency prediction was obtained. The tiered system showed a higher sensitivity (from 88 to 96%) compared with h-CLAT alone. Further studies are needed to identify the best integrated testing strategy or strategies necessary to cover the different regulatory goals and risk assessments (hazard identification, classification, potency assessment, etc.).

3. Informative background to the Mandate and Opinion

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 for those intended for topical application (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 currently 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, in particular as regards to cosmetics.

The current view therefore is to combine different test methods in order to address different key mechanisms of skin sensitisation: skin bioavailability, haptenation (the protein binding of chemicals which triggers immunological responses), epidermal inflammation, dendritic cell activation and migration, T cell proliferation. Test methods are currently under development which have been specifically designed to address these key mechanistic steps involved in skin sensitisation. Before these test methods can be routinely used, e.g. in ITSs, their capacity to produce reproducible results needs to be demonstrated as a first step. There is ample evidence showing that maturation markers in general, and CD86 and CD54 in specific, are generally up-regulated in response to challenges that cause inflammation and cytotoxicity. There is however a window in which only sensitizers (or the

majority of them) activate dendritic cells (DCs). Cellular stress induced by allergens is different from the one triggered by irritants. Furthermore, hypersensitivity reactions are the result of normally beneficial immune responses acting inappropriately against benign antigens, causing inflammatory reactions and tissue damage. Just for clarification, DCs are recognized as important antigen presenting cells in adaptive immunity because of their capacity to stimulate naïve lymphocytes (Banchereau et al., 2000). Langerhans cells (LC) are resident immature DCs in the skin capable to take up and process contact allergens. During this process LC differentiate into mature immunostimulatory cells up-regulating the expression of co-stimulatory molecules such as CD80, CD86 and CD40 and adhesion molecules including CD2, CD11a, CD54, CD58 (Quah and O'Neill, 2005). Activated LC move from the epidermis into the dermis, and into the regional lymphatic system. In the lymph node, LC differentiate into mature dendritic cells and present antigen to specific T lymphocyte using MHC class II molecules to hold the processed antigen in place. Adhesion molecules on both the antigen-presenting cell (i.e. CD86) and the T-cell (i.e. CD28) ensure appropriate contact and co-stimulation. Following appropriate stimulus, a clone of T cells with the ability to react to the antigen, which caused their expansion, is produced. The h-CLAT it is a test method that allows for quantitative analysis of a chemical's potential to induce activation of THP-1 cells (used as a surrogate for human myeloid dendritic cells). This method has been initially proposed by Ashikaga et al. (2002) to identify sensitizers, and Yoshida et al. (2003) reported that naïve THP-1 could respond to sensitizers specifically through augmented expression of co-stimulatory molecules, CD54 and CD86, and considered this as a possible tool to be used as an *in vitro* sensitization test.

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- Dr. Michael WOOLHISER (external expert)
- Prof. Yong HEO (external expert, ICATM nomination)

ESAC / ESAC WG Coordination

Dr. Claudius GRIESINGER

ANNEX 2

ECVAM REQUEST FOR ESAC ADVICE

on

an ECVAM-coordinated study concerning the transferability and reliability of the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing

Title page information	
Abbreviated title of ESAC request	h-CLAT test method for skin sensitisation testing
ESAC REQUEST Nr.	2013-02
Template used for preparing request	EP 2.01
Date of finalising request	3/6/2013
Date of submitting request to ESAC	3/6/2013 for discussion at ESAC38 18/19 June 2013
Request discussed through	ESAC 38
Opinion expected at (date)	Q4 of 2013: ESAC plenary meeting or through written procedure
File name of this request	**ESAC REQUEST 2013-03 h-CLAT-final.doc**

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1. TYPE OF REQUEST

Request Type	Identify request ("YES")
R1 ESAC Peer Review **of a Prevalidation Study or Validation Study**	**YES**: Validation study addressing mainly reliability
If R1)applies please specify further:	
▶**Prevalidation Study**	
▶**Prospective Validation Study**	In the period between 2010 and 2012 EURL ECVAM coordinated a **validation study focusing on an assessment of reliability** of three test methods for skin sensitisation testing: 1) the *Direct Peptide Reactivity Assay (DPRA)*, 2) the *human Cell Line Activation Test (h-CLAT)*, 3) the *Myeloid U937 Skin Sensitisation Test* (MUSST). This request focuses on the h-CLAT test method.
▶**Retrospective Validation Study**	
▶**Validation Study based on Performance Standards**	
R2 Scientific Advice on a test method submitted to ECVAM for validation **(e.g. the test method's biological relevance etc.)**	
R3 Other Scientific Advice **(e.g. on test methods, their use; on technical issues such as cell culturing, stem cells, definition of performance standards etc.)**	

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

Validation of the reliability of the Human Cell Line Activation Test (h-CLAT)

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 skin allergy, leading to the disease called allergic contact dermatitis (ACD) in humans.

The identification of the skin sensitisation potential represents an important component of the safety assessment of new and existing substances including cosmetic ingredients. Current regulatory predictive tests for skin sensitisation rely on the use of animals. These include: guinea-pig tests (Buehler Test and Guinea-pig Maximisation Test) (TG 406, OECD 1992; TM B06, EC 2008a), the murine Local Lymph Node Assay (LLNA) (TG 429, OECD 2010a; TM B42, EC 2008a) and its non-radio-isotopic variants (TG 422a, OECD 2010b; TG 422b, OECD 2010c).

The key events underlying of the induction of skin sensitisation are well understood and have been recently documented by the OECD in its report on: "The Adverse Outcome Pathway (AOP) for Skin Sensitisation Initiated by Covalent Binding to Proteins" (OECD 2012a; 2012b). These include: 1) the ability of the chemical to penetrate the skin and reach the site of haptenation (skin bioavailability), 2) the covalent binding of the chemical to skin proteins (haptenation), 3) the release of pro-inflammatory signals and the induction of cyto-protective cellular pathways in keratinocytes 4) the activation and maturation of Dendritic cells (DC) the skin immunocompetent cells, 5) the migration of DC from skin to the regional lymph nodes, 6) the presentation by DC of the haptenated protein to T cells and the clonal expansion of memory T cells (lymphocytes capable of being stimulated and activated specifically by the haptenated protein).

Progress has been made in recent years in the development of mechanistically-based alternative methods for hazard identification some of which might also be able to contribute to potency prediction. However, none of these tests is currently regarded to have the potential to function as a stand-alone method to fully replace the animal tests. Instead, it is proposed that a combination of in *in silico*, *in chemico* and *in vitro* tests, addressing the key biological events of skin sensitisation, will be needed to achieve this goal.

Proposals on how to use these methods in Integrated Testing Strategies (ITS)/Integrated Approaches to Testing and Assessment (IATA) for both hazard identification and potency prediction are emerging.

2) The Human Cell Line Activation Test (h-CLAT).

The **Human Cell Line Activation Test** addresses the role that Langerhans cell (LC) and dermal dendritic cells (DC) play in the induction of skin sensitization. These cells are important mediators in the skin sensitization process since they are capable of presenting the hapten-protein conjugate to responsive T lymphocytes in the lymph nodes draining the site of exposure (Kimber and Cumberbatch, 1992). The maturation process of LC and DC from antigen processing cells to antigen presenting cells is considered a key event in the acquisition of skin sensitisation. This maturation process involves the modulation of the expression of cell surface phenotypic markers, those most commonly reported being CD54, CD80, CD86 and major histocompatibility complex (MHC) class II (Galvao dos Santos et al., 2009). This knowledge has been exploited in the development of *in vitro* tests based on the use of DC-like immortalized cell-lines to screen the skin sensitization potential of chemicals.

The h-CLAT measures the modulation of CD86 and CD54 protein markers on the surface of THP-1 cells (human monocytic cell line) by flow cytometric analysis, following 24 hour cell exposure to 8 concentrations of a test substance. The concentrations used in the main experiment are selected on the basis of the CV75 value, the estimated concentration of test substance yielding 75% cell viability, previously determined with a propidium iodide viability assay. A chemical is classified as sensitiser if the expression of either the CD86 and/or the CD54 is equal or exceeds a defined threshold in at least 2 of 3 independent evaluations.

The h-CLAT test method was jointly developed by Kao Corporation and Shiseido. Extensive development/optimisation/evaluation work including assessment of the test method's performance in multi-laboratory ring trials was conducted prior submission to ECVAM. The submission to ECVAM reported results for 100 chemicals with an accuracy of 84% for distinguishing sensitisers from non-sensitisers compared to LLNA data.

3) Study objectives and design

The validation of the h-CLAT test method was part of larger validation study involving the assessment of two other test methods, the Direct Peptide Reactivity Assay (DPRA) and the Myeloid U937 Skin Sensitisation Test (MUSST). The validation study was coordinated by ECVAM in the period between 2010 and 2012 with the primary objective of assessing the test methods' transferability and within and between laboratory reproducibility in view of their potential future use in integrated non-animal approaches intended to reduce and replace the currently used animal tests for skin sensitisation hazard identification.

As a secondary goal of the study, the experimental data were used to perform:

a) A preliminary evaluation of the ability of the three tests to reliably discriminate skin sensitising (S) from non-sensitising (NS) chemicals as defined by the Globally Harmonised System (GHS) of classification and labelling of substances (category 1; no category) (UN, 2011) and as implemented in the European Commission Regulation on classification, labelling and packaging (CLP) of substances and mixtures (EC, 2008b).

b) Where possible, a preliminary consideration of the ability of the three tests to contribute to potency categorisation e.g. GHS sub-category 1A (strong sensitisers) and 1B (other sensitisers) as defined in the fourth revised edition of GHS (2011).

24 coded test items were tested by each of the four laboratories participating in the study for the evaluation of the h-CLAT (Kao and Shiseido as the lead laboratories, Biossay and EURL ECVAM as the naïve laboratories) to generate information on the between-laboratory reproducibility. A subset of 15 chemicals was tested two additional times in each laboratory for the evaluation of the within-laboratory reproducibility.

With respect to the ECVAM's modular approach to validation (Hartung et al., 2004) the study generated information on modules 1) test definition, 2) within laboratory reproducibility, 3) transferability and 4) between laboratory reproducibility. In addition, the experimental data contributed to modules 5) predictive capacity and 6) applicability domain. **However, the number of chemicals used in this validation study, which was based on statistical considerations related to the evaluation of the reproducibility only, was not sufficient on its own to conclude on the last two modules.**

4) Study results

The main results for the study's primary goal are summarised in the table below:

Module	Results
Module 2 WLR	Evaluation of the WLR for a subset (n=15) of the validation study chemicals in each laboratory focused on the concordance of predictions (sensitizer versus non-sensitiser) as determined by the results of three independent experiments. Kao Laboratory WLR=86.7% Shiseido Laboratory WLR=80% EURL ECVAM Laboratory WLR=80% Bioassay Laboratory WLR=73.3%
Module 3 Transferability	Both naïve laboratories (EURL ECVAM and Bioassay) succeeded in transferring the protocol to their testing facilities.
Module 4 BLR	Evaluation of the BLR for the 24 chemicals focused on the concordance of the predictions (sensitiser versus non-sensitiser) and was calculated by comparing the two naïve laboratories with each of the two lead laboratories separately. Naïve and Kao BLR=83.3% Naïve and Shiseido BLR=79.2% Overall BLR=79.2%

5) Conclusions of the VMG

The VMG concluded that the information generated in this validation study demonstrates that the h-CLAT is a robust test method that can be easily transferred to properly equipped laboratories sufficiently experienced in cell culture and flow cytometry analysis. In addition the study results support the fact that the h-CLAT is a reproducible test method that can contribute to the determination of the sensitization potential of substances.

References

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EC 2008b. Regulation No 1272/2008 of 16 December 2008 of the European Parliament and of the Council 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 of the European Union L 353, p. 1-1355.

Galvao dos Santos G, Reinders J, Ouwehand K, Rustemeyer T, Schepper RJ & Gibbs S (2009) Progress on the development of human in vitro dendritic cell based assays for assessment of the sensitizing potential of a compound. Toxicology and Applied Toxicology 236(3); 372-382.

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

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Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins; Part 2: Use of the AOP to Develop Chemical Categories and Integrated Assessment and Testing Approaches Series on Testing and Assessment No.168 ENV/JM/MONO(2012)10/PART2. Paris.

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4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

Objective *Why does ECVAM require advice on the current issue?*	The opinion of ESAC should support EURL ECVAM with respect to the development of an EURL ECVAM recommendation on the h-CLAT assay outlining (1) the scientific basis of the assay, (2) its overall performance as assessed during the study and based on other (e.g. published) information, (3) its applicability and limitations. Furthermore, the advice of ESAC should support ECVAM with respect to the analysis of possible data gaps that need to be addressed in view determining the test method's potential use and usefulness within integrated approaches for skin sensitisation hazard and risk assessment.

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 study was conducted appropriately in view of the objective of the study. The study objective was to assess (1) the reproducibility of the h-CLAT method within one laboratory (WLR) (2) its transferability to other laboratories (3) its reproducibility between laboratories (BLR) (4) Furthermore, the study aimed at assessing, in a preliminary manner, the predictive capacity of the test method for distinguishing between sensitisers and non-sensitisers and, where possible, to appraise its potential to contribute to a further sub-categorisation of sensitisers into two subcategories (1A and 1B). When reviewing the design and conduct of the study, the following issues should be addressed in particular: (a) Clarity of the test definition (module 1) (b) Clarity of the definition of the study objective and study management (c) Appropriateness of the study design & execution in view of the study objectives, *inter alia:* o Is the **number of tested chemicals (24)** 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? (d) Appropriateness of the **study execution** (e.g. were there pre-defined test acceptance criteria, were these respected? How were exceptions / deviations handled? Were provisions specified for retesting? Was the number of repetitions sufficient? etc.) (e) Appropriateness of the **statistical analysis** used for analysing WLR, transferability, BLR and (preliminary) predictive capacity. 2) **CONCLUSIONS OF STUDY:** The ESAC is requested to assess whether the conclusions, as presented in the Validation Study Report, are substantiated by the information generated in the study and are plausible with respect to existing information and current views (e.g. literature). In particular: (a) Are the conclusions on **reproducibility** (WLR and BLR) as well as transferability justified and plausible? (b) Are the conclusions on preliminary **predictive capacity** justified and plausible with respect to existing information (c) 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)? (d) Do the data generated with this defined set of chemicals **together** with available existing data provide sufficient information on the **applicability and possible limitations** of the test method, in particular in view of its potential use within an ITS for sensitisation? 3) **SUGGESTED USE OF THE TEST METHOD:** The ESAC is requested (a) to evaluate, on the basis of the data summarised in the validation study report, the possible use of the test 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, technical limitations etc.) and (c) to identify possible further information required (i.e. are there gaps) to be able to determine the potential use and usefulness of the test method within integrated approaches.

4.3 TIMELINES

Timelines concerning this request *When does ECVAM require the advice?*	**Timeline**	**Indication**
	Finalised ESAC Opinion required by:	4Q 2013 (probably through written procedure)
	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	YES Final request presented at ESAC 38, 18/19 June 2013

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. However, no WG needs to be established, as EURL ECVAM has taken the decision to employ the existing *ESAC WG "Sensitisation"* (set up in 2011) also for the h-CLAT review. The WG has already prepared detailed reviews/draft opinions on the DPRA and the Keratinosens test methods. This will add consistency to the review of these three sensitisation test methods and expedite progress as, at the time of issuing this request (June 2013), the VSR is already available and the WG can therefore commence with the review work. **Present ESAC WG:** • Dr. Erwin ROGGEN (ESAC member, Chair of ESAC WG and rapporteur; *3Rs Management and Consultancy, Denmark*) • Prof. A. Wallace HAYES (external expert; *Harvard University, USA*) • Dr. Maja ALECSIC (external expert, *Unilever, UK*) • Dr. Emanuela CORSINI (external expert;

		Dipartimento di scienze farmacologiche e biomoleculari, Università Degli Studi di Milano, Italy) • Dr. David LOVELL (external expert; *University of Surrey, UK*) • Dr. Michael WOOLHISER (external expert; *Dow Chemical Company, USA*) • Prof. Yong HEO (external expert, ICATM nomination (KoCVAM)*; College of Natural Sciences, Catholic University of Deagu, South Korea)*
	S3 Invited Experts	
	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):	

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	
	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	Already available? (YES/NO)	File name
0	EURL ECVAM Validation Study Report	YES	h-CLAT Validation Study Report.pdf
2	Appendices 1-15 to EURL ECVAM Validation Study Report	YES	h-CLAT appendices to VSR.pdf
3	EURL ECVAM Strategy for Replacement of Animal Testing for Skin Sensitisation Hazard Identification and Classification	YES	EURL ECVAM strategy .pdf
4	OECD Report: The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1	YES	OECD AOP-part1.pdf
5	Publication: Progress on the development of human in vitro dendritic cell based assays for assessment of the sensitizing potential of a compound	YES	dos Santos 2009.pdf
6	Publication: A Comparative Evaluation of In Vitro Skin Sensitisation Tests: The Human Cell-line Activation Test (h-CLAT) versus the Local Lymph Node Assay (LLNA)	YES	Ashikaga 2010.pdf
7	Publication: Predicting skin sensitization potential and inter-laboratory reproducibility of a human Cell Line Activation Test (h-CLAT) in the European Cosmetics Association (COLIPA) ring trials	YES	Sakaguchi 2010.pdf
8	Publication Predictive performance for human skin sensitizing potential of the human cell line activation test (h-CLAT)	YES	Nukada 2011
9	Publication: Prediction of skin sensitization potency of chemicals by human Cell Line Activation Test (h-CLAT) and an attempt at classifying skin sensitization potency	YES	Nukada 2012a.pdf
10	Publication: Data integration of non-animal tests for the development of a test battery to predict the skin sensitizing potential and potency of chemicals	YES	Nukada 2012b.pdf
11	Publication: Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with high octanol-water partition coefficients	NO Will be made available as soon as possible	Takenouchi et al.- Submitted for publication

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

During its 38th meeting on 18/19 June 2013 the ESAC plenary decided to employ the ESAC Working Group "Sensitisation" for preparing a detailed scientific review of the study on the h-CLAT test method for skin sensitisation testing.

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 WG is requested to conduct a scientific review of the ECVAM-coordinated validation study focusing on an assessment of reliability of the h-CLAT test method. 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) 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 ESAC Chair and the ESAC Coordinator 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	***July 2013***	1. Discussion of the mandate and first appraisal of the VSR. 2. Agreement on further timelines and possible work distribution	
2	***Friday 20. September 2013***	Forwarding of initial	

		observations (within ESAC WG template) to ECVAM	
3	***1 & 2 October 2013***	ESAC WG meeting at JRC campus in Ispra, Italy	Draft ESAC WG report
4	***End of November/mid December 2013***	Forwarding final report to ESAC Chair and ESAC Coordinator	Final report, adopted by WG

7.6 QUESTIONS WHICH SHOULD BE ADDRESSED BY THE ESAC WG

The ESAC WG is requested to address the **questions posed to the ESAC** which have been broken down further in more **specific questions** (see section 4.2).

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. The template allows moreover for addressing the issues specific studies outlined in section 4.2. The Coordinator will provide guidance if necessary.

European Commission
EUR 27022 EN – Joint Research Centre – Institute for Health and Consumer Protection

Title: **EURL ECVAM Recommendation on the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing**

Author(s): European Union Reference Laboratory for Alternatives to Animal Testing

Luxembourg: Publications Office of the European Union

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